# REGENERATION AND BRAIN REPAIR

**EDITED BY: Daniella Rylander Ottosson, Andreas Heuer and Sofia Grade**

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# REGENERATION AND BRAIN REPAIR

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# Editorial: Regeneration and Brain Repair

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**Editorial on the Research Topic**

**Regeneration and Brain Repair**

Brain disorders such as Parkinson's disease (PD), Alzheimer's disease, Multiple sclerosis, Huntington's disease (HD), and Stroke currently lack effective treatments and represent major healthcare challenges. With the aim to develop strategies for brain repair, the field of neural regeneration is constantly exploring exciting new and refined strategies. The current Research Topic has gathered new evidence and compiled past and recent efforts to achieve this goal. Altogether, four main subtopics are showcased (1) neuronal replacement strategies, mainly through cell transplantation, (2) neuroprotective approaches, (3) studies into the disease pathology to efficiently design the former strategies, and (4) adult neurogenesis as a valuable lens to neuronal development in an adult brain.

In contrast to many other mammalian organs, the brain lacks regenerative capacity (with some exceptions) and therefore, neurons can only be restored through an exogenous route, e.g., via cell transplantation. The past years have witnessed impressive developments in obtaining novel cell sources for cell transplantation ranging from the patients' own cells through reprogramming of skin fibroblasts to the generation of chimeric animals for xenotransplantation. The interest and efforts laid in this field are enormous and have led to the fascinating development of hESC (human embryonic stem cell)- and iPSC (induced pluripotent stem cell)-derived neuronal subtypes of clinical relevance. Indeed, human PSC-derived dopaminergic neurons for PD are now in clinical trials and similar studies using human PSC-derived striatal neurons in patients of HD are likely to follow. Further progress in this field will critically depend on a close interaction between experimental and clinical research. In the future, cell replacement strategies might also involve *in situ* cell type conversion e.g., via forced expression of neurogenic transcription factors in non-neuronal cells. This so-called neuronal reprogramming has been evolving at a fast pace and holds great potential since it relies on patients' own cells.

This Research Topic includes two unique reviews in the field of cell therapy for PD and HD. One in the shape of a 40-year perspective piece on circuitry repair in basal ganglia, presenting an overview of past and current efforts to restore neurotransmission or damaged connectivity in the adult mammalian brain. Here, pioneers in the field of cell replacement therapy using primary fetal tissue (Björklund) and hESCs (Parmar) cover the historical development of the field from its beginnings in the 1970s to those of today using alternative cell sources as donor tissue. The second review by Osborn et al. describes cell therapy for PD with a focus on donor cell types, engaging into a revision of advantages and drawbacks of autologous cells, and careful considerations on costs and benefits.

Despite great progress, there are still many efforts needed to validate that the lab-grown cells are *en pair* with the gold-standard primary tissue from fetal brain as well as to elucidate the exact mechanism of repair in a given paradigm. A new era of brain repair has arrived, where advances in single cell profiling help define cell identities and lineages progression, going hand-in-hand with rapidly expanding methods for *in vivo* reprogramming. On this theme, the review from the Brüstle laboratory covered major achievements and future prospects on transcription factor-guided differentiation and forward programming of PSCs (Flitsc et al.). The authors beautifully cover the most representative progress to derive clinically relevant specialized neuronal subtypes as well as glial cells and highlight the remaining challenges. With similar motivation, the Takahashi lab used a newly identified cell surface marker of corticospinal motor neurons progenitor cells L1CAM to enrich the donor cell suspension obtained from fetal tissue, into the cell type of interest for transplantation into rodents (Samata et al.). They report enhanced survival and ability to extend axonal projections in the corticospinal tract using L1CAM+ sorted fetal grafts cells as compared with non-sorted, reiterating the need for an identity match also in models of acute brain injury.

Besides cell transplantation or local reprogramming strategies, brain repair approaches also include disease-modifying strategies designed to retard the ongoing degeneration by neuroprotection, ultimately slowing down disease progression. On this theme, leucine-rich repeat kinase 2 (LRRK2) appears as a promising target to counteract multiple pathogenic processes underlying PD development as described by Calabresi and co-authors in the appraisal from Mancini et al. LRRK2 mutations are responsible for the majority of inherited familial PD cases, can also be found in sporadic PD, and might underlie early pathological phenomena. Along the same lines, Manfredsson et al. share an opinion article that assembles the points of discussion on glial derived-neurotrophic factor (GDNF) delivery in PD raised at the American Society of Neural Therapy and Repair and recommendations calling for improved preclinical models and methods of GDNF delivery, early diagnostics, and clinical trial design.

Furthermore, the present Research Topic includes contributions that provide fundamental insights into the biology of brain pathology. Prodromidou and Matsas illuminate the emergent role of miRNAs as master regulators of gene expression that rewire transcriptional landscapes during human brain development and neurological disease, a finding that instigated their use in therapeutics. From gene expression to cellular processes and extracellular matrix (ECM) alterations, a hybrid article from the Götz lab provides original results of proteome profiling after traumatic brain injury, framed into an elegant review of the field of glial scar biology, with a focus on ECM composition and wound healing (Kjell and Götz). A recurrent theme on various contributions was that not only disease mechanisms need to be better understood but also disease models need to be improved. Ermine et al. describe a model of endothelin-induced cortical ischemia in rats with a temporal progression of the behavioral deficits and of atrophy that better resemble those in stroke patients.

This collection also highlights research on adult neurogenesis that constitutes unarguably a hot topic in neurosciences and with implications for repair. In a review from Petrik and Encinas, we can follow the debate and controversy on the existence of adult neurogenesis in the human brain and its similarity to rodent neurogenesis. This review brings up the issues of technical criteria to identify adult neurogenesis in humans and entertains considerations about the temporal differences in neurogenesis decline in rodents vs. humans and the need to re-evaluate the existence of human neurogenesis out of the hippocampal niche. Given the current landscape on this topic we can certainly expect more studies to be added in the future and the discussion to continue. Indeed, the field of neural regeneration will continue to be explored further in the quest for brain repair.

## AUTHOR CONTRIBUTIONS

DR, SG, and AH decided the layout, wrote the manuscript, acted Editors to this Research Topic, and selected the articles described therein. All authors contributed to the article and approved the submitted version.

# frontiers in Neuroscience

# Perspective: Of Mice and Men – How Widespread Is Adult Neurogenesis?

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These are exciting times for research on adult hippocampal neurogenesis (AHN). Debate and controversy regarding the existence of generation of new neurons in the adult, and even diseased human brain flourishes as articles against and in favor accumulate. Adult neurogenesis in the human brain is a phenomenon that does not share the qualities of quantum mechanics. The scientific community should agree that human AHN exists or does not, but not both at the same time. In this commentary, we discuss the latest research articles about hAHN and what their findings imply for the neurogenesis field.

Keywords: neuroscience, neural stem / progenitor cells, hippocampus, human neurogenesis, adult neurogenesis

*"The best-laid schemes o' mice an' men gang aft agley"* (The best laid schemes of mice and men go often askew)

Robert Burns (1785)

It is common that new concepts are doubted and re-doubted. We already overcame the once disbelieve in the existence of adult neurogenesis in the mammalian brain. However, a new controversy arose recently about the existence of human AHN (hAHN), the process of generating adult-born neurons from neural stem cells (NSCs). While it is not the first time that the existence of adult neurogenesis has been discredited (Rakic, 1985), the findings that adult neurogenesis may not exist in adult human hippocampus (Cipriani et al., 2018; Sorrells et al., 2018) come at a time when research on adult neurogenesis constitutes a major field in neurosciences due to the importance to the functions (memory, learning and mood control) associated with this phenomenon in animal models (Eisch and Petrik, 2012). The findings by Cipriani et al. (2018) and Sorrells et al. (2018) (**Figure 1**) are in direct conflict with another three major recent studies demonstrating hAHN (Boldrini et al., 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019) following the path of previous reports (Fahrner et al., 2007; Knoth et al., 2010). This most recent controversy does not only stir up the research community but also examines its conceptual and structural complexion.

hAHN has been proposed to exist using a plethora of techniques ranging from immunohistochemistry for native or synthetic markers of proliferation (Eriksson et al., 1998), cell markers of neuroblasts and immature neurons (Knoth et al., 2010), to unique radioactive carbon-based cell-birth dating (Spalding et al., 2013) and non-invasive imaging approaches (Manganas et al., 2007). All of these, however, focused on the hippocampus, whereas human neurogenesis in the walls of lateral ventricles has remained far less studied. Thus, when a second wave of controversy on adult neurogenesis in humans had appeared, driven by the findings of Alvarez-Buylla lab (Sanai et al., 2011), most of the adult neurogenesis researchers were (and still are) disconcerted, largely because ventricular neurogenesis lies outside of the predominant

**FIGURE 1 |** Schematic summary of recent research articles on human adult hippocampal neurogenesis. **(A)** In mice, a population of well-characterized neural stem cells (NSCs) generates neuronal-fate committed precursors that amplify their numbers through cell divisions and then differentiate into neuroblasts that maturate into neurons. **(B)** In healthy humans, cell-division dependent neurogenesis has been reported using radioactive carbon-based cell-birth dating (Spalding et al., 2013) and BrdU incorporation (Eriksson et al., 1998). **(C)** The abundant presence of both neural precursor like-cells and immature neurons (Tobin et al., 2019) or immature neurons (Moreno-Jiménez et al., 2019) has been shown, coexisting with cell division markers. **(D)** Total absence of cell proliferation, neural precursor like-cells and immature neurons has been demonstrated in the adult hippocampus (Sorrells et al., 2018). **(E)** Present, but reduced number of neural progenitor-like cells and immature neurons has been reported (Cipriani et al., 2018; Boldrini et al., 2018). In Alzheimer's disease, neural precursor like-cells and immature neurons are greatly reduced (Cipriani et al., 2018; Moreno-Jiménez et al., 2019) **(F)** or still exist in more prominent cell populations (Tobin et al., 2019) **(G)**. Orange nuclei indicate cell division while nuclei indicate not proliferative state. (Human shape Designed Freepik-Vilmosvarga).

hippocampus-focused interest. The findings of Sanai et al. (2011) that adult humans do not show neurogenesis derived from the subventricular zone of the lateral ventricles contradict articles claiming its presence (Curtis et al., 2007; Ernst et al., 2014). This report that the subventricular NSCs quickly disappear from human brain during infancy should have been perceived with a greater urgency that similar studies will be published about hAHN. The lack of reaction in the research field says a lot about its structure and the way it sees its own subject of study. Such hippocampus-heavy tendency can be further appreciated in the fields' disinterest in so called non-canonical adult neurogenesis in the hypothalamus, where unique adult NSCs generate diet-responsive adult born neurons (Yoo and Blackshaw, 2018). Interestingly, hypothalamic neurogenesis regulates ventricular neurogenesis (Paul et al., 2017) and therefore now more than ever it is important to investigate if the hypothalamic neurogenesis also exists in adult humans and to what degree (Pellegrino et al., 2018).

Adult neurogenesis has been confirmed in the majority of species of terrestrial mammals, but it seems to be absent in cetaceans, reviewed in detail in Amrein (2015), Patzke et al. (2015), Lipp and Bonfanti (2016). Markers for cell proliferation, stem cells and immature neurons were identified in adult hippocampus of mammals with small, lissencephalic brains such as rodents, but also in large, gyrencephalic brains of phylogenetically distant species such as the cows (Rodriguez-Perez et al., 2003), the African elephants (Patzke et al., 2014), or the dogs (Hwang et al., 2007). Furthermore, adult neurogenesis has been found in hippocampus of various different primate species including marmosets (Bunk et al., 2011), lemurs (Fasemore et al., 2018), macaques (Gould et al., 2001; Jabes et al., 2010) and baboons, where adult neurogenesis is required for the antidepressant action (Perera et al., 2011; Wu et al., 2014). The prevalence of adult neurogenesis in primates suggests that it should be found also in humans. However, phylogenetics may not be the most reliable predictor of adult neurogenesis existence even in related taxonomic ranks. For example, some species of bats do have active neurogenesis, while others do not (Amrein, 2015). This could be caused by natural differences in closely related taxons or it could stem from technical reasons, which may not be the case for well prepared specimens of bat brain (Amrein et al., 2007) but could apply to more complicated autopsies of human tissue. Indeed, some native cell markers for neurogenesis are sensitive to fast degradation and specific tissue fixation, which can be the most likely factor to explain the disagreement in the results regarding the human data (Lucassen et al., 2019).

The immunohistochemical detection of individual cell markers may not support the existence of adult neurogenesis, however, their combination could (as summarized elsewhere (Kempermann et al., 2018). For example, Moreno-Jiménez et al. (2019) reported PSA-NCAM or doublecortin (DCX+) positive cells in human hippocampus as an evidence of adult neurogenesis, because these markers label neuroblasts or immature adult-born neurons in mice (Kempermann et al., 2004) and other mammals. On the other hand, Sorrells et al. (2018) reported a lack of DCX+ and PSA-NCAM+ neurons as well as the sharp decline of proliferating cells labeled by Ki67, the

endogenous marker of cell cycle. Because Moreno-Jiménez et al. (2019) did not stained for proliferation markers, an argument could be made that the observed DCX+ neurons are not a direct product of adult neurogenesis but rather a unique subset of neurons expressing markers associated with neurogenesis. However, both immature neurons and proliferating Ki67+ or PCNA+ cells or proliferating Ki67+ Nestin+ putative progenitor cells have been demonstrated in the other most recent studies (Boldrini et al., 2018; Tobin et al., 2019) or in previous studies on adult hippocampus neurogenesis in humans that used either endogenous (Liu et al., 2008; Knoth et al., 2010; Dennis et al., 2016; Mathews et al., 2017) or synthetic markers of proliferation (Eriksson et al., 1998; Ernst et al., 2014).

In our opinion two major questions arise from these recent data. First, how do we actually define adult neurogenesis? Based on the literature consensus, adult neurogenesis is the generation, through cell division of neural progenitors, of new neuronal fate-committed precursors that undergo a process of neuronal differentiation and maturation (**Figure 1A**). Second, what is needed in terms of biomarker expression to accept this definition of adult neurogenesis? Expression of DCX and or PSA-NCAM may not be sufficient. The existence of very slowly maturing neurons which maintain the expression of these immature markers but were actually generated during development has been demonstrated in the brain of rodents and sheep (Piumatti et al., 2018; La Rosa et al., 2019). This process that represents another fascinating form of brain plasticity supports the argument that exploring cell divisions should be a requisite for confirmation of adult neurogenesis in humans. On the other hand, presence of cell division together with the presence of neuroblasts or immature neurons may not be sufficient criteria for claiming neurogenesis. Even in the neurogenic niches, there are other actively dividing cell types such as astrocytes, microglia, pericytes, endothelial cells and oligodendrocyte progenitors (OPCs). Some of these cell types share specific cell markers with neural precursors. For example, nestin is present in OPCs and perycites (Encinas et al., 2011) and Sox2 is expressed in all astrocytes in the hippocampus (Komitova and Eriksson, 2004). This shared expression of certain cell markers is of special relevance in aging. One of the hallmarks of astrocytes in the aged brain is the gradual acquisition of a reactive-like and even proliferative phenotype (Clarke et al., 2018), which is further characterized by the expression of nestin. Thus, expression of nestin and Sox2 may not constitute a valid marker combination to exclusively identify neural progenitors. Instead, the stem cells and progenitors should be described by more recent specific biomarkers such as Lunatic fringe (*Lfng*) (Semerci et al., 2017) or the lysophosphatidic acid receptor 1 (*LPAR1*) (Walker et al., 2016) and by exclusion of expression of S100ß, a marker of mature astrocytes. Finally, another strategy could be utilized to strengthen the conclusions about hAHN – a correlation between levels of cell division in progenitors and levels of DCX or PSA-NCAM in immature neurons. Even though correlation does not imply causation, a positive correlation would point toward the existence of a neurogenic cascade, adding up to the earlier works by Spalding et al. (2013) and Eriksson et al. (1998). These works suggest the existence of neurogenesis in the adult human brain

(**Figure 1B**) by detecting in neurons markers that would have been incorporated, arguably (see Duque and Spector, 2019. for critical technical analysis), of those cells only through mitosis.

This newest controversy on the existence of adult neurogenesis in human hippocampus highlights other aspects than just definition of cellular stages by specific markers. First, there is the issue of time (reviewed in detail in Snyder, 2019). Mice live about 50 times shorter than humans, yet their adult neurogenesis declines more rapidly with age, whereas human neurogenesis could persist for up to 80 decades (Knoth et al., 2010; Moreno-Jiménez et al., 2019). If hAHN exists, what are the mechanisms that allow humans to maintain active putative neural progenitors for so much longer? What could be the key molecular determinants for such long-term cellular "stemness"? Importantly, when we look at studies using human samples, opposite results on the existence of neural progenitors emerge. Neural progenitors are harder to be determined in human samples due to limited technical toolbox as explained above and when addressed, opposite results have been found. While Sorrells et al. (2018) report a drastic reduction of neural stem and progenitor-like cells that would thus explain the absence of adult neurogenesis, Boldrini et al. (2018) and Tobin et al. (2019) report their abundant existence. Half way, Cipriani et al. (2018) showed persistence of neural stem and progenitor-like cells in the adult brain, but absence of actual neurogenesis. In any case, the properties of these neural progenitors are yet poorly studied and could be different from those of the mouse. For instance, according to the published data, in human samples putative NSCs would have to have a more stellate morphology than a radial one (Boldrini et al., 2018; Cipriani et al., 2018; Tobin et al., 2019) (**Figures 1C–E**). Second, the most recent studies by Moreno-Jiménez et al. (2019) and Tobin et al. (2019) again confirm abundant adult neurogenesis, or at least the abundant presence of immature neurons, in healthy human hippocampus but limited or absent neurogenesis in patients with Alzheimer's Disease (AD) (**Figure 1F**). This finding that AD alters adult neurogenesis does not only underscore the necessity of proper triage of diseased tissue specimens in human studies but is in line with conclusions from AD mouse models (Hamilton et al., 2015). However, as with many compounded topics such as modeling of the AD in rodents, it is apparent that the small, lissencephalic brains of mice may not be the best proxy for the large and complex human brains (Jankowsky and Zheng, 2017). To put it bluntly: mice are not small humans. And yet, sort of automatic assumptions are drawn from rodent models to functional implications in humans. As commented before, it could be that the neuronal maturation process is much slower in humans and that the ratio between cell proliferation and maturing neurons is much weaker than in mice. Very slowly maturing neurons would read out as an apparent higher-than-real amount of neurogenesis (which implies birth of neurons). On the other hand, if humans lack adult neurogenesis, how would all the adult neurogenesis-dependent brain functions described in rodents operate in humans without adult neurogenesis? And what are the reasons why humans have diverged in evolution from other primates that contain AHN?

We should use the current debate to re-evaluate the *status quo* of the neurogenesis field with respect to the laboratory models, quality controls and theoretical concepts to move the topic and the field forward. In other words, humans are not large mice; disease, metabolism and life style can negatively affect the tissue and lead us to inaccurate conclusion; and molecular mechanisms driving NSCs in hippocampus (Petrik et al., 2015) may not be the same in the other neurogenic niches (Ninkovic et al., 2013). Furthermore, this recent surge in interest in human adult neurogenesis should be employed to re-evaluate if adult neurogenesis is prevalent in parts of human brain other than the hippocampus. How well established is the fact that adult neurogenesis is actually absent in the ventricular system of the adult human brain? It is possible that the evolutionary pressures for greater complexity in human brain did not strip it from the subventricular neurogenesis (Sanai et al., 2011), but rather made neurogenesis more prevalent in regions of the central nervous system where we have not yet looked in both physiological and pathophysiological conditions? In conclusion, first we have to stipulate what technical criteria are essential to identify adult neurogenesis. And then we should not only ask whether adult neurogenesis does exist in the human brain or not, but we should also ask whether it occurs as a process similar to rodent neurogenesis, or whether it is more wide-spread than we originally thought.

## AUTHOR CONTRIBUTIONS

Both authors contributed equally to the writing of this manuscript.

## REFERENCES

Amrein, I. (2015). Adult hippocampal neurogenesis in natural populations of mammals. *Cold Spring Harb. Perspect. Biol.* 7:a021295. doi: 10.1101/cshperspect.a021295

Amrein, I., Dechmann, D. K., Winter, Y., and Lipp, H. P. (2007). Absent or low rate of adult neurogenesis in the hippocampus of bats (Chiroptera). *PLoS One* 2:e455. doi: 10.1371/journal.pone.0000455

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., et al. (2018). Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell* 22, 589–599.e5. doi: 10.1016/j.stem.2018.03.015

Bunk, E. C., Stelzer, S., Hermann, S., Schafers, M., Schlatt, S., and Schwamborn, J. C. (2011). Cellular organization of adult neurogenesis in the common marmoset. *Aging Cell* 10, 28–38. doi: 10.1111/j.1474-9726.2010.00639.x

Cipriani, S., Ferrer, I., Aronica, E., Kovacs, G. G., Verney, C., Nardelli, J., et al. (2018). Hippocampal radial glial subtypes and their neurogenic potential in human fetuses and healthy and Alzheimer's Disease Adults. *Cereb. Cortex* 28, 2458–2478. doi: 10.1093/cercor/bhy096

Clarke, L. E., Liddelow, S. A., Chakraborty, C., Munch, A. E., Heiman, M., and Barres, B. A. (2018). Normal aging induces A1-like astrocyte reactivity. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1896–E1905. doi: 10.1073/pnas.1800165115

Curtis, M. A., Kam, M., Nannmark, U., Anderson, M. F., Axell, M. Z., Wikkelso, C., et al. (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 315, 1243–1249. doi: 10.1126/science.1136281

Dennis, C. V., Suh, L. S., Rodriguez, M. L., Kril, J. J., and Sutherland, G. T. (2016). Human adult neurogenesis across the ages: an immunohistochemical study. *Neuropathol. Appl. Neurobiol.* 42, 621–638. doi: 10.1111/nan.12337

Duque, A., and Spector, R. (2019). A balanced evaluation of the evidence for adult neurogenesis in humans: implication for neuropsychiatric disorders. *Brain Struct. Funct.* 224, 2281–2295. doi: 10.1007/s00429-019-01917-6

Eisch, A. J., and Petrik, D. (2012). Depression and hippocampal neurogenesis: a road to remission? *Science* 338, 72–75. doi: 10.1126/science.1222941

Encinas, J. M., Michurina, T. V., Peunova, N., Park, J. H., Tordo, J., Peterson, D. A., et al. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* 8, 566–579. doi: 10.1016/j.stem.2011.03.010

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., et al. (1998). Neurogenesis in the adult human hippocampus. *Nat. Med.* 4, 1313–1317.

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., et al. (2014). Neurogenesis in the striatum of the adult human brain. *Cell* 156, 1072–1083. doi: 10.1016/j.cell.2014.01.044

Fahrner, A., Kann, G., Flubacher, A., Heinrich, C., Freiman, T. M., Zentner, J., et al. (2007). Granule cell dispersion is not accompanied by enhanced neurogenesis in temporal lobe epilepsy patients. Exp. Neurol. 203, 320–332. doi: 10.1016/j.expneurol.2006.08.023

Fasemore, T. M., Patzke, N., Kaswera-Kyamakya, C., Gilissen, E., Manger, P. R., and Ihunwo, A. O. (2018). The distribution of Ki-67 and doublecortin-immunopositive cells in the brains of three strepsirrhine primates: galago demidoff, perodicticus potto, and Lemur catta. *Neuroscience* 372, 46–57. doi: 10.1016/j.neuroscience.2017.12.037

Gould, E., Vail, N., Wagers, M., and Gross, C. G. (2001). Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10910–10917. doi: 10.1073/pnas.181354698

Hamilton, L. K., Dufresne, M., Joppe, S. E., Petryszyn, S., Aumont, A., Calon, F., et al. (2015). Aberrant lipid metabolism in the forebrain niche suppresses adult neural stem cell proliferation in an animal model of Alzheimer's Disease. *Cell Stem Cell* 17, 397–411. doi: 10.1016/j.stem.2015.08.001

Hwang, I. K., Yoo, K. Y., Li, H., Choi, J. H., Kwon, Y. G., Ahn, Y., et al. (2007). Differences in doublecortin immunoreactivity and protein levels in the hippocampal dentate gyrus between adult and aged dogs. *Neurochem. Res.* 32, 1604–1609. doi: 10.1007/s11064-007-9366-1

Jabes, A., Lavenex, P. B., Amaral, D. G., and Lavenex, P. (2010). Quantitative analysis of postnatal neurogenesis and neuron number in the macaque monkey dentate gyrus. *Eur. J. Neurosci.* 31, 273–285. doi: 10.1111/j.1460-9568.2009.07061.x

Jankowsky, J. L., and Zheng, H. (2017). Practical considerations for choosing a mouse model of Alzheimer's disease. *Mol. Neurodegener.* 12:89. doi: 10.1186/s13024-017-0231-7

Kempermann, G., Gage, F. H., Aigner, L., Song, H., Curtis, M. A., Thuret, S., et al. (2018). Human adult neurogenesis: evidence and remaining questions. *Cell Stem Cell* 23, 25–30. doi: 10.1016/j.stem.2018.04.004

Kempermann, G., Jessberger, S., Steiner, B., and Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* 27, 447–452. doi: 10.1016/j.tins.2004.05.013

Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., et al. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One* 5:e8809. doi: 10.1371/journal.pone.0008809

Komitova, M., and Eriksson, P. S. (2004). Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. *Neurosci. Lett.* 369, 24–27. doi: 10.1016/j.neulet.2004.07.035

La Rosa, C., Ghibaudi, M., and Bonfanti, L. (2019). Newly generated and non-newly generated "Immature" neurons in the mammalian brain: a possible reservoir of young cells to prevent brain aging and disease? *J. Clin. Med.* 8:E685. doi: 10.3390/jcm8050685

Lipp, H. P., and Bonfanti, L. (2016). Adult neurogenesis in mammals: variations and confusions. *Brain Behav. Evol.* 87, 205–221. doi: 10.1159/000446905

Liu, Y. W., Curtis, M. A., Gibbons, H. M., Mee, E. W., Bergin, P. S., Teoh, H. H., et al. (2008). Doublecortin expression in the normal and epileptic adult human brain. *Eur. J. Neurosci.* 28, 2254–2265. doi: 10.1111/j.1460-9568.2008.06518.x

Lucassen, P. J., Toni, N., Kempermann, G., Frisen, J., Gage, F. H., and Swaab, D. F. (2019). Limits to human neurogenesis-really? *Mol. Psychiatry* doi: 10.1038/s41380-018-0337-5 [Epub ahead of print].

Manganas, L. N., Zhang, X., Li, Y., Hazel, R. D., Smith, S. D., Wagshul, M. E., et al. (2007). Magnetic resonance spectroscopy identifies neural progenitor cells in the live human brain. *Science* 318, 980–985. doi: 10.1126/science.1147851

Mathews, K. J., Allen, K. M., Boerrigter, D., Ball, H., Shannon Weickert, C., and Double, K. L. (2017). Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers. *Aging Cell* 16, 1195–1199. doi: 10.1111/acel.12641

Moreno-Jiménez, E. P., Flor-García, M., Terreros-Roncal, J., Rábano, A., Cafini, F., Pallas-Bazarra, N., et al. (2019). Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nat. Med.* 25, 554–560. doi: 10.1038/s41591-019-0375-9

Ninkovic, J., Steiner-Mezzadri, A., Jawerka, M., Akinci, U., Masserdotti, G., Petricca, S., et al. (2013). The BAF complex interacts with Pax6 in adult neural progenitors to establish a neurogenic cross-regulatory transcriptional network. *Cell Stem Cell* 13, 403–418. doi: 10.1016/j.stem.2013.07.002

Patzke, N., Olaleye, O., Haagensen, M., Hof, P. R., Ihunwo, A. O., and Manger, P. R. (2014). Organization and chemical neuroanatomy of the African elephant (*Loxodonta africana*) hippocampus. *Brain Struct. Funct.* 219, 1587–1601. doi: 10.1007/s00429-013-0587-6

Patzke, N., Spocter, M. A., Karlsson, K. A. E., Bertelsen, M. F., Haagensen, M., Chawana, R., et al. (2015). In contrast to many other mammals, cetaceans have relatively small hippocampi that appear to lack adult neurogenesis. *Brain Struct. Funct.* 220, 361–383. doi: 10.1007/s00429-013-0660-1

Paul, A., Chaker, Z., and Doetsch, F. (2017). Hypothalamic regulation of regionally distinct adult neural stem cells and neurogenesis. *Science* 356, 1383–1386. doi: 10.1126/science.aal3839

Pellegrino, G., Trubert, C., Terrien, J., Pifferi, F., Leroy, D., Loyens, A., et al. (2018). A comparative study of the neural stem cell niche in the adult hypothalamus of human, mouse, rat and gray mouse lemur (*Microcebus murinus*). *J. Comp. Neurol.* 526, 1419–1443. doi: 10.1002/cne.24376

Perera, T. D., Dwork, A. J., Keegan, K. A., Thirumangalakudi, L., Lipira, C. M., Joyce, N., et al. (2011). Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates. *PLoS One* 6:e17600. doi: 10.1371/journal.pone.0017600

Petrik, D., Latchney, S. E., Masiulis, I., Yun, S., Zhang, Z., Wu, J. I., et al. (2015). Chromatin remodeling factor Brg1 supports the early maintenance and late responsiveness of nestin-lineage adult neural stem and progenitor cells. *Stem Cells* 33, 3655–3665. doi: 10.1002/stem.2215

Piumatti, M., Palazzo, O., La Rosa, C., Crociara, P., Parolisi, R., Luzzati, F., et al. (2018). Non-Newly generated, "Immature" neurons in the sheep brain are not restricted to cerebral cortex. *J. Neurosci.* 38, 826–842. doi: 10.1523/JNEUROSCI.1781-17.2017

Rakic, P. (1985). Limits of neurogenesis in primates. *Science* 227, 1054–1056. doi: 10.1126/science.3975601

Rodriguez-Perez, L. M., Perez-Martin, M., Jimenez, A. J., and Fernandez-Llebrez, P. (2003). Immunocytochemical characterisation of the wall of the bovine lateral ventricle. *Cell Tissue Res.* 314, 325–335. doi: 10.1007/s00441-003-0794-1

Sanai, N., Nguyen, T., Ihrie, R. A., Mirzadeh, Z., Tsai, H. H., Wong, M., et al. (2011). Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* 478, 382–386. doi: 10.1038/nature10487

Semerci, F., Choi, W. T., Bajic, A., Thakkar, A., Encinas, J. M., Depreux, F., et al. (2017). Lunatic fringe-mediated Notch signaling regulates adult hippocampal neural stem cell maintenance. *eLife* 6:e24660. doi: 10.7554/eLife.24660

Snyder, J. S. (2019). Recalibrating the relevance of adult neurogenesis. *Trends Neurosci.* 42, 164–178. doi: 10.1016/j.tins.2018.12.001

Sorrells, S. F., Paredes, M. F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K. W., et al. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555, 377–381. doi: 10.1038/nature25975

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153, 1219–1227. doi: 10.1016/j.cell.2013.05.002

Tobin, M. K., Musaraca, K., Disouky, A., Shetti, A., Bheri, A., Honer, W. G., et al. (2019). Human hippocampal neurogenesis persists in aged adults and Alzheimer's disease patients. *Cell Stem Cell* 24, 974–982.e3. doi: 10.1016/j.stem.2019.05.003

Walker, T. L., Overall, R. W., Vogler, S., Sykes, A. M., Ruhwald, S., Lasse, D., et al. (2016). Lysophosphatidic acid receptor is a functional marker of adult hippocampal precursor cells. *Stem Cell Rep.* 6, 552–565. doi: 10.1016/j.stemcr.2016.03.002

Wu, M. V., Shamy, J. L., Bedi, G., Choi, C. W., Wall, M. M., Arango, V., et al. (2014). Impact of social status and antidepressant treatment on neurogenesis in the baboon hippocampus. *Neuropsychopharmacology* 39, 1861–1871. doi: 10.1038/npp.2014.33

Yoo, S., and Blackshaw, S. (2018). Regulation and function of neurogenesis in the adult mammalian hypothalamus. *Prog. Neurobiol.* 170, 53–66. doi: 10.1016/j.pneurobio.2018.04.001

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# Long-Term Motor Deficit and Diffuse Cortical Atrophy Following Focal Cortical Ischemia in Athymic Rats

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Development of new stroke therapies requires animal models that recapitulate the pathophysiological and functional consequences of ischemic brain damage over time-frames relevant to the therapeutic intervention. This is particularly relevant for the rapidly developing area of stem cell therapies, where functional replacement of circuitry will require maturation of transplanted human cells over months. An additional challenge is the establishment of models of ischemia with stable behavioral phenotypes in chronically immune-suppressed animals to allow for long-term survival of human cell grafts. Here we report that microinjection of endothelin-1 into the sensorimotor cortex of athymic rats results in ischemic damage with a sustained deficit in function of the contralateral forepaw that persists for up to 9 months. The histological post-mortem analysis revealed chronic and diffuse atrophy of the ischemic cortical hemisphere that continued to progress over 9 months. Secondary atrophy remote to the primary site of injury and its relationship with long-term cognitive and functional decline is now recognized in human populations. Thus, focal cortical infarction in athymic rats mirrors important pathophysiological and functional features relevant to human stroke, and will be valuable for assessing efficacy of stem cell based therapies.

Keywords: diaschisis, staircase test, neurodegeneration, infarction, sensorimotor, stroke

## INTRODUCTION

Pre-clinical development of new therapies for stroke is critically dependent on animal models of ischemic brain damage that produce functional impairments relevant to human stroke outcomes. The stability of these impairments and sensitivity to different behavioral tests can vary widely depending on the nature of the ischemic event, the timing of the testing after damage and the animal species and strain (Carmichael and Chesselet, 2002; Schaar et al., 2010; Trueman et al., 2016). This can have an important baring on the design and interpretation of pre-clinical tests of efficacy for novel therapies. Importantly, functional deficits targeted by new therapies should be stable over a time-course relevant to the therapeutic mechanism and distinguishable from those that resolve spontaneously and independently of any treatment.

This presents particular challenges for stem cell therapies, which aim to restore function in stroke patients through intra-cerebral transplantation of cells that can replace damaged neuronal circuitry. Recent studies using human pluripotent stem cells have shown that grafted neurons require months, rather than weeks, to acquire the mature electrophysiological properties necessary to replace functional neurons (for review see, Thompson and Bjorklund, 2015). Thus transient functional deficits limited to the acute phase after stroke, such as gross motor function assessed by rotarod performance in certain rodent stroke models (Hunter et al., 2000; Zhang et al., 2000; Bouet et al., 2007), are unsuitable as tests of the potential benefit of cell replacement

therapies. Furthermore, preclinical work in this area requires the use of chronically immune-compromised animals in order to prevent rejection of xeno-grafted human cells.

Here we sought to establish a model of ischemia in athymic ('nude') rats resulting in motor deficits that persist over a time frame that is clinically meaningful for assessment of efficacy of human cell-based restorative therapies. We chose a model of focal cortical ischemia induced through local injection of the vasoconstrictor endothelin-1 (ET-1). Previous studies have shown that cortical injection of ET-1 recapitulates important pathophysiological aspects of human ischemia including significant reduction in cortical blood flow, persisting up to 23 h (Schirrmacher et al., 2016), leading to hypoperfused tissue and development of an infarcted area associated with neuronal cell loss (Windle et al., 2006; Nguemeni et al., 2015; Weishaupt et al., 2016).

Behavioral studies in rodents have reported impairment in both motor (Adkins et al., 2004; Gilmour et al., 2004; Soleman et al., 2010) and cognitive function (Cordova et al., 2014; Deziel et al., 2015; Livingston-Thomas et al., 2015) after microinjection of ET-1 into the sensorimotor cortex. Tests of learning and memory and executive function have shown impairment in certain tasks that persist up to 18 weeks after ischemia (Livingston-Thomas et al., 2015). Motor performance has been investigated more extensively, where a number of studies have shown deficits in a range of motor tasks, however, the follow-up time has typically been limited to 2–4 weeks post-ischemia, although impairment in a forelimb reaching task up to 12 weeks post-ischemia has been reported (Gilmour et al., 2004).

The aim of this study was to assess the stability of motor impairment over a significantly longer timeframe and feasibility of the model in immune compromised rats. We also report results of post-mortem histological analysis showing chronically progressive atrophy of the infarcted cortical hemisphere.

## MATERIALS AND METHODS

### Animals

All procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

A total of 58 male athymic (CBH$^{rnu}$) rats at 8 weeks of age were used at the beginning of this study. The animals were group housed in individually ventilated cages with Alpha-dri paper bedding material (Abel Scientific, Perth) to reduce skin and eye irritation and housed on a 12 h light/dark cycle with *ad libitum* access to food and water.

The study design involved the establishment of a group of 44 animals with focal cortical ischemia induced by local injection of ET-1 and a control group of 14 animals injected with saline at the same location. Animals were tested for motor function at 1, 2, 4, 8, 16, 24, and 36 weeks after ET-1/Saline injection. The staircase pellet retrieval test was used as the primary measure and

a subset of animals were also tested for gross motor function on the accelerating rotarod. At the completion of the study at 9 months, we also elected to test forepaw function using the adjusted stepping test. A separate cohort was used for histological analysis at each corresponding time-point. Four animals were also taken at 3 days in order to measure infarct volume. All of the saline injected animals were taken for post-mortem histology at 9 months. Long-term experiments with athymic rats present particular challenges with respect to maintaining the health and well-being of the animals.

Spontaneous development of skin irritation and respiratory complications are not unusual, even in certified clean facilities. This lead us to euthanize 19 animals at various time-points beyond 3 weeks and these were excluded from histological and behavioral analysis. The experimental design is presented below.
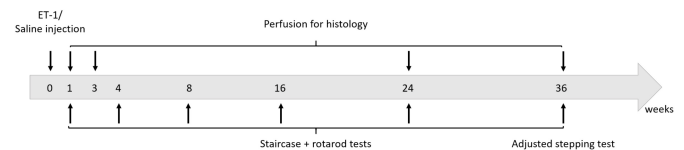


### Endothelin Induced Ischemia

All surgeries were performed under general anesthesia using 3% isoflurane delivered in $O_2$. The rats were fixed in a flat skull position in a stereotaxic frame (Kopf, Germany) and 0.5 µl of either 0.9% sterile saline ($n = 14$) or 800 pmol/µl ET-1 (AusPep, Melbourne) in sterile saline ($n = 44$) was delivered at each of two sites in the frontal cortex (a total of 1 µl delivered) using a glass capillary attached to a 5 µl micro-syringe as previously described (Windle et al., 2006). The stereotaxic co-ordinates were: 0.5 and 2 mm rostral to Bregma; 2.8 mm lateral to Bregma (right hemisphere) and 1.5 mm below the dural surface. The solution was delivered at a rate of 0.5 µl/min. There was consistently reflux of some solution up the cannula and the solution was allowed to sit on the surrounding cortical surface.

### Rotarod Test

Gross motor function was assessed on an accelerating rotarod within a 5 min test period. Before testing, a training period was conducted with one steady session at 16 rpm and two ramping sessions at 4–40 rpm over 5 min with 10 min rest intervals in between each. Testing was conducted with two sessions at 4–40 rpm over 5 min with a 10 min rest interval and the average latency to fall recorded (sec) was recorded. Animals were tested at 1 week and 4, 8, 16, 24, and 36 weeks after injection of saline ($n = 5$) or ET-1 ($n = 7$). All tests were performed blinded to saline or ET-1 treatment.

### Staircase Test

Skilled forepaw use was assessed using the staircase test originally described by Montoya et al. (1991) and modified by Winkler et al. (1999). Briefly, the animals were placed in a staircase apparatus (Campden Instruments, United Kingdom) in a dark room where each forepaw had unilateral access to sugar pellets (35 mm, Able Scientific, Canning Vale) positioned on an ascending set of steps. Ten pellets were placed on each of steps 2–6 for a

total of 50 accessible pellets per forelimb. The total number of pellets consumed was scored for each forelimb over a 20 min test period. All animals were placed on a food-restricted diet such that weight during the test period was 80–90% of the pre-test free-feeding weight. A training period was required to achieve a stable level of performance for the unimpaired forelimb (contralateral to saline/ET-1 injection) so that animals were tested once a day over 7–10 days. Animals that were not able to retrieve a minimum of 20 pellets with the unimpaired forelimb were not included for further testing. The number of pellets consumed was recorded as the average performance over the last 3 days of testing. The first test was initiated 4 days after surgery and completed by 2 weeks post-surgery (represented as '2 week' time-point, **Figure 1**). Animals were again tested at 4, 8, 16, 24, and 36 weeks – for these later time-points the weeks indicate the initiation of testing. All tests were performed blinded to saline ($n = 8$) or ET-1 treatment ($n = 18$).

## Adjusting Stepping

This test was only included at the final, long-term time-point of 36 weeks post-surgery as an additional measure of motor function. Based on procedures originally described by Schallert et al. (1979) and modified by Olsson et al. (1995) and Winkler et al. (1999), rats were assessed for their ability to make stepping adjustments to a weight-bearing forelimb as it is moved laterally along a smooth surface. Rats were held by the experimenter so that one forelimb was allowed to make weight-bearing contact with the bench and the rats were moved laterally in both directions (forehand and backhand) over a 1 m distance. This was repeated for each forelimb and the number of adjustment steps was recorded. This test required a training period for stable performance by the unimpaired forelimb. Rats were tested twice per day for 7–10 consecutive days and the final performance reported is the average score over the last 2 days of testing (4 sessions). All tests were performed blinded to saline ($n = 6$) or ET-1 treatment ($n = 7$).

## Tissue Processing and Histology

Animals injected with ET-1 were taken for histological assessment at 1, 3, 24, and 36 weeks after surgery. The saline group was taken at 36 weeks. Animals received an overdose of pentobarbitone (100 mg/kg) and were perfused with 50 ml of phosphate buffered saline followed by 250 ml of paraformaldehyde (4% w/v in 0.1M PBS) via transcardiac perfusion. The brains were post-fixed for 2 h in 4% paraformaldehyde and cryo-protected overnight in sucrose (30% w/v in 0.1M PBS) before sectioning on the coronal plane using a freezing microtome (Leica). Sections were collected in 12 series at a thickness of 40 μm. Immunohistochemical detection of NeuN was performed on free-floating sections as previously described in Thompson et al. (2005). Briefly, the sections were incubated overnight with the primary antibody (NeuN, raised in mouse, Millipore) diluted 1:200 in 0.1M PBS with 0.5% Triton X-100 (Amereso, United States) and 5% normal donkey serum (NDS). After washed in PBS the sections were blocked with 2% NDS for 15 min and then incubated for 2 h with biotinylated-donkey-anti-mouse secondary (Jackson labs) diluted 1:400 in PBS with

0.5% TritonX-100 and 2% NDS. The sections were again washed in PBS before incubation with a streptavidin-peroxidase complex (VECTASTAIN ABC system, Vector Labs) for 1 h. Detection of the peroxidase labeled antibody complex was via $H_2O_2$ catalyzed precipitation of the diaminobenzidine (DAB) chromagen. The DAB-labeled sections were dehydrated in alcohol and xylene, and cover-slipped with DePex mounting medium (BDH Chemicals, United Kingdom).

## Quantification of Cortical Volume

A 1:12 series of sections immuno-labeled for NeuN were used to quantify cortical volumes in saline injected animals at 36 weeks and in ET-1 injected animals at 1, 3, 24, and 36 weeks. A Leica (DM6000) microscope equipped with a motorized X–Y stage was used to capture photomontages of whole coronal sections. Cortical area was measured in each hemisphere of every consecutive section beginning 1.7 mm rostral and extending 0.7 mm caudal to Bregma (6 sections). Cortical volumes were calculated from the sum of the area, the section thickness and interval according to the principle of Cavalieri (1966).

## Stereological Quantification of Cortical Neurons

To assess neuronal density in the cortex, the number of NeuN labeled cells were estimated in a defined region of interest immediately adjacent to the infarcted area (lateral and ventral, see boxed area shown in **Figure 2A**) in order to avoid the vacuous tissue associated with the infarction, which was associated with a high degree of non-specific labeling at the earlier time-points after ET-1 injection. Two sections were used corresponding to approximately 0.78 and 1.74 mm rostral to Bregma. A stereological counting approach with systematic random sampling within the region of interest according to optical dissector rules (Gundersen et al., 1988; Mayhew, 1991) was used to estimate total NeuN cell numbers in that region. Counting frame grid dimensions and fractionator x, y coordinates were determined using the grid overlay program (Stereoinvestigator v7.0, MicroBrightField, Williston, VT, United States). Guard zones were set at 1 μm (top and bottom) and NeuN-labeled nuclei quantified within the counting frame (dimensions used were 40 μm × 40 μm) at periodic intervals (x = 200 μm, y = 200 μm) in the delineated region of interest. Tissue volume was calculated according to the principle of Cavalieri within the Steroinvestigator software in order to determine the density of NeuN cells. The accuracy of the stereological estimations was determined by the coefficients of error and coefficients of variance. Estimations were deemed acceptable if coefficients were > 0.1 (West et al., 1991).

## Statistical Analysis

Unless stated otherwise, all quantitative data is expressed as the mean ± SEM of the mean and an alpha value of < 0.05 was used to define statistical significance. Prism software was used to determine statistical differenced in the means between groups.

Comparison of motor performance between the control and ET-1 treated groups was performed by two-way ANOVA
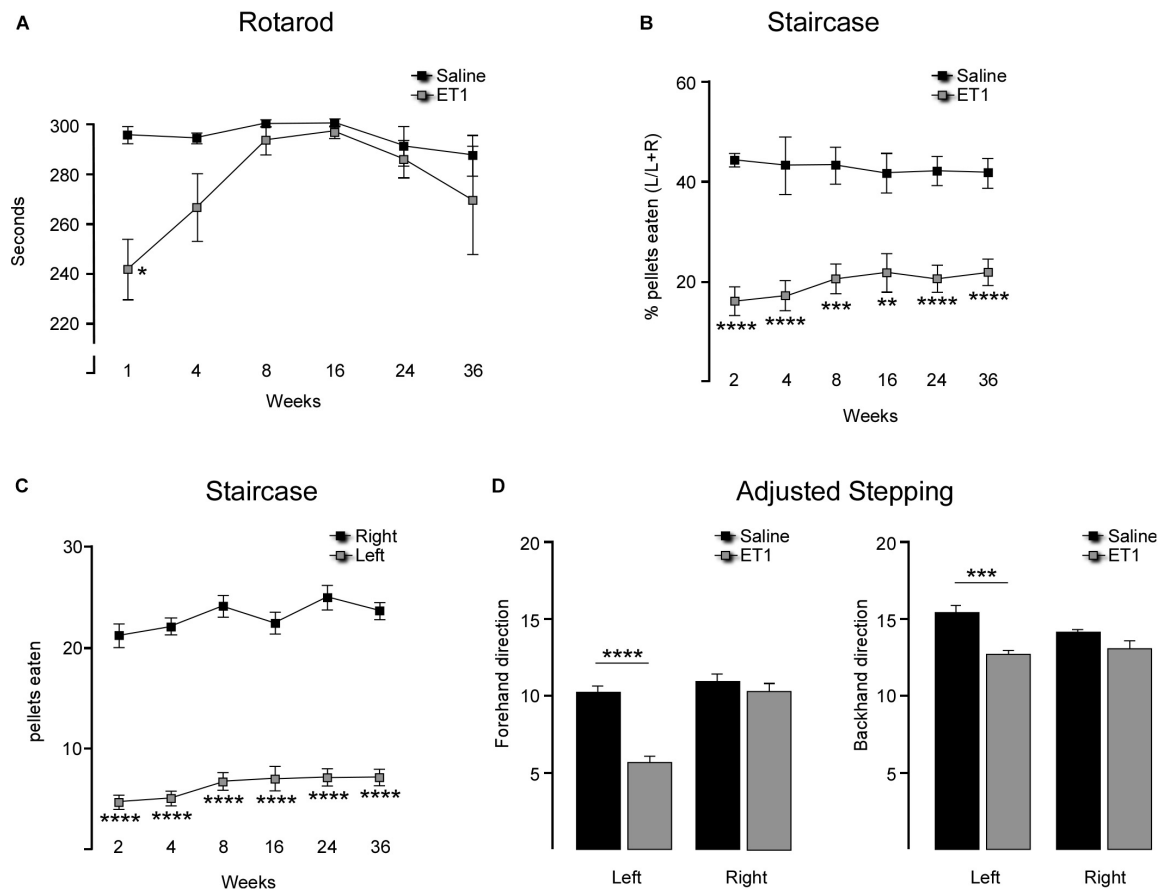
**FIGURE 1 |** Longitudinal comparison of motor performance in rats injected with saline or ET-1. **(A)** Mean latency to fall (±SEM) on the accelerating rotarod was significantly shorter for ET-1 treated animals ($n = 7$) at 1 week but not significantly different from saline controls ($n = 6$) from 4 weeks and later [two way ANOVA with Sidak's multiple comparison: Interaction factor $F_{5,63} = 1.817$, $p = 0.111$, Time factor $F_{5,63} = 2.536$, $p = 0.0373$, Group factor $F_{1,63} = 10.42$, $p = 0.002$; $t(63) = 3.829$; **$p < 0.0018$]. **(B)** Successful pellet retrieval using the forelimb contralateral to the injected hemisphere (left forelimb) was significantly lower for ET-1 treated animals ($n = 18$) compared to the saline controls ($n = 8$) at all time-points tests up to 36 weeks (two-way ANOVA with Sidak's multiple comparison: Interaction factor $F_{5,121} = 0.5086$, $p = 0.7693$, Time factor $F_{5,121} = 0.1052$, $p = 0.9909$, Group factor $F_{1,121} = 129.6$; $p < 0.0001$; **$p = 0.0012$, ***$p < 0.001$, ****$p < 0.0001$). **(C)** Total pellets retrieved with each forelimb shows a significant reduction in skilled use of the left forelimb in ET-1 animals ($n = 18$) compared to the saline controls ($n = 8$) at all time-points tests up to 36 weeks (two-way ANOVA with Sidak's multiple comparison: Interaction factor $F_{5,194} = 0.3998$, $p = 0.8485$, Time factor $F_{5,194} = 3.171$, $p = 0.0089$, Group factor $F_{1,194} = 911.6$, $p < 0.0001$; ****$p < 0.001$). **(D)** At the 36 week time-point, ET-1 treated animals ($n = 7$) displayed a significant reduction in their capacity to make adjusted, weight-baring steps with the left forelimb compared saline controls ($n = 7$) in both the backhand and forehand directions ($t$-test for each forelimb; ***$p = 0.0002$; ****$p < 0.0001$) while there was no difference in performance for the right forelimb between treatment groups. All data shown as the group mean ± SEM.

and the Holm-Sidak method was used to correct for multiple comparisons. For comparison of atrophy between the sham group and ET-1 group at multiple time-points and at each Bregma point, one-way ANOVA with Dunnett's test for multiple comparisons and two-way ANOVA were performed. Only differences between treatment groups at each individual Bregma point were assessed.

# RESULTS

## Motor Function

Animals were tested for gross motor co-ordination using an accelerating rotarod. One week after surgery the ET-1 treated animals showed a significant impairment in their capacity to

remain on the rotarod (**Figure 1A** and **Table 1**). This resolved by 4 weeks such that the rotarod performance was not significantly different from the saline treated animals for the remainder of the study (**Table 1**).

Staircase testing showed that the ET-1 treated animals were significantly impaired in skilled use of the forepaw contralateral to the injected hemisphere relative to the saline treated group (**Figures 1B,C**). This was evident at the end of the first 10 day testing period (2 weeks after surgery) and was maintained throughout all later testing periods initiated at 4, 8, 16, 24, and 36 weeks. The saline treated animals retrieved similar amounts of pellets with both the right (ipsilateral to injected hemisphere) and left (contralateral) forelimbs at all time-points, while the ET-1 treated animals only retrieved ~20% of the total pellets retrieved using the contralateral forelimb (**Figure 1B**) (**Table 1**).

**TABLE 1 |** Statistical details for behavior tests.

| Test | Time points, weeks | Saline Mean ± SEM | ET-1 Mean± SEM | |
|---|---|---|---|---|
| Rotarod | 1 | 295.7 ± 3.28s | 241.8 ± 12s | $t(11) = 4.025, p = 0.002$ |
| | 4 | 294.3 ± 1.91s | 266.5 ± 13.52s | $t(11) = 1.875, p = 0.088$ |
| | 8 | 300 ± 0s | 293.4 ± 5.69s | $t(11) = 1.062, p = 0.31$ |
| | 16 | 300 ± 0s | 296.5 ± 2.36s | $t(10) = 1.221, p = 0.25$ |
| | 24 | 291.2 ± 7.88s | 286 ± 7.37s | $t(11) = 0.476, p = 0.64$ |
| | 36 | 287.5 ± 8.12s | 269.5 ± 21.63s | $t(9) = 0.5995, p = 0.56$ |
| Staircase % pellets eaten (L/L+R) | 2 | 44.38 ± 1.16 | 16.94 ± 2.11 | $t(21) = 6.676, p < 0.0001$ |
| | 4 | 43.22 ± 5.7 | 18.04 ± 2.24 | $t(21) = 4.874, p < 0.0001$ |
| | 8 | 43.26 ± 3.7 | 21.37 ± 2.20 | $t(21) = 4.731, p < 0.001$ |
| | 16 | 41.72 ± 3.98 | 22.58 ± 3.1 | $t(21) = 3.019, p = 0.0065$ |
| | 24 | 42.16 ± 2.87 | 21.4 ± 1.94, | $t(21) = 5.182, p < 0.0001$ |
| | 36 | 41.66 ± 2.93 | 22.68 ± 1.88 | $t(16) = 5.356, p < 0.0001$ |
| | | **Right side** | **Left side** | |
| Staircase pellets eaten | 2 | 21.2 ± 1.15 | 4.7 ± 0.70 | $t(34) = 12.13, p < 0.0001$ |
| | 4 | 22.1 ± 0.70 | 5.1 ± 0.70 | $t(34) = 17.07, p < 0.0001$ |
| | 8 | 24.1 ± 1.07 | 6.8 ± 0.83 | $t(34) = 12.75, p < 0.0001$ |
| | 16 | 22.4 ± 1.06 | 7.1 ± 1.16 | $t(34) = 9.747, p < 0.0001$ |
| | 24 | 24.9 ± 1.19 | 7.2 ± 0.84 | $t(34) = 12.18, p < 0.0001$ |
| | 36 | 23.6 ± 0.75 | 7.2 ± 0.77 | $t(24) = 15.29, p < 0.0001$ |
| Adjusted stepping | Forehand left | 10.27 ± 0.4 | 5.671 ± 0.42 | $t(12) = 7.966, p < 0.0001$ |
| | Forehand right | 10.8 ± 0.5944 | 10.27 ± 0.4799 | $t(12) = 0.697, p = 0.4994$ |
| | Backhand left | 15.2 ± 0.4072 | 12.49 ± 0.3247 | $t(12) = 5.205, p = 0.0002$ |
| | Backhand right | 15.14 ± 0.1956 | 14.01 ± 0.4638 | $t(12) = 1.891, p = 0.0830$ |

This equated to on average ∼4–5 pellets compared to > 20 pellets retrieved using the ipsilateral forelimb across all time-points (**Figure 1C** and **Table 1**). There was no significant difference in the average number of ipsilateral and contralateral pellet retrievals in the saline injected group at any time-point (not shown).

At the final 36-week time-point for motor testing, we elected to include adjusted stepping as an additional test of forelimb use. Compared to the saline control group, animals treated with ET-1 were significantly impaired in their ability to adjust the placement of a weight-baring limb in order to maintain balance as the limb was moved laterally across a smooth surface in the backhand or forehand direction (**Figure 1D** and **Table 1**).

## Changes in Brain Volume

Histological analysis 3 days after ET-1 delivery allowed us to calculate the size of the initial cortical infarction, defined as complete absence of NeuN + neurons, as $(9.0 ± 2.7 \text{ mm}^3; n = 4)$. The remaining ET-1 treated animals were taken for histological assessment at 1, 3, 24, and 36 weeks after injection in order to quantify changes in cortical volume over time. Representative coronal sections immuno-labeled for NeuN at each time-point illustrate the cyto-architectural changes over time (**Figure 2A**). At the early 1-week time-point an infarcted area around the injected cortical site was clearly apparent, including vacuous tissue architecture characterized by NeuN + cell loss with areas resembling necrosis and edema. The NeuN cell loss extended into the dorsal aspect of the underlying striatum. By 3 weeks the overtly necrotic, infarcted area had largely resolved in most

animals and there was gross morphological evidence for glial scarring around the injection site. This persisted at the later 24 and 36-week time-points where there were persistent areas of scarring, including NeuN + cell loss in deep cortical layers proximal to the corpus callosum.

Quantification of total cortical volume between +1.7 to −0.7 mm relative to bregma showed no changed in the ischemic hemisphere relative to saline controls at the early 1 week time-point, but a significant level of global atrophy from 3 weeks that progressed further to 24 and 36 weeks (**Figure 2B**). Cortical volume analyzed with one way ANOVA, $F_{8,41} = 16.87, p < 0.001$ and Dunnett's multiple comparison test: Saline mean ± SEM = 58.98 ± 1.67; 3 weeks ET1 ipsilateral mean ± SEM = 48.67 ± 3.05, p = 0.0177; 24 weeks ET1 ipsilateral mean ± SEM = 43.63 ± 2.95, p = 0.0001; 36 weeks ET1 ipsilateral mean ± SEM = 46.72 ± 2.03, p = 0.0003. Interestingly, there was a progressive increase in contralateral cortical volume, which reached significance compared to saline controls by 36 weeks (36 weeks ET1 contralateral mean ± SEM = 68.7 ± 2.49, p = 0.0046) (**Figure 2B**).

Representation of cortical volume in the ipsilateral hemisphere as a percentage of saline injected animals highlighted a volumetric loss of 26.03 ± 4.99% (p = 0.0021) and 20.8 ± 3.44% (p = 0.0069) hemispheric volume at the 24 and 36 time-points, respectively (**Figure 2C**). Inspection of cortical area at specific coronal levels revealed a significant loss in area at later time-points that extended well-beyond the initial injury site to include the most rostral and caudal sections examined (**Figure 2D**); two way ANOVA, time factor:

**FIGURE 2 |** Progressive cortical atrophy after focal ischemia. **(A)** Representative coronal sections labeled for NeuN illustrate gross morphology for a saline injected animal and 1, 3, 24, and 36 weeks after ET-1 injection (note enlarged versions of the boxed area are illustrated in **Figure 3**). **(B)** Total hemispheric cortical volume (bregma −2.1 to +2.7) in saline injected animals ($n = 8$, pooled data for contralateral and ipsilateral hemispheres shown) and ET-1 animals 1 ($n = 4$), 3 ($n = 4$), 24 ($n = 5$) and 36 ($n = 8$) weeks after ET-1 injection, showing a significant loss of volume in the ipsilateral cortex at 24 and 36 weeks (one way ANOVA, $F_{8,41} = 16.87$, $p < 0.001$ with Dunnett's multiple comparisons tests; $^*p = 0.0177$; $^{**}p = 0.0046$; $^{***}p = 0.0003$; $^{****}p = 0.0001$). **(C)** Progression of ipsilateral cortical atrophy over time, expressed as% atrophy relative to saline treated animals, shows significant atrophy at 24 and 36 weeks (one-way ANOVA $F_{4,20} = 5.65$, $p = 0.0033$, Dunnet's multiple comparison test; $^{**}p < 0.01$). **(D)** Ipsilateral cortical area at specific rostra-caudal levels shows that compared to saline injected animals there was a significant reduction in area initially closest to the ET-1 injection site and then extending to more distal rostro-caudal levels at later time-points [two-way ANOVA with a significance with time $F_{4,144} = 28.83$, $p < 0.0001$; saline ($n = 8$), ET-1 3 weeks ($n = 4$), ET-1 24 weeks ($n = 5$), ET-1 36 weeks ($n = 8$); $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.001$]. All data shown as the group mean ± SEM. Scale bar: **(A)** 500 μm.

**FIGURE 3 |** Cortical neuronal cell density does not change in areas of atrophy. **(A)** Representative images of immune-labeling for NeuN in a cortical area adjacent to the injection of saline or 1, 3, 24, or 36 weeks after ET-1 injection. **(B)** Counting of NeuN + cells within a defined area showed that cortical neuronal density was not significantly different in atrophied cortical regions after ischemia [one-way ANOVA $F_{4,19}$ = 3.059, $p$ = 0.042, Dunnett's *post hoc*; saline ($n$ = 6), ET-1 1 week ($n$ = 4), ET-1 3 weeks ($n$ = 4), ET-1 24 weeks ($n$ = 5), ET-1 36 weeks ($n$ = 5)]. Scale bar: **(A)** 500 μm.
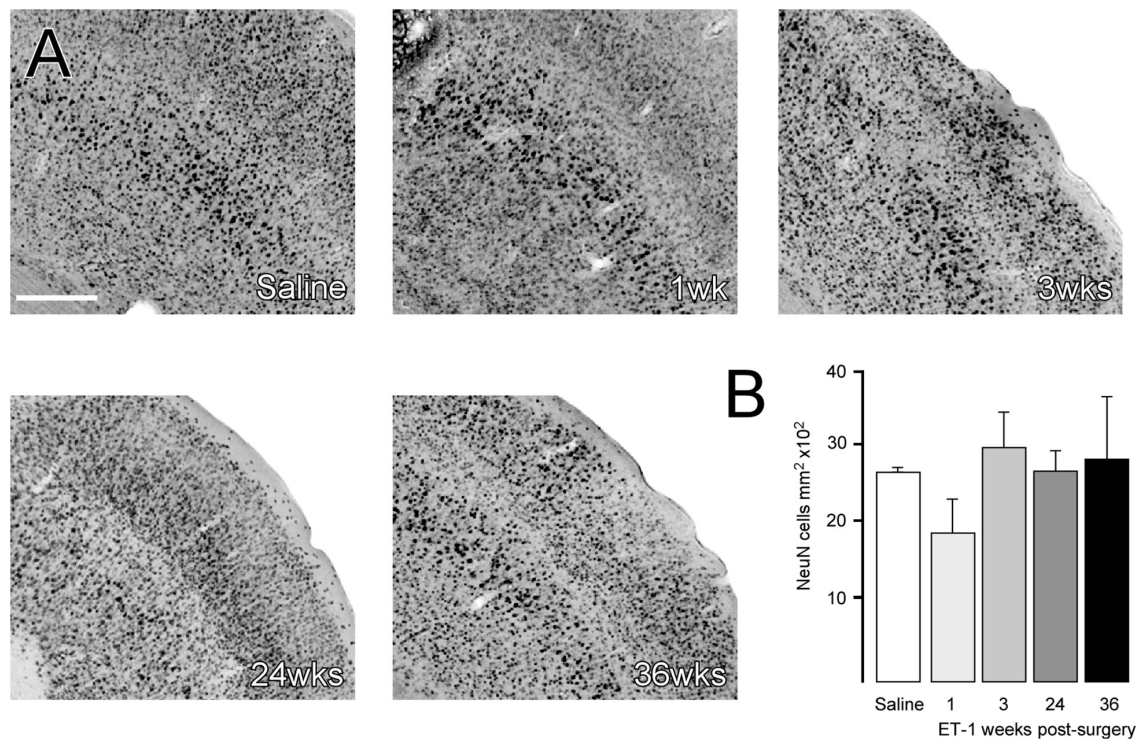
$F_{4,144}$ = 28.83, $p$ < 0.0001 and multiple comparison at each section distal to Bregma: at 1.7 mm: Saline mean ± SEM: 19.91 ± 0.6, 36 week ET-1 mean ± SEM = 16.28 ± 0.95, $p$ = 0.0342; at 1.22 mm: Saline mean ± SEM: 21.39 ± 0.51, 24 weeks ET-1 mean ± SEM = 15.36 ± 0.41, $p$ = 0.0004; 36 weeks ET-1 mean ± SEM = 15.23 ± 1.21, $p$ < 0.0001; at 0.74 mm: Saline mean ± SEM: 21.27 ± 0.48, 24 weeks ET-1 mean ± SEM = 14.56 ± 1.64, $p$ < 0.0001, 36 weeks ET-1 mean ± SEM = 16.68 ± 0.92, $p$ = 0.0032; at 0.26 mm: Saline mean ± SEM: 20.53 ± 0.52, 24 weeks ET-1 mean ± SEM = 14.65 ± 1.58, $p$ = 0.0006; and at −0.7 mm: Saline mean ± SEM: 21.46 ± 0.34, $p$ = 0.0490, 24 weeks ET-1 mean ± SEM = 14.49 ± 1.43, $p$ < 0.0001, 36 weeks ET-1 mean 36 week ± SEM = 15.15 ± 0.89, $p$ < 0.0001).

To determine if changes in brain volume were associated with neuronal loss, stereological counting of NeuN-labeled cortical neurons was performed to determine neuronal density. The area quantified is indicated as a boxed region in the coronal section from the saline group in **Figure 2A** and representative higher magnification images from each group are shown in **Figure 3A**. The results showed that neuronal density in the cortical area immediately adjacent to the infarcted area was not significantly different from saline treated animals at any time-point, despite what appeared to be a small reduction at the 1-week time-point (**Figure 3B**) [one way ANOVA $F_{4,19}$ = 3.059, $p$ = 0.042. Dunnett's multiple

comparisons tests: NeuN density: Saline mean ± SEM = 2687 ±, $n$ = 6; ET-1 1 week mean ± SEM = 1896 ±, $n$ = 4; ET-1 3 weeks mean ± SEM = 2995 ±, $n$ = 4; ET-1 24 weeks mean ± SEM = 2688 ±, $n$ = 5; ET-1 36 weeks mean ± SEM = 2852 ±, n-5; $p$-value (saline − ET-1 1 week) = 0.0708; $p$-value (saline − ET-1 3 week) = 0,7365; $p$-value (saline − ET-1 24 week) = 0.999; $p$-value (saline − ET-1 36 week) = 0.9486].

# DISCUSSION

These results show that focal ischemic damage to the frontal cortex through injection of ET-1 can result in impairment of forelimb function that persists for up to 9 months. Gross motor co-ordination, assessed on the accelerating rotarod, was significantly impaired in the acute phase, 1 week after ischemia, but recovered to be similar to control levels at subsequent testing beyond 4 weeks. This is consistent with other pre-clinical studies in various rodent stroke models reporting rapid and sometimes complete recovery in rotarod performance between 4 and 14 days after ischemia (Hunter et al., 2000; Zhang et al., 2000; Bouet et al., 2007). In the first 3 months following human stroke, significant recovery of gross motor function is observed, but patients often report sustained deficits in fine motor function, especially in the

upper limb (Jorgensen et al., 1995; Rothrock et al., 1995; Kreisel et al., 2007).

Tests of skilled motor function, particularly paw reaching tasks including the staircase test, have been shown to be more sensitive for detection of motor impairments that persist beyond the spontaneous recovery phase in both mouse (Bouet et al., 2007; Balkaya et al., 2013; Roome et al., 2014) and rat (Gilmour et al., 2004; Windle et al., 2006; Soleman et al., 2010; Trueman et al., 2016) models. The study by Gilmour et al. (2004) has the longest follow up period post-stroke, reporting persistent impairment in a forelimb-reaching task 12 weeks after injection of ET-1 into the sensorimotor cortex. Here we report that impairments of forelimb use in both the staircase and lateralized stepping tests are present up to 9 months after focal cortical ischemia. This is significant for the development of cell replacement based therapies, where therapeutic impact may manifest after months rather than weeks (for review see, Thompson and Bjorklund, 2015). Importantly also in this context, these results were obtained in athymic rats, which allow for the long-term survival of xeno-grafted human cells without immune-rejection.

A conspicuous feature of the histological analysis was the chronically progressive nature of cortical atrophy. The cortical atrophy was extensive and remote from brain infarct site, progressively extending throughout the ipsilesional hemisphere and continuing to progress between the 3 week and 6-month time-points. Counting of NeuN + cell density in the peri-infarct area revealed an initial drop in density at 1 week, while density at the later time-points was similar to saline injected cortex. This suggests *neuronal cell loss* as an underlying feature of the progressive atrophy, rather than a more passive re-organization and shrinkage of the extra-cellular compartment, which would necessarily result in increased neuronal density.

Diffuse and progressive atrophy beyond the penumbra region is increasingly becoming recognized as an important feature of stroke pathophysiology. This is particularly evident from recent clinical imaging studies. Seghier et al. (2014) report progression of 'whole brain' atrophy that persists for years after ischemia, and is accelerated in the ipsilesional hemisphere based on T1-weighted imaging of 56 patients 2 months to 6 years after stroke. Other studies suggest patterns of connectivity between remote nuclei and the primary site of damage may well be a major determinant of seemingly diffuse and chronic secondary degeneration. For example, thinning of remote cortical areas has been linked to secondary degeneration within associated white matter tracts of connectivity with the site of infarction (Cheng et al., 2015; Duering et al., 2015). This was also reported in a recent study in rats where ET-1 induced focal ischemia in prefrontal cortex resulted in a pattern of secondary inflammation and white matter damage that matched well with anatomical connectivity to the infarcted area (Weishaupt et al., 2016). In patients, contralesional thalamic volume has also been shown as significantly reduced, with the degree of atrophy proportional to the severity of stroke (Brodtmann et al., 2012; Yassi et al., 2015). The relationship between the development of dementia after stroke and progression of atrophy in brain regions remote to the site of damage is becoming an increasingly important area (Sun et al., 2014). Interestingly, the increased contralateral cortical volume seen here has also been reported in clinical imaging studies (Brodtmann et al., 2012) and may represent a homeostatic, compensatory response.

In summary, we report here that a focal model of cortical ischemia in athymic rats recapitulates important aspects of human stroke relevant for the development of therapies. The stable nature of motor deficit over 9 months on an athymic background forms a valuable model for the assessment of human cell based therapies, where therapeutic impact related to functional integration of mature neurons will likely take months, rather than weeks. Furthermore, the progressive cortical atrophy is in line with findings from recent clinical imaging studies and may be important substrates for stroke-related dementia. Despite the gathering clinical data, this phenomenon has not been well-captured and described in animal models. The utilization of focal ischemic injury models will allow for a systematic approach to developing a better understanding of the relationship between primary sites of injury, the pattern of subsequent secondary degeneration and functional impact, and opportunities for therapeutic intervention, including both protective and regenerative cell-based therapies.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

## AUTHOR CONTRIBUTIONS

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# REFERENCES

Adkins, D. L., Voorhies, A. C., and Jones, T. A. (2004). Behavioral and neuroplastic effects of focal endothelin-1 induced sensorimotor cortex lesions. *Neuroscience* 128, 473–486. doi: 10.1016/j.neuroscience.2004.07.019

Balkaya, M., Krober, J. M., Rex, A., and Endres, M. (2013). Assessing post-stroke behavior in mouse models of focal ischemia. *J. Cereb. Blood Flow Metab.* 33, 330–338. doi: 10.1038/jcbfm.2012.185

Bouet, V., Freret, T., Toutain, J., Divoux, D., Boulouard, M., Schumann-Bard, P., et al. (2007). Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. *Exp. Neurol.* 203, 555–567. doi: 10.1016/j.expneurol.2006.09.006

Brodtmann, A., Pardoe, H., Li, Q., Lichter, R., Ostergaard, L., and Cumming, T. (2012). Changes in regional brain volume three months after stroke. *J. Neurol. Sci.* 322, 122–128. doi: 10.1016/j.jns.2012.07.019

Carmichael, S. T., and Chesselet, M. F. (2002). Synchronous neuronal activity is a signal for axonal sprouting after cortical lesions in the adult. *J. Neurosci.* 22, 6062–6070. doi: 10.1523/jneurosci.22-14-06062.2002

Cavalieri, B. (1966). *Geometric degl: Indivisible*. Turin: Unione Tipografico-Editrice.

Cheng, B., Schulz, R., Bonstrup, M., Hummel, F. C., Sedlacik, J., Fiehler, J., et al. (2015). Structural plasticity of remote cortical brain regions is determined by connectivity to the primary lesion in subcortical stroke. *J. Cereb. Blood Flow Metab.* 35, 1507–1514. doi: 10.1038/jcbfm.2015.74

Cordova, C. A., Jackson, D., Langdon, K. D., Hewlett, K. A., and Corbett, D. (2014). Impaired executive function following ischemic stroke in the rat medial prefrontal cortex. *Behav. Brain Res.* 258, 106–111. doi: 10.1016/j.bbr.2013.10.022

Deziel, R. A., Ryan, C. L., and Tasker, R. A. (2015). Ischemic lesions localized to the medial prefrontal cortex produce selective deficits in measures of executive function in rats. *Behav. Brain Res.* 293, 54–61. doi: 10.1016/j.bbr.2015.07.003

Duering, M., Righart, R., Wollenweber, F. A., Zietemann, V., Gesierich, B., and Dichgans, M. (2015). Acute infarcts cause focal thinning in remote cortex via degeneration of connecting fiber tracts. *Neurology* 84, 1685–1692. doi: 10.1212/WNL.0000000000001502

Gilmour, G., Iversen, S. D., O'Neill, M. F., and Bannerman, D. M. (2004). The effects of intracortical endothelin-1 injections on skilled forelimb use: implications for modelling recovery of function after stroke. *Behav. Brain Res.* 150, 171–183. doi: 10.1016/j.bbr.2003.07.006

Gundersen, H. J., Bagger, P., Bendtsen, T. F., Evans, S. M., Korbo, L., Marcussen, N., et al. (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 96, 857–881. doi: 10.1111/j.1699-0463.1988.tb00954.x

Hunter, A. J., Hatcher, J., Virley, D., Nelson, P., Irving, E., Hadingham, S. J., et al. (2000). Functional assessments in mice and rats after focal stroke. *Neuropharmacology* 39, 806–816. doi: 10.1016/s0028-3908(99)00262-2

Jorgensen, H. S., Nakayama, H., Raaschou, H. O., Vive-Larsen, J., Stoier, M., and Olsen, T. S. (1995). Outcome and time course of recovery in stroke. Part II: time course of recovery. The copenhagen stroke study. *Arch. Phys. Med. Rehabil.* 76, 406–412. doi: 10.1016/s0003-9993(95)80568-0

Kreisel, S. H., Hennerici, M. G., and Bazner, H. (2007). Pathophysiology of stroke rehabilitation: the natural course of clinical recovery, use-dependent plasticity and rehabilitative outcome. *Cerebrovasc. Dis.* 23, 243–255. doi: 10.1159/000098323

Livingston-Thomas, J. M., Jeffers, M. S., Nguemeni, C., Shoichet, M. S., Morshead, C. M., and Corbett, D. (2015). Assessing cognitive function following medial prefrontal stroke in the rat. *Behav. Brain Res.* 294, 102–110. doi: 10.1016/j.bbr.2015.07.053

Mayhew, T. M. (1991). The new stereological methods for interpreting functional morphology from slices of cells and organs. *Exp. Physiol.* 76, 639–665. doi: 10.1113/expphysiol.1991.sp003533

Montoya, C. P., Campbell-Hope, L. J., Pemberton, K. D., and Dunnett, S. B. (1991). The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. *J. Neurosci. Methods* 36, 219–228. doi: 10.1016/0165-0270(91)90048-5

Nguemeni, C., Gomez-Smith, M., Jeffers, M. S., Schuch, C. P., and Corbett, D. (2015). Time course of neuronal death following endothelin-1 induced focal ischemia in rats. *J. Neurosci. Methods* 242, 72–76. doi: 10.1016/j.jneumeth.2015.01.005

Olsson, M., Nikkhah, G., Bentlage, C., and Bjorklund, A. (1995). Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *J. Neurosci.* 15(5 Pt 2), 3863–3875. doi: 10.1523/jneurosci.15-05-03863.1995

Roome, R. B., Bartlett, R. F., Jeffers, M., Xiong, J., Corbett, D., and Vanderluit, J. L. (2014). A reproducible Endothelin-1 model of forelimb motor cortex stroke in the mouse. *J. Neurosci. Methods* 233, 34–44. doi: 10.1016/j.jneumeth.2014.05.014

Rothrock, J. F., Clark, W. M., and Lyden, P. D. (1995). Spontaneous early improvement following ischemic stroke. *Stroke* 26, 1358–1360. doi: 10.1161/01.str.26.8.1358

Schaar, K. L., Brenneman, M. M., and Savitz, S. I. (2010). Functional assessments in the rodent stroke model. *Exp. Transl. Stroke Med.* 2:13. doi: 10.1186/2040-7378-2-13

Schallert, T., De Ryck, M., Whishaw, I. Q., Ramirez, V. D., and Teitelbaum, P. (1979). Excessive bracing reactions and their control by atropine and L-DOPA in an animal analog of Parkinsonism. *Exp. Neurol.* 64, 33–43. doi: 10.1016/0014-4886(79)90003-7

Schirrmacher, R., Dea, M., Heiss, W. D., Kostikov, A., Funck, T., Quessy, S., et al. (2016). Which aspects of stroke do animal models capture? A multitracer Micro-PET study of focal ischemia with Endothelin-1. *Cerebrovasc. Dis.* 41, 139–147. doi: 10.1159/000442997

Seghier, M. L., Ramsden, S., Lim, L., Leff, A. P., and Price, C. J. (2014). Gradual lesion expansion and brain shrinkage years after stroke. *Stroke* 45, 877–879. doi: 10.1161/STROKEAHA.113.003587

Soleman, S., Yip, P., Leasure, J. L., and Moon, L. (2010). Sustained sensorimotor impairments after endothelin-1 induced focal cerebral ischemia (stroke) in aged rats. *Exp. Neurol.* 222, 13–24. doi: 10.1016/j.expneurol.2009.11.007

Sun, J. H., Tan, L., and Yu, J. T. (2014). Post-stroke cognitive impairment: epidemiology, mechanisms and management. *Ann. Transl. Med.* 2:80. doi: 10.3978/j.issn.2305-5839.2014.08.05

Thompson, L., Barraud, P., Andersson, E., Kirik, D., and Björklund, A. (2005). Identification of dopaminergic neurons of nigral and ventral tegmental area subtypes in grafts of fetal ventral mesencephalon based on cell morphology, protein expression, and efferent projections. *J. Neurosci. Jul.* 25, 6467–6477. doi: 10.1523/JNEUROSCI.1676-05.2005

Thompson, L. H., and Bjorklund, A. (2015). Reconstruction of brain circuitry by neural transplants generated from pluripotent stem cells. *Neurobiol. Dis.* 79, 28–40. doi: 10.1016/j.nbd.2015.04.003

Trueman, R. C., Diaz, C., Farr, T. D., Harrison, D. J., Fuller, A., Tokarczuk, P. F., et al. (2016). Systematic and detailed analysis of behavioural tests in the rat middle cerebral artery occlusion model of stroke: tests for long-term assessment. *J. Cereb. Blood Flow Metab.* 37, 1349–1361. doi: 10.1177/0271678X16654921

Weishaupt, N., Zhang, A., Deziel, R. A., Tasker, R. A., and Whitehead, S. N. (2016). Prefrontal ischemia in the rat leads to secondary damage and inflammation in remote gray and white matter regions. *Front. Neurosci.* 10:81. doi: 10.3389/fnins.2016.00081

West, M. J., Slomianka, L., and Gundersen, H. J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* 231, 482–497. doi: 10.1002/ar.1092310411

Windle, V., Szymanska, A., Granter-Button, S., White, C., Buist, R., Peeling, J., et al. (2006). An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. *Exp. Neurol.* 201, 324–334. doi: 10.1016/j.expneurol.2006.04.012

Winkler, C., Bentlage, C., Nikkhah, G., Samii, M., and Bjorklund, A. (1999). Intranigral transplants of GABA-rich striatal tissue induce behavioral recovery

in the rat Parkinson model and promote the effects obtained by intrastriatal dopaminergic transplants. *Exp. Neurol.* 155, 165–186. doi: 10.1006/exnr.1998.6916

Yassi, N., Malpas, C. B., Campbell, B. C., Moffat, B., Steward, C., Parsons, M. W., et al. (2015). Contralesional thalamic surface atrophy and functional disconnection 3 months after ischemic stroke. *Cerebrovasc. Dis.* 39, 232–241. doi: 10.1159/000381105

Zhang, L., Chen, J., Li, Y., Zhang, Z. G., and Chopp, M. (2000). Quantitative measurement of motor and somatosensory impairments after mild (30 min) and severe (2 h) transient middle cerebral artery occlusion in rats. *J. Neurol. Sci.* 174, 141–146. doi: 10.1016/s0022-510x(00)00268-9

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# Species-Specific miRNAs in Human Brain Development and Disease

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Identification of the unique features of human brain development and function can be critical towards the elucidation of intricate processes such as higher cognitive functions and human-specific pathologies like neuropsychiatric and behavioral disorders. The developing primate and human central nervous system (CNS) are distinguished by expanded progenitor zones and a protracted time course of neurogenesis, leading to the expansion in brain size, prominent gyral anatomy, distinctive synaptic properties, and complex neural circuits. Comparative genomic studies have revealed that adaptations of brain capacities may be partly explained by human-specific genetic changes that impact the function of proteins associated with neocortical expansion, synaptic function, and language development. However, the formation of complex gene networks may be most relevant for brain evolution. Indeed, recent studies identified distinct human-specific gene expression patterns across developmental time occurring in brain regions linked to cognition. Interestingly, such modules show species-specific divergence and are enriched in genes associated with neuronal development and synapse formation whilst also being implicated in neuropsychiatric diseases. microRNAs represent a powerful component of gene-regulatory networks by promoting spatiotemporal post-transcriptional control of gene expression in the human and primate brain. It has also been suggested that the divergence in miRNA expression plays an important role in shaping gene expression divergence among species. Primate-specific and human-specific miRNAs are principally involved in progenitor proliferation and neurogenic processes but also associate with human cognition, and neurological disorders. Human embryonic or induced pluripotent stem cells and brain organoids, permitting experimental access to neural cells and differentiation stages that are otherwise difficult or impossible to reach in humans, are an essential means for studying species-specific brain miRNAs. Single-cell sequencing approaches can further decode refined miRNA-mRNA interactions during developmental transitions. Elucidating species-specific miRNA regulation will shed new light into the mechanisms that control spatiotemporal events during human brain development and disease, an important step towards fostering novel, holistic and effective therapeutic approaches for neural disorders. In this review, we discuss species-specific regulation of miRNA function, its contribution to the evolving features of the human brain and in neurological disease, with respect also to future therapeutic approaches.

**Keywords: RNA sequencing (RNAseq), primate, neurogenesis, evolution, gene networks, brain, miRNAs, human**

# INTRODUCTION

Human brain development presents unique features and underlies the intricately coordinated spatiotemporal expression of thousands of genes. This elaborate mechanism entails the timely acquisition of diverse cellular identities further orchestrating regional specialization and inter-connectivity in the brain. microRNAs (miRNAs) are powerful post-transcriptional regulators, increasingly recognized as important components of fundamental neurodevelopmental processes and related disorders (Adlakha and Saini, 2014). miRNAs act to repress the translation or degrade their mRNA targets to modulate and fine-tune gene expression levels. One of the early roles ascribed to miRNAs was their contribution to developmental transitions by suppressing transcripts associated with the previous stage. An alternative, but complementary hypothesis suggests that miRNAs act reduce the variance in the expression level of their target genes, conferring increased robustness to signaling decisions during development (Hornstein and Shomron, 2006; Ebert and Sharp, 2012). Importantly, it is appreciated that miRNA-driven regulation contributed critically to gene expression changes on the human evolutionary lineage affecting genes involved in progenitor proliferation and neuronal generation and function (Nowakowski et al., 2013; Arcila et al., 2014). miRNA-mediated regulation in the developing brain presents primate distinct aspects, including over 100 primate-specific and 14 human-specific miRNAs that have been identified (Berezikov, 2011; Hu et al., 2011).

In this review we present current data on species-specific miRNA regulation and discuss miRNAs as hubs of critical brain transcriptional processes during human neural development. Finally, we stress the requirement to delineate the functional significance of miRNA-driven transcriptome changes at the single-cell level, as an important step towards resolving the complex regulatory network operating during human neurogenesis. Towards this direction, brain organoids and application of single-cell sequencing methodologies constitute invaluable tools to address causality between the emergence of novel miRNAs and rewiring of transcriptional programs during the evolution of brain complexity.

## DISTINCT FEATURES OF HUMAN BRAIN DEVELOPMENT

Although brain development follows the same principles across mammals, the primate and human central nervous system (CNS) is distinguished by highly derived features. These include expanded progenitor zones (Smart et al., 2002) accompanied by enhanced and tightly controlled proliferative and/or neurogenic potential of progenitor cells (Otani et al., 2016; Sousa et al., 2017a,b), further associated with molecular changes and an increased diversity of neural cell types (Bystron et al., 2006; Lui et al., 2011; Gulden and Šestan, 2014; Taverna et al., 2014; Bae et al., 2015; Dehay et al., 2015). Human brain development is characterized by a relatively protracted time course of neurogenesis, followed by an extraordinary numeric expansion of the neuronal cell population (Rockel et al., 1980;

Hutsler et al., 2005; Marín-Padilla, 2014; Otani et al., 2016) and the emergence of sophisticated neural circuits of connectivity reflected in prominent changes in gyral anatomy (Rogers et al., 2010; Hofman, 2012; **Figure 1**).

Brain evolution produced changes in morphology, abundance, and function of cell types. For example, differences between rodent and human neuronal features include distinctive membrane (Wang et al., 2015; Eyal et al., 2016) and synaptic properties Molnár et al., 2008, 2016; Testa-Silva et al., 2010; Verhoog et al., 2013; Szegedi et al., 2016). Deviations between humans and non-human primates (NHPs) have also been reported regarding the morphology and number of glial cells in the brain (Oberheim et al., 2012; Bianchi et al., 2013; Herculano-Houzel et al., 2016). In addition, excitatory projection neurons in humans show more elaborate dendritic arborization, and contain a greater number and density of spines compared to non-human primates (NHPs; Duan et al., 2003; Elston et al., 2011). Along this line, a subgroup of modified pyramidal neurons, known as spindle or von Economo neurons, mainly found in the fronto-insular and anterior cingulate cortex, are larger and more numerous in humans than in other apes (Allman et al., 2010). Although their function remains elusive, they have been implicated in brain disorders with social-emotional deficits, such as autism spectrum disorders (ASDs), schizophrenia (SCZ), frontotemporal dementia and Alzheimer's disease (AD; Yang et al., 2019). Evidence also exists for species-specific differences in serotonergic transmission, dopamine innervation and the regional localization and abundance of certain subclasses of inhibitory neurons and their axonal projections in the neocortex of humans and NHPs (Sherwood et al., 2004; Raghanti et al., 2008, 2016). Similarly, a specialized GABAergic neuron subtype has recently been identified with a molecular and anatomical signature specific to humans (Boldog et al., 2018).

## GENE NETWORKS ASSOCIATED WITH PRIMATE- AND HUMAN-SPECIFIC BRAIN ADAPTATIONS

Comparative genomic studies revealed that adapted brain specializations and capacities may be partly explained by human-specific gene conversions that impact the function of proteins associated with neocortical expansion, synaptic function, and language development. Characteristic examples include genes that have undergone human-specific duplication like the cortical development gene Slit-Robo Rho GTPase activating protein 2 (*SRGAP2*) which induces branching of neurons and neurite outgrowth (Dennis et al., 2012). Another case is ARHGAP11 which is expressed in basal progenitors and promotes neocortical expansion by increasing neuron numbers and brain folding (Florio et al., 2015). *FOXP2* is also a gene that has undergone surprisingly rapid evolution in the primate lineage leading to humans and encodes a homeodomain protein essential for normal human speech (Lai et al., 2001). Similarly, *ASPM* (Gai et al., 2016), *Microcephalin* (Evans et al., 2004) and *AHI1* genes (Ferland et al., 2004) that have undergone "positive
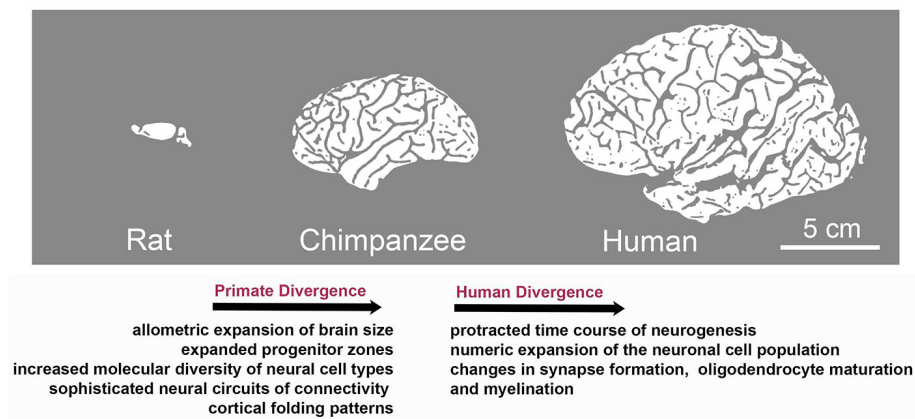
**FIGURE 1 |** Species-specific evolution of traits associated with brain development during the divergence to primate and human lineages. Although brain development follows the same basic principles across mammals, evolution has resulted in the appearance of species-specific features. Characteristics of primate divergence include allometric increase in brain size, expansion of progenitor zones along with increased diversity of neural cell types and sophistication of neural circuits reflected in enhanced gyral anatomy. Human brain development is further distinguished by a relatively protracted period of neurogenesis, followed by an extraordinary numeric expansion of the neuronal cell population and by the heterochronic or heterotopic expression of genes associated with synapse formation and myelination in brain regions including the prefrontal cortex, which is central to human cognition and behavior.

selection," encode for human proteins that associate with normal cerebral cortical size and axon guidance.

Importantly, gene co-expression analyses have revealed that transcriptional regulation and complexity in the neocortex have dramatically increased on the human lineage (Konopka et al., 2012; Silbereis et al., 2016). The allometric expansion of the brain is primarily accompanied by changes in patterns of gene networks and neuronal activity leading to the structural reorganization of the connectome and possibly harboring new behavioral and cognitive phenotypes (Buckner and Krienen, 2013). In this regard, recent transcriptome studies have provided crucial information on divergent patterns of molecular expression that are most relevant in evolutionary terms and can be used to uncover human specializations of brain structure and function.

Specifically, fetal gene co-expression modules have been identified that showed substantial regional differences in the developing human neocortex (Johnson et al., 2009; Miller et al., 2014; Pletikos et al., 2014). Enriched genes were shown to be critical for neuronal processes, such as differentiation, maturation, axonal projection, and synapse formation. These molecular networks also displayed divergence between humans and rhesus macaques (**Figure 1**; Pletikos et al., 2014), highlighting evolving biological processes involved in the patterning and differentiation of neural circuits in discrete areas (Miller et al., 2014; Hoerder-Suabedissen and Molnár, 2015).

Further work revealed human-distinct temporal progression of neurodevelopmental processes predominantly during the early and mid-fetal period. The identified gene modules were linked to synapse formation, neuronal differentiation, oligodendrocyte maturation, and myelination, and exhibited heterochronic or heterotopic expression in brain regions including the prefrontal cortex, which is central to human cognition and behavior (**Figure 1**). Interestingly, divergent

spatiotemporal expression patterns included also genes associated with ASDs and schizophrenia (Konopka et al., 2012; Zhu et al., 2018). These studies highlight gene expression regulation in space and time as an evolution-modified feature that impacts on neurodevelopmental processes as well as in brain complexity and disease. Diverse expression patterns responsible for interspecies differences can be attributed to changes in DNA methylation, histone modifications (Maze et al., 2014), alternative splicing events, promoter-driven transcription regulation (Davuluri et al., 2008; Nilsen and Graveley, 2010) and non-coding RNAs.

## microRNAs ARE POWERFUL POST-TRANSCRIPTIONAL REGULATORS OF GENE EXPRESSION IN THE BRAIN

microRNAs (miRNAs) are a class of short non-coding RNAs of ~22 nucleotides in length that constitute an important component of the regulatory circuitry determining expression patterns. Mature miRNAs mediate post-transcriptional regulation of gene expression through direct degradation of their target mRNA and/or suppression of translation. miRNAs bind to their mRNA targets by partial complementarity between the mRNA's 3′UTR and a 6–8 nucleotides long "seed" sequence at the 5′ end of the microRNA. Therefore, a single microRNA can target multiple mRNAs simultaneously, while a single mRNA may be regulated by different microRNAs (Klein et al., 2005; Kosik, 2006; Saliminejad et al., 2019).

miRNA biogenesis begins with the transcription of double-stranded primary miRNA (pri-miRNA) short hairpin structures by RNA polymerase II. The pri-miRNA is then cleaved by the RNase-III enzyme, Drosha, producing ~70-bp pre-miRNAs that are exported from the nucleus into the cytoplasm by Exportin 5 (Exp5), a Ran-GTP dependent Nucleo/cytoplasmic cargo

transporter. The Dicer enzyme cleaves pre-miRNA sequences into 21–23 nt mature miRNA double-stranded duplexes which are loaded into a pre-RISC (pre-miRNA-induced silencing complex) containing Argonaute (Ago) and other proteins. In the mature miRISC complex the "passenger" strand (complementary strand) is removed leaving just the "guide" strand (mature miRNA strand) which will bind to the mRNA target and instigate inhibition of its expression, reviewed in Winter et al. (2009) and Davis et al. (2015).

The concentration of miRNAs within cells is regulated at different levels. It has been shown that Ago proteins are not only critical for miRNA biogenesis and function, but they also regulate the abundance of mature miRNAs by increasing their stability (Grishok et al., 2001; Diederichs and Haber, 2007; Winter and Diederichs, 2011). Association with mRNA targets also enhances miRNA stability, a phenomenon known as target-mediated miRNA protection, while the introduction of additional target sites can also promote miRNA accumulation (Chatterjee et al., 2011). On the other hand, miRNAs are subject to degradation. It has been shown that miRNA-mRNA interactions not only stabilize but can also destabilize the miRNA and promote its degradation through a process known as target RNA directed miRNA degradation (Ameres et al., 2010; Fuchs Wightman et al., 2018). The degree of sequence complementarity infers the outcome of the miRNA-mRNA interaction, with higher complementarity favoring miRNA degradation and lower complementarity favoring miRNA stabilization. As demonstrated, target mRNAs promote posttranscriptional modifications to the 3′ end of the miRNA involving either the addition of non-templated nucleotides, a process known as "3′-end tailing" or elimination of nucleotides via 3′-to-5′ trimming, both of which control the rate of miRNA decay (Baccarini et al., 2011; Marcinowski et al., 2012).

The ability of miRNAs to fine-tune gene expression levels (Schratt, 2009) constitutes a critical property for controlling spatiotemporal events during brain development. Of the 2,500 mature miRNAs that have been identified in humans (Friedländer et al., 2014), an estimated 70% is expressed in the nervous system (Adlakha and Saini, 2014). miRNAs have emerged as important post-transcriptional regulators of gene expression involved in neurogenesis and neural function in mammalian species (Davis et al., 2015; Nowakowski et al., 2018). A relatively small number of brain miRNAs are well characterized, including miR-92 which targets EOMES (TBR2), a T-box transcription factor that is preferentially expressed in cortical intermediate progenitors and regulates cortical neuron production and expansion, thereby affecting the thickness of the cerebral cortex (Nowakowski et al., 2013). miR-124 and miR-9 have also been shown to affect neural lineage differentiation by downregulating multiple mRNAs (Krichevsky et al., 2006). MiR-9 is a highly brain enriched miRNA that is involved in a negative feedback loop with TLX, a nuclear receptor (Zhao et al., 2009) which controls stem cell proliferation in developing and adult brain (Shi et al., 2004; Liu et al., 2008; Zhang et al., 2008). MiR-9 together with miR-124, one of the most abundant miRNAs in the brain, target REST which opposes

neuronal differentiation (Conaco et al., 2006; Visvanathan et al., 2007), while REST itself acts as an inhibitor of miR-124 expression. Moreover, both miRNAs act synergistically to repress BAF53a, a subunit of the neural-progenitor-specific BAF (npBAF) chromatin-remodeling complex, operating during the post-mitotic phase of neuronal development (Yoo et al., 2009). Finally, miR-124 downregulates the RNA-binding protein Ptbp1, a repressor of neuron-specific splicing (Makeyev et al., 2007).

Additionally, miRNAs have been shown to be essential for neural subtype specification. For example, miR-7a promotes oligodendrocyte generation by targeting Pax6 and NeuroD4 (Zhao et al., 2012), while miR-218 is required to establish motor neuron fate (Thiebes et al., 2015). miRNAs have also important roles in synapse formation and plasticity. These include miR-125 which targets the post-synaptic protein PSD-95 in cortical neurons (Muddashetty et al., 2011), the neuron-specific miR-129 which represses Kv1.1, a voltage-gated potassium channel that regulates excitability (Sosanya et al., 2013), and miR-219 which downregulates CamKII, a the major mediator of Long Term Potentiation (LTP) and N-methyl-D-aspartate receptor (NMDA) signaling (Kocerha et al., 2009). Other miRNAs modulate synaptic function upon activation. miR-485 expression is increased following neuronal stimulation to regulate the pre-synaptic protein SV2A and inhibit neurotransmitter release (Cohen et al., 2011). miR-132, on the other hand, accumulates in response to activity in forebrain neurons to regulate dendritic growth, activity-induced spine growth and spine morphology (Magill et al., 2010; Nudelman et al., 2010).

# THE EVOLVING ROLE OF miRNAs IN THE PRIMATE AND HUMAN BRAIN

## The Evolution of miRNAs

Evolution of miRNAs is an ongoing process and experimental evidence suggests that along with highly conserved miRNAs, a number of new brain miRNAs have emerged. Many of these are not conserved beyond primates, indicating their recent origin (Berezikov et al., 2006). Following evolutionary adaptations, more than 100 primate-specific miRNAs (that is, miRNAs present only in humans and non-human primates) and 14 human-specific miRNAs (**Table 1**) have been identified in the developing brain (Berezikov, 2011). Novel miRNAs arise either by the appearance of transcribed hairpin structures or by mutations in the miRNA seed region (Lu et al., 2008). The genomic sources for the acquisition of novel miRNAs are reviewed in detail in "Evolution of microRNA diversity and regulation in animals" (Berezikov, 2011). Briefly, novel miRNAs can emerge by duplication of existing miRNA genes. Alternatively, introns are a frequent source of unstructured transcripts that can gradually evolve into novel intronic miRNAs. De novo emergence of miRNAs can also occur, where an evolved transcriptional unit provides a source of the initially unstructured transcript that transitions through the miRNA-like hairpin stage and evolves into a novel miRNA gene. In addition, transposable elements or structured transcripts, such as tRNA and small nucleolar RNA (snoRNA), can provide novel transcriptional units for the evolution of miRNA-like hairpins into novel miRNA

List of human-specific miRNAs identified so far according to the study of Hu et al. (2012).

| Precursor id | Chromosome |
| --- | --- |
| hsa-mir-1302-10 | chr15 |
| hsa-mir-1302-11 | chr19 |
| hsa-mir-1302-2 | chr1 |
| hsa-mir-3156-3 | chr21 |
| hsa-mir-3648 | chr21 |
| hsa-mir-3673 | chr8 |
| hsa-mir-3690 | chrX |
| hsa-mir-4487 | chr11 |
| hsa-mir-4739 | chr17 |
| hsa-mir-5095 | chr1 |
| hsa-mir-659 | chr22 |
| hsa-mir-941-1 | chr20 |
| hsa-mir-941-3 | chr20 |
| hsa-mir-941-4 | chr20 |

genes. Finally, antisense transcription of existing miRNA loci can lead to the formation of miRNA hairpins with novel mature miRNA sequences.

## miRNAs as Key Regulators in Shaping Patterns of Gene Expression in the Developing and Adult Primate and Human Brain

A series of observations support the notion that miRNAs are instrumental contributors to the alterations that may account for the accelerated evolution of the human brain. First, elevated production of mature miRNAs has been observed in the human brain compared to other species, which is attributed to the higher processing efficiency of miRNA precursors in humans (Chakraborty et al., 2018).

It has been proposed that the evolution of miRNA-mediated regulatory networks has contributed to organismal complexity (Berezikov, 2011). This can mechanistically be explained by the ability of miRNAs to advance network functionalities by delivering the extra precision required to constrain the intrinsically noisy gene expression process (Raj and van Oudenaarden, 2008; Herranz and Cohen, 2010). In support, the work of Arcila et al. (2014) on primate-specific miRNAs suggests that integration of novel miRNAs into ancient gene circuitry exerted additional regulation over-proliferation of neural progenitors in cortical germinal areas (**Figure 2**), a region that demonstrates significant expansion across brain evolution (Arcila et al., 2014).

Furthermore, evolving miRNA-mediated regulation shows critical implications in shaping gene networks and even determining anatomical regions during brain development. Work on primates has revealed that miRNA profiles can resolve discrete areas within the developing cortex while dominant differences were observed between the germinal zone and the differentiated cells in the cortical plate (Arcila et al., 2014). Consistently, studies on the transition from infant to adolescent human brain show differential expression of miRNAs within and between brain regions, with the prefrontal cortex, the region mostly connected with human
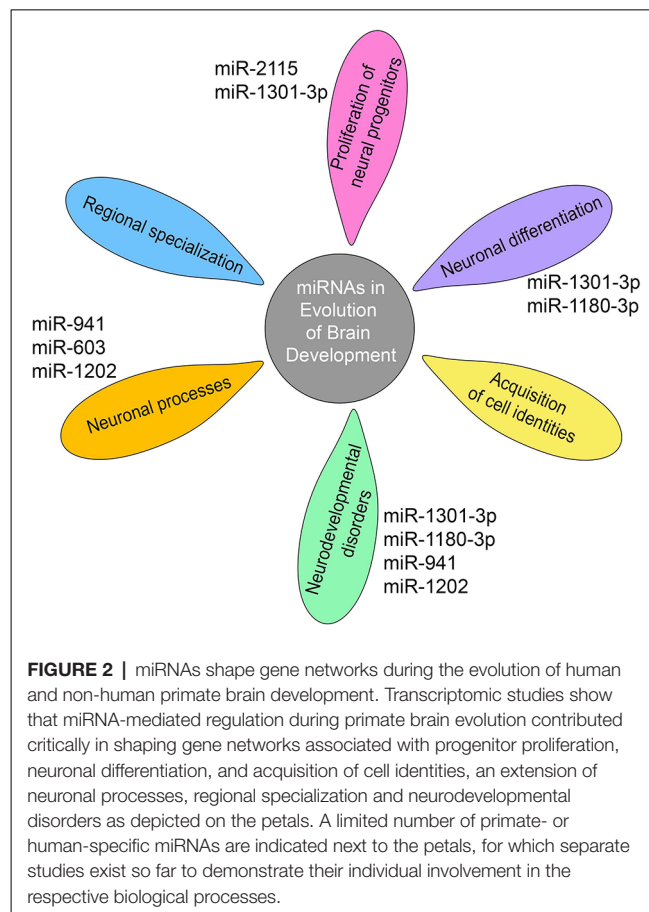


**FIGURE 2 |** miRNAs shape gene networks during the evolution of human and non-human primate brain development. Transcriptomic studies show that miRNA-mediated regulation during primate brain evolution contributed critically in shaping gene networks associated with progenitor proliferation, neuronal differentiation, and acquisition of cell identities, an extension of neuronal processes, regional specialization and neurodevelopmental disorders as depicted on the petals. A limited number of primate- or human-specific miRNAs are indicated next to the petals, for which separate studies exist so far to demonstrate their individual involvement in the respective biological processes.

cognition, exhibiting the greatest number of differentially expressed miRNAs (Ziats and Rennert, 2014). Moreover, miRNA expression displays increased deviations between brain regions over time, indicating the implication of miRNAs in the regional specialization as the brain matures (Ziats and Rennert, 2014). Importantly, common neurodevelopmental disorders associated with genes targeted by these miRNAs (**Figure 2**; Ziats and Rennert, 2014).

Recent elegant work highlights the impact of miRNAs in regulating regionally divergent transcriptional states in the developing human cortex. To characterize the landscape of miRNA–mRNA interactions during human brain development, *Nowakovski et al* developed a new single-cell approach for combined mRNA and miRNA profiling in the same cell across human fetal tissue samples corresponding to peak neurogenesis [Gestational week (GW) 15 and 16.5] and early gliogenesis (GW 19–20.5). Their study revealed that major regulatory molecules like transcription factors, chromatin modifiers, and signaling components are enriched among miRNA targets. Reconstruction of gene-regulatory networks uncovered that miRNA-mRNA interactions often correspond to the acquisition of cell-type identities and undergo dynamic transitions even among closely related cell types during neuronal differentiation and maturation (**Figure 2**). Further strengthening previous studies, the authors demonstrate that different pathways driven by brain-specific miRNAs are

related to developmental stage and cortical area specificity (Nowakowski et al., 2018).

Transcriptomic studies founded the notion that miRNA divergence correlates with the divergence of gene expression patterns in the prefrontal cortex and cerebellum among humans, chimpanzees, and macaques. Accordingly, it has been reported that a significant inverse relationship exists between human and chimpanzee miRNA expression divergence and expression divergence of their predicted target genes at both mRNA and protein levels (Hu et al., 2011). Inline, Svante Pääbo and colleagues placed miRNAs among the key regulators that remodeled cortical development (Somel et al., 2011). Using a computational method of analysis, they show that trans-acting regulators and particularly miRNAs drive the pronounced gene expression changes observed in the human prefrontal cortex. As deduced, the developmental profiles of miRNAs, as well as their target genes, show the fastest rates of human-specific evolutionary change.

# HUMAN- AND PRIMATE-SPECIFIC miRNAs IN NEURAL DEVELOPMENT, PHYSIOLOGY AND PATHOLOGY

Expression, even if at low levels, has been detected in the prefrontal cortex and cerebellum for the majority of the 14 human-specific miRNAs identified so far (Hu et al., 2012). Enrichment analysis of the predicted target genes of human-specific miRNAs attests that these relate to neuronal components and processes, including cytoskeletal elements, metal ion binding, and postsynaptic, dendritic, and somatic functions (Barbash et al., 2014). Separate studies on individual primate- and human-specific miRNAs are limited and confined in human tissue examination complemented by functional analysis using cell lines or mouse models in one case (**Table 2**). Nevertheless, data so far show that primate-specific miRNAs are principally involved in the regulation of cell cycle dynamics operating during progenitor proliferation and neuronal differentiation while they further associate with neurodevelopmental disorders (**Figure 2**).

A great ape specific miRNA, miR-2115, is enriched in radial glia and becomes prominently upregulated at GW19–20 in the human germinal zones. It controls cell-cycle dynamics during human cortical development by fine-tuning the expression of ORC4, a known regulator of DNA replication (Nowakowski et al., 2018). miR-1301-3p and miR-1180-3p are primate-specific miRNAs that have been identified in the germinal zones of the visual cortex of the macaque developing brain. miR-1301-3p regulates the mRNA for histone-lysine N-methyltransferases mll1 and mll2 (MixedLineage, Leukemia) that function by resolving silenced bivalent loci in neural precursors for induction of neurogenesis. Another target of miR-1301-3p is the transcription factor TCF4 which patterns progenitor cells in the developing CNS and has been linked to schizophrenia and intellectual disability (Arcila et al., 2014). miR-1180-3p targets kansl1 and dlx1. Kansl1 is a chromatin regulator that when haploinsufficient, causes intellectual disability, hypotonia, and distinctive facial features associated with the

**TABLE 2 |** A summary of the studies on individual primate- and human-specific miRNAs.

| miRNA | Species conservation | mRNA target(s) | Detection of brain expression | Methodology | Proposed associated function and neurodevelopmental disorder | Reference |
|---|---|---|---|---|---|---|
| miR-2115 | Primate specific | ORC4 | Upregulated at GW19– GW 20 in the human germinal zones | Single-cell RNA sequencing for identification. A mouse model for functional studies | Cell cycle dynamics during human cortical development | Nowakowski et al. (2018) |
| mir-1301-3p | Primate specific | histone-lysine N-methyl-transferase TCF4 | Fetal brain of cynomolgus monkeys (Macaca fascicularis, gestation period 165 days) | RNA Sequencing for identification Target validation and luciferase assays in human cell lines | Neurogenesis, schizophrenia, intellectual disability | Arcila et al. (2014) |
| miR-1180-3p | Primate specific | col3a1 and col1a1 KANSL1 and DLX1 | Fetal brain of cynomolgus monkeys (Macaca fascicularis, gestation period 165 days) | RNA Sequencing for identification Target validation and luciferase assays in human cell lines | GABAergic neurogenesis, autism, intellectual disability | Arcila et al. (2014) |
| miR-1202 | Primate specific | GRM4 | Elevated in the brain of depressed individuals | Functional analysis in cell lines and human NPCs. Drug testing in human NPCs. | Pathophysiology of depression | Lopez et al. (2014) |
| miR-603 | Primate specific | E2F1 and LRPAP1 | | Target validation and luciferase assays in human cell lines | Association with AD risk | Zhang et al. (2016) |
| miR-941 | Human-specific | SMO, SUFU, GLI1, IRS1, PPARGC1A and FOXO1 | High expression in the human prefrontal cortex and cerebellum | RNA sequencing for expression analysis. Target validation in human cell lines | Human longevity, neurotransmitter signaling, language and speech | Hu et al. (2012) |

17q21.31microdeletion syndrome, while dlx1 is a homeodomain transcription factor that controls GABAergic neurogenesis and has been associated with autism (Arcila et al., 2014).

In addition, a number of species-specific miRNAs associate with human cognition and neurodegenerative diseases (**Table 2**). miR-941 is the only human-specific miRNA expressed highly in the prefrontal cortex and cerebellum and has been proposed to be associated with longevity and neurotransmitter signaling. Individuals containing a microdeletion in the chromosomal region containing pre-miR-941 display developmental delay and disruption of cognitive functions including language and speech (**Figure 2**; Hu et al., 2012). miR-1202, a miRNA specific to primates, and enriched in the human brain is associated with the pathophysiology of depression. It is differentially expressed in depressed individuals and has been shown to target the Metabotropic Glutamate Receptor 4 (GRM4; Lindsley and Hopkins, 2012), a synaptic molecule modulating neurotransmission that also constitutes an attractive therapeutic target for Parkinson's disease and schizophrenia (**Figure 2**; Lopez et al., 2014).

Another primate-specific miRNA, miR-603, is a novel intronic miRNA of the gene KIAA1217, which is highly expressed in human brain. miR-603 directly downregulates the key neuronal apoptotic component E2F1 and can prevent cells from undergoing apoptosis. In addition, miR-603 targets LRPAP1 involved in Aβ amyloid peptide clearance and the pathogenesis of AD. Finally, the rs11014002 SNP in precursor pre-miR-603 increases the expression of mature miR-603, which may account for its association with reduced risk for AD (Zhang et al., 2016; **Figure 2**).

## HUMAN-SPECIFIC REGULATION OF miRNA FUNCTION IN THE BRAIN

Evolutionary changes and consequently species distinct features also exist for the binding partners of miRNAs. The evolution of miRNA regulation is intimately intertwined with the evolution of their targets, as newly emerging miRNAs integrate into preexisting gene expression circuitries (Arcila et al., 2014). This is particularly pronounced in the brain where neuronal transcripts not only have longer 3′-untranslated regions (UTRs) which are the main target region for miRNAs (Meunier et al., 2013), but also display an increased density of potential binding sites, enhancing their selective advantage for acquiring miRNA-mediated regulation (Cacchiarelli et al., 2008; Barbash et al., 2014). These findings in conjunction with the appearance of increased new variants around target genes of human-specific miRNAs are consistent with the theory that the speciation of hominids was accompanied by an enhancement in the capacity of newly evolved miRNAs to modulate gene expression (Barbash et al., 2014).

Interestingly Hu et al identified five miRNAs, namely miR-184, miR-487a, miR-383, miR-34c-5p, and miR-299-3p, with high sequence conservation among species, which nevertheless show significantly different levels of expression in humans, while two of these (miR-299-3p and miR-

184) show preferential expression in cortical neurons (Hu et al., 2012). Functional analysis combining the targets of all five miRNAs revealed enrichment in genes associated with neural processes and specifically with cell proliferation and differentiation, synaptic transmission and neuronal function. Moreover, miR-299-3p targets associate with axon guidance, while miR-184 targets are related to long-term potentiation, which is directly linked to learning and memory formation (Hu et al., 2011). Human-specific miRNA-mediated regulation of certain genes with a critical role in brain function has also been reported. For instance, miR-483–5p, binds in a sequence-exclusive manner to the human epigenetic regulator methyl CpG binding protein 2 (MeCP2) that controls proper neurological function, to modulate its levels in the fetal cortex (Han et al., 2013).

## HUMAN EXPERIMENTAL MODELS FOR INVESTIGATION OF miRNA-SPECIFIC FEATURES OF BRAIN DEVELOPMENT AND ASSOCIATED DISORDERS

Access to human tissue in combination with high-throughput sequencing techniques provided important insight into the significance of the miRNA-driven transcriptome changes across brain development and evolution. However, it is necessary to validate predicted targets and clarify cell type association, stage-specificity and mode of action for individual miRNAs. As experimental animals cannot fully simulate human brain development and disease, human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) represent valuable means for advancing human studies (Chambers et al., 2009; Shi et al., 2012). Directed cortical differentiation of human, chimpanzee, and macaque iPSCs (Otani et al., 2016) clearly depicted the divergence in the timing of key developmental events and highlighted the extended proliferation of chimpanzee and human progenitors as compared to macaque, emphasizing the validity of such systems in reproducing species-specific features.

Further studies on hESCs accompanied by miRNA profiling during differentiation of various neuronal subtypes underlined the effect of known miRNAs, such as let-7, miR-124, miR-7, miR-125 and miR-9 in progenitor proliferation, cell fate specification and neuronal commitment and maturation (Delaloy et al., 2010; Boissart et al., 2012; Liu et al., 2012; Cimadamore et al., 2013; Tu et al., 2018). miRNA research on hESC models has also uncovered new targets and roles for these miRNAs. For example, miR-9 which has been described in mouse to promote neuronal lineage differentiation by targeting REST, was shown to also target Stathmin, a protein involved in microtubule stability, and to coordinate proliferation and migration during the early stage of maturation of neural progenitors. Similarly, miR-125 with reported neuronal function, was shown to act in earlier stages to promote exit from pluripotency and potentiate neural specification by targeting SMAD4. Moreover, research on patient induced pluripotent stem cells and their neuronal derivatives uncovered the role

of certain miRNAs in autism and schizophrenia (Halevy et al., 2015; Murai et al., 2016; Mellios et al., 2018).

Notably, given the complexity of the human brain, 3D tissue culture models that embody cellular diversity and spatial organization that mimics brain architecture, are more relevant to understand the critical input of miRNAs in shaping intricate brain gene networks. Brain organoids that grow as 3D aggregates from pluripotent stem cells comprise an exciting new tool for modeling human brain physiology and pathology, for uncovering human-specific traits and for drug discovery and testing. Despite current limitations, particularly batch heterogeneity that impedes consistency, organoids resemble human brain not only at the cellular level, but also in terms of general tissue structure, developmental trajectories and neuronal functionalities (Lancaster et al., 2013; Paşca et al., 2015; Qian et al., 2016; Giandomenico et al., 2019). Cortical organoids in particular that have been more extensively studied, recapitulate the organization of neural progenitor zones to a considerable degree, reflecting developmental events during embryonic stages *in vivo* (Lancaster et al., 2013; Qian et al., 2016). Such systems can be valuable for functional gain- or loss-of-function studies to elucidate the contribution of ancient or novel miRNAs in primate and/or human-specific processes. Nevertheless, studying neuronal network formation among different brain regions, especially distant ones, remains a challenge. Recent advances in the generation of fused brain organoids from co-culture of individual ones with distinct regional identities may provide a *closer-to-the-in-vivo-situation* model to investigate the basis of human neural circuitry formation in health and disease (Birey et al., 2017; Xiang et al., 2017, 2019). Alternatively, it is possible to exploit the inherent intrinsic heterogeneity observed within single organoids, as for example in brain organoids containing retinal regions that can respond to light stimuli (Quadrato et al., 2017). However in all cases absence of vascularization restricts oxygen and nutrient supplies limiting organoid survival, consequently affecting the time-scale of such studies. Therefore a combination of different approaches and technologies, including 2D cultures derived from pluripotent stem cells, brain organoids, single-cell transcriptomics, potentially along with miRNA target degradation kinetics analysis to elucidate the miRNA-driven evolutionary variants of the neurodevelopmental program should prove more rewarding.

In relevance, single-cell RNA-sequencing technology has greatly facilitated research efforts in resolving the diversity and developmental trajectories of human brain cell types providing transformative insights into the developmental lineages and functional states of individual cells (Tasic et al., 2016, 2018; Nowakowski et al., 2017; Paul et al., 2017; Hrvatin et al., 2018; Lake et al., 2018; Zhong et al., 2018). Importantly, a number of studies have illustrated the potential to integrate spatial information at single-cell resolution (Ke et al., 2013; Lubeck et al., 2014; Chen et al., 2015; Satija et al., 2015; Salmén et al., 2018). However, when designing such experiments, consideration of the tradeoff between a number of cells sequenced and the read depth per cell is a factor that has to be seriously considered, not the least because of budgetary constraints (Menon, 2018). Additionally, such developments still

necessitate the formulation of new computational modalities to integrate the rapidly expanding data sets generated from diverse sources so that biological sense is made (Butler et al., 2018; Stuart and Satija, 2019). Improvements in algorithm design and data analysis are essential to allow unsupervised methods for tracking gene expression and correctly reconstructing trajectories that map cell lineages and developmental transition of cells.

## Towards miRNA Based Therapeutics

The more we understand miRNA biology and its contribution to the intricate regulatory networks of gene expression during development, the better will we be able to uncover related disturbances underlying the origin of various neuropsychiatric and behavioral disorders. Indeed, compelling evidence regarding the involvement of miRNAs in human disease has instigated attention into their potential use as therapeutics (van Rooij et al., 2012). miRNA-based strategies include miRNA mimics and inhibitors (antagomiRs) to respectively increase and decrease their expression or, conversely, their target genes. The field currently progresses rapidly as in 2018 the FDA approved the first therapy based on miRNA administration for the treatment of rare progressive polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (Adams et al., 2018; Wood, 2018). Understanding the functions of miRNAs in specific cell types and during different stages throughout life under normal and pathological conditions could improve the specificity and efficacy of miRNA therapeutic strategies. Moreover, delivery to the brain is challenging and the pleiotropic nature of miRNA functions undeniably makes off-target biological effects an important limitation (Junn and Mouradian, 2012). Novel approaches are in the pipeline to design new chemical formulations in order to increase miRNA stability and improve their specificity and permeability, while delivery methods are also being developed to target the brain and decrease unwanted side effects (van Rooij et al., 2012; Søkilde et al., 2015; Rupaimoole and Slack, 2017). In this regard, decoding species-specific regulation of miRNA function will prove an important step for fostering novel, holistic and effective therapeutic approaches for human disorders.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Accumulating evidence highlights the emergent role of miRNAs as critical regulators that influence the overall transcriptional landscape in the human brain. In addition, primate- and human-specific regulation by miRNAs during neurodevelopment argues in favor of their involvement in the establishment of species-specific features during brain evolution. A comprehensive understanding of the spatiotemporal miRNA mediated regulation will help resolve the intricate regulatory network operating during human neurogenesis and will grant important cues for the causality of neurodevelopmental disorders. Down this line, 2D- and 3D-human pluripotent-derived experimental setups constitute a useful system for modeling species-specific features of development and for

capturing miRNA mediated rewiring of the transcriptional program. In conjunction, single-cell sequencing approaches can decode the refined miRNA-mRNA interactions during cell developmental transitions. Taken together, miRNAs constitute an indispensable component of the extended gene regulatory network and evaluating their functional significance in the evolving brain can determine key features of human neuronal development and disease.

## AUTHOR CONTRIBUTIONS

KP and RM planned and wrote the manuscript.

## REFERENCES

Adams, D., Gonzalez-Duarte, A., O'Riordan, W. D., Yang, C. C., Ueda, M., Kristen, A. V., et al. (2018). Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* 379, 11–21. doi: 10.1056/NEJMoa1716153

Adlakha, Y. K., and Saini, N. (2014). Brain microRNAs and insights into biological functions and therapeutic potential of brain enriched miRNA-128. *Mol. Cancer* 13:33. doi: 10.1186/1476-4598-13-33

Allman, J. M., Tetreault, N. A., Hakeem, A. Y., Manaye, K. F., Semendeferi, K., Erwin, J. M., et al. (2010). The von Economo neurons in frontoinsular and anterior cingulate cortex in great apes and humans. *Brain Struct. Funct.* 214, 495–517. doi: 10.1007/s00429-010-0254-0

Ameres, S. L., Horwich, M. D., Hung, J. H., Xu, J., Ghildiyal, M., Weng, Z., et al. (2010). Target RNA-directed trimming and tailing of small silencing RNAs. *Science* 328, 1534–1539. doi: 10.1126/science.1187058

Arcila, M. L., Betizeau, M., Cambronne, X. A., Guzman, E., Doerflinger, N., Bouhallier, F., et al. (2014). Novel primate miRNAs coevolved with ancient target genes in germinal zone-specific expression patterns. *Neuron* 81, 1255–1262. doi: 10.1016/j.neuron.2014.01.017

Baccarini, A., Chauhan, H., Gardner, T. J., Jayaprakash, A. D., Sachidanandam, R., and Brown, B. D. (2011). Kinetic analysis reveals the fate of a microRNA following target regulation in mammalian cells. *Curr. Biol.* 21, 369–376. doi: 10.1016/j.cub.2011.01.067

Bae, B. I., Jayaraman, D., and Walsh, C. A. (2015). Genetic changes shaping the human brain. *Dev. Cell* 32, 423–434. doi: 10.1016/j.devcel.2015.01.035

Barbash, S., Shifman, S., and Soreq, H. (2014). Global coevolution of human microRNAs and their target genes. *Mol. Biol. Evol.* 31, 1237–1247. doi: 10.1093/molbev/msu090

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nat. Rev. Genet.* 12, 846–860. doi: 10.1038/nrg3079

Berezikov, E., Thuemmler, F., van Laake, L. W., Kondova, I., Bontrop, R., Cuppen, E., et al. (2006). Diversity of microRNAs in human and chimpanzee brain. *Nature genetics* 38, 1375–1377. doi: 10.1038/ng1914

Bianchi, S., Stimpson, C. D., Bauernfeind, A. L., Schapiro, S. J., Baze, W. B., McArthur, M. J., et al. (2013). Dendritic morphology of pyramidal neurons in the chimpanzee neocortex: regional specializations and comparison to humans. *Cereb. Cortex* 23, 2429–2436. doi: 10.1093/cercor/bhs239

Birey, F., Andersen, J., Makinson, C. D., Islam, S., Wei, W., Huber, N., et al. (2017). Assembly of functionally integrated human forebrain spheroids. *Nature* 545, 54–59. doi: 10.1038/nature22330

Boissart, C., Nissan, X., Giraud-Triboult, K., Peschanski, M., and Benchoua, A. (2012). miR-125 potentiates early neural specification of human embryonic stem cells. *Development* 139, 1247–1257. doi: 10.1242/dev.073627

Boldog, E., Bakken, T. E., Hodge, R. D., Novotny, M., Aevermann, B. D., Baka, J., et al. (2018). Transcriptomic and morphophysiological evidence for a specialized human cortical GABAergic cell type. *Nat. Neurosci.* 21, 1185–1195. doi: 10.1038/s41593-018-0205-2

Buckner, R. L., and Krienen, F. M. (2013). The evolution of distributed association networks in the human brain. *Trends Cogn. Sci.* 17, 648–665. doi: 10.1016/j.tics.2013.09.017

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Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. doi: 10.1038/nbt.4096

Bystron, I., Rakic, P., Molnar, Z., and Blakemore, C. (2006). The first neurons of the human cerebral cortex. *Nat. Neurosci.* 9, 880–886. doi: 10.1038/nn1726

Cacchiarelli, D., Santoni, D., and Bozzoni, I. (2008). MicroRNAs as prime players in a combinatorial view of evolution. *RNA Biol.* 5, 120–122. doi: 10.4161/rna.5.3.6569

Chakraborty, S., Islam, M. R., Ali, M. M., and Nabi, A. (2018). Evolutionary divergence of brain-specific precursor miRNAs drives efficient processing and production of mature miRNAs in human. *Neuroscience* 392, 141–159. doi: 10.1016/j.neuroscience.2018.09.010

Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat. Biotechnol.* 27, 275–280. doi: 10.1038/nbt.1529

Chatterjee, S., Fasler, M., Büssing, I., and Grosshans, H. (2011). Target-mediated protection of endogenous microRNAs in *C. elegans. Dev. Cell* 20, 388–396. doi: 10.1016/j.devcel.2011.02.008

Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S., and Zhuang, X. (2015). RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 348:aaa6090. doi: 10.1126/science.aaa6090

Cimadamore, F., Amador-Arjona, A., Chen, C., Huang, C. T., and Terskikh, A. V. (2013). SOX2-LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors. *Proc. Natl. Acad. Sci. U S A* 110, E3017–E3026. doi: 10.1073/pnas.1220176110

Cohen, J. E., Lee, P. R., Chen, S., Li, W., and Fields, R. D. (2011). MicroRNA regulation of homeostatic synaptic plasticity. *Proc. Natl. Acad. Sci. U S A* 108, 11650–11655. doi: 10.1073/pnas.1017576108

Conaco, C., Otto, S., Han, J. J., and Mandel, G. (2006). Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc. Natl. Acad. Sci. U S A* 103, 2422–2427. doi: 10.1073/pnas.0511041103

Davis, G. M., Haas, M. A., and Pocock, R. (2015). MicroRNAs: not "fine-tuners" but key regulators of neuronal development and function. *Front. Neurol.* 6:245. doi: 10.3389/fneur.2015.00245

Davuluri, R. V., Suzuki, Y., Sugano, S., Plass, C., and Huang, T. H. (2008). The functional consequences of alternative promoter use in mammalian genomes. *Trends Genet.* 24, 167–177. doi: 10.1016/j.tig.2008.01.008

Dehay, C., Kennedy, H., and Kosik, K. S. (2015). The outer subventricular zone and primate-specific cortical complexification. *Neuron* 85, 683–694. doi: 10.1016/j.neuron.2014.12.060

Delaloy, C., Liu, L., Lee, J. A., Su, H., Shen, F., Yang, G. Y., et al. (2010). MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors. *Cell Stem Cell* 6, 323–335. doi: 10.1016/j.stem.2010.02.015

Dennis, M. Y., Nuttle, X., Sudmant, P. H., Antonacci, F., Graves, T. A., Nefedov, M., et al. (2012). Evolution of human-specific neural SRGAP2 genes by incomplete segmental duplication. *Cell* 149, 912–922. doi: 10.1016/j.cell.2012.03.033

Diederichs, S., and Haber, D. A. (2007). Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* 131, 1097–1108. doi: 10.1016/j.cell.2007.10.032

Duan, H., Wearne, S. L., Rocher, A. B., Macedo, A., Morrison, J. H., and Hof, P. R. (2003). Age-related dendritic and spine changes in corticocortically projecting neurons in macaque monkeys. *Cereb. Cortex* 13, 950–961. doi: 10.1093/cercor/13.9.950

Ebert, M. S., and Sharp, P. A. (2012). Roles for microRNAs in conferring robustness to biological processes. *Cell* 149, 515–524. doi: 10.1016/j.cell.2012.04.005

Elston, G. N., Benavides-Piccione, R., Elston, A., Manger, P. R., and Defelipe, J. (2011). Pyramidal cells in prefrontal cortex of primates: marked differences in neuronal structure among species. *Front. Neuroanat.* 5:2. doi: 10.3389/fnana.2011.00002

Evans, P. D., Anderson, J. R., Vallender, E. J., Choi, S. S., and Lahn, B. T. (2004). Reconstructing the evolutionary history of microcephalin, a gene controlling human brain size. *Hum. Mol. Genet.* 13, 1139–1145. doi: 10.1093/hmg/ddh126

Eyal, G., Verhoog, M. B., Testa-Silva, G., Deitcher, Y., Lodder, J. C., Benavides-Piccione, R., et al. (2016). Unique membrane properties and enhanced signal processing in human neocortical neurons. *Elife* 5:e16553. doi: 10.7554/eLife.16553

Ferland, R. J., Eyaid, W., Collura, R. V., Tully, L. D., Hill, R. S., Al-Nouri, D., et al. (2004). Abnormal cerebellar development and axonal decussation due to mutations in AHI1 in Joubert syndrome. *Nat. Genet.* 36, 1008–1013. doi: 10.1038/ng1419

Florio, M., Albert, M., Taverna, E., Namba, T., Brandl, H., Lewitus, E., et al. (2015). Human-specific gene ARHGAP11B promotes basal progenitor amplification and neocortex expansion. *Science* 347, 1465–1470. doi: 10.1126/science.aaa1975

Friedländer, M. R., Lizano, E., Houben, A. J., Bezdan, D., Banez-Coronel, M., Kudla, G., et al. (2014). Evidence for the biogenesis of more than 1,000 novel human microRNAs. *Genome Biol.* 15:R57. doi: 10.1186/gb-2014-15-4-r57

Fuchs Wightman, F., Giono, L. E., Fededa, J. P., and de la Mata, M. (2018). Target RNAs strike back on MicroRNAs. *Front. Genet.* 9:435. doi: 10.3389/fgene.2018.00435

Gai, M., Bianchi, F. T., Vagnoni, C., Vernì, F., Bonaccorsi, S., Pasquero, S., et al. (2016). ASPM and CITK regulate spindle orientation by affecting the dynamics of astral microtubules. *EMBO Rep.* 17, 1396–1409. doi: 10.15252/embr.201541823

Giandomenico, S. L., Mierau, S. B., Gibbons, G. M., Wenger, L. M. D., Masullo, L., Sit, T., et al. (2019). Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat. Neurosci.* 22, 669–679. doi: 10.1038/s41593-019-0350-2

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., et al. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* 106, 23–34. doi: 10.1016/s0092-8674(01)00431-7

Gulden, F. O., and Šestan, N. (2014). Neurobiology: building a bigger brain. *Nature* 515, 206–207. doi: 10.1038/515206a

Halevy, T., Czech, C., and Benvenisty, N. (2015). Molecular mechanisms regulating the defects in fragile X syndrome neurons derived from human pluripotent stem cells. *Stem Cell Rep.* 4, 37–46. doi: 10.1016/j.stemcr.2014.10.015

Han, K., Gennarino, V. A., Lee, Y., Pang, K., Hashimoto-Torii, K., Choufani, S., et al. (2013). Human-specific regulation of MeCP2 levels in fetal brains by microRNA miR-483–5p. *Genes Dev.* 27, 485–490. doi: 10.1101/gad.207456.112

Herculano-Houzel, S., Kaas, J. H., and de Oliveira-Souza, R. (2016). Corticalization of motor control in humans is a consequence of brain scaling in primate evolution. *J. Comp. Neurol.* 524, 448–455. doi: 10.1002/cne.23792

Herranz, H., and Cohen, S. M. (2010). MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. *Genes Dev.* 24, 1339–1344. doi: 10.1101/gad.1937010

Hoerder-Suabedissen, A., and Molnár, Z. (2015). Development, evolution and pathology of neocortical subplate neurons. *Nat. Rev. Neurosci.* 16, 133–146. doi: 10.1038/nrn3915

Hofman, M. A. (2012). Design principles of the human brain: an evolutionary perspective. *Prog. Brain Res.* 195, 373–390. doi: 10.1016/B978-0-444-53860-4.00018-0

Hornstein, E., and Shomron, N. (2006). Canalization of development by microRNAs. *Nat. Genet.* 38, S20–S24. doi: 10.1038/ng1803

Hrvatin, S., Hochbaum, D. R., Nagy, M. A., Cicconet, M., Robertson, K., Cheadle, L., et al. (2018). Single-cell analysis of experience-dependent transcriptomic states in the mouse visual cortex. *Nat. Neurosci.* 21, 120–129. doi: 10.1038/s41593-017-0029-5

Hu, H. Y., Guo, S., Xi, J., Yan, Z., Fu, N., Zhang, X., et al. (2011). MicroRNA expression and regulation in human, chimpanzee and macaque brains. *PLoS Genet.* 7:e1002327. doi: 10.1371/journal.pgen.1002327

Hu, H. Y., He, L., Fominykh, K., Yan, Z., Guo, S., Zhang, X., et al. (2012). Evolution of the human-specific microRNA miR-941. *Nat. Commun.* 3:1145. doi: 10.1038/ncomms2146

Hutsler, J. J., Lee, D. G., and Porter, K. K. (2005). Comparative analysis of cortical layering and supragranular layer enlargement in rodent carnivore and primate species. *Brain Res.* 1052, 71–81. doi: 10.1016/j.brainres.2005.06.015

Johnson, M. B., Kawasawa, Y. I., Mason, C. E., Krsnik, Z., Coppola, G., Bogdanovic, D., et al. (2009). Functional and evolutionary insights into human brain development through global transcriptome analysis. *Neuron* 62, 494–509. doi: 10.1016/j.neuron.2009.03.027

Junn, E., and Mouradian, M. M. (2012). MicroRNAs in neurodegenerative diseases and their therapeutic potential. *Pharmacol. Ther.* 133, 142–150. doi: 10.1016/j.pharmthera.2011.10.002

Ke, R., Mignardi, M., Pacureanu, A., Svedlund, J., Botling, J., Wahlby, C., et al. (2013). *in situ* sequencing for RNA analysis in preserved tissue and cells. *Nat. Methods* 10, 857–860. doi: 10.1038/nmeth.2563

Klein, M. E., Impey, S., and Goodman, R. H. (2005). Role reversal: the regulation of neuronal gene expression by microRNAs. *Curr. Opin. Neurobiol.* 15, 507–513. doi: 10.1016/j.conb.2005.08.011

Kocerha, J., Faghihi, M. A., Lopez-Toledano, M. A., Huang, J., Ramsey, A. J., Caron, M. G., et al. (2009). MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction. *Proc. Natl. Acad. Sci. U S A* 106, 3507–3512. doi: 10.1073/pnas.0805854106

Konopka, G., Friedrich, T., Davis-Turak, J., Winden, K., Oldham, M. C., Gao, F., et al. (2012). Human-specific transcriptional networks in the brain. *Neuron* 75, 601–617. doi: 10.1016/j.neuron.2012.05.034

Kosik, K. S. (2006). The neuronal microRNA system. *Nat. Rev. Neurosci.* 7, 911–920. doi: 10.1038/nrn2037

Krichevsky, A. M., Sonntag, K. C., Isacson, O., and Kosik, K. S. (2006). Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells* 24, 857–864. doi: 10.1634/stemcells.2005-0441

Lai, C. S., Fisher, S. E., Hurst, J. A., Vargha-Khadem, F., and Monaco, A. P. (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature* 413, 519–523. doi: 10.1038/35097076

Lake, B. B., Chen, S., Sos, B. C., Fan, J., Kaeser, G. E., Yung, Y. C., et al. (2018). Integrative single-cell analysis of transcriptional and epigenetic states in the human adult brain. *Nat. Biotechnol.* 36, 70–80. doi: 10.1038/nbt.4038

Lancaster, M. A., Renner, M., Martin, C. A., Wenzel, D., Bicknell, L. S., Hurles, M. E., et al. (2013). Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373–379. doi: 10.1038/nature12517

Lindsley, C. W., and Hopkins, C. R. (2012). Metabotropic glutamate receptor 4 (mGlu4)-positive allosteric modulators for the treatment of Parkinson's disease: historical perspective and review of the patent literature. *Expert Opin. Ther. Pat.* 22, 461–481. doi: 10.1517/13543776.2012.679437

Liu, H. K., Belz, T., Bock, D., Takacs, A., Wu, H., Lichter, P., et al. (2008). The nuclear receptor tailless is required for neurogenesis in the adult subventricular zone. *Genes Dev.* 22, 2473–2478. doi: 10.1101/gad.479308

Liu, J., Githinji, J., McLaughlin, B., Wilczek, K., and Nolta, J. (2012). Role of miRNAs in neuronal differentiation from human embryonic stem cell-derived neural stem cells. *Stem Cell Rev. Rep.* 8, 1129–1137. doi: 10.1007/s12015-012-9411-6

Lopez, J. P., Lim, R., Cruceanu, C., Crapper, L., Fasano, C., Labonte, B., et al. (2014). miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment. *Nat. Med.* 20, 764–768. doi: 10.1038/nm.3582

Lu, J., Shen, Y., Wu, Q., Kumar, S., He, B., Shi, S., et al. (2008). The birth and death of microRNA genes in *Drosophila*. *Nat. Genet.* 40, 351–355. doi: 10.1038/ng.73

Lubeck, E., Coskun, A. F., Zhiyentayev, T., Ahmad, M., and Cai, L. (2014). Single-cell *in situ* RNA profiling by sequential hybridization. *Nat. Methods* 11, 360–361. doi: 10.1038/nmeth.2892

Lui, J. H., Hansen, D. V., and Kriegstein, A. R. (2011). Development and evolution of the human neocortex. *Cell* 146, 18–36. doi: 10.1016/j.cell.2011.06.030

Magill, S. T., Cambronne, X. A., Luikart, B. W., Lioy, D. T., Leighton, B. H., Westbrook, G. L., et al. (2010). microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. *Proc. Natl. Acad. Sci. U S A* 107, 20382–20387. doi: 10.1073/pnas.1015691107

Makeyev, E. V., Zhang, J., Carrasco, M. A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* 27, 435–448. doi: 10.1016/j.molcel.2007.07.015

Marcinkowski, A., Tanguy, M., Krmpotic, A., Radle, B., Lisnic, V. J., Tuddenham, L., et al. (2012). Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication *in vivo*. *PLoS Pathog.* 8:e1002510. doi: 10.1371/journal.ppat.1002510

Marín-Padilla, M. (2014). The mammalian neocortex new pyramidal neuron: a new conception. *Front. Neuroanat.* 7:51. doi: 10.3389/fnana.2013.00051

Maze, I., Chaudhury, D., Dietz, D. M., Von Schimmelmann, M., Kennedy, P. J., Lobo, M. K., et al. (2014). G9a influences neuronal subtype specification in striatum. *Nat. Neurosci.* 17, 533–539. doi: 10.1038/nn.3670

Mellios, N., Feldman, D. A., Sheridan, S. D., Ip, J. P. K., Kwok, S., Amoah, S. K., et al. (2018). MeCP2-regulated miRNAs control early human neurogenesis through differential effects on ERK and AKT signaling. *Mol. Psychiatry* 23, 1051–1065. doi: 10.1038/mp.2017.86

Menon, V. (2018). Clustering single cells: a review of approaches on high-and low-depth single-cell RNA-seq data. *Brief. Funct. Genomics* 17, 240–245. doi: 10.1093/bfgp/elx044

Meunier, J., Lemoine, F., Soumillon, M., Liechti, A., Weier, M., Guschanski, K., et al. (2013). Birth and expression evolution of mammalian microRNA genes. *Genome Res.* 23, 34–45. doi: 10.1101/gr.140269.112

Miller, J. A., Ding, S. L., Sunkin, S. M., Smith, K. A., Ng, L., Szafer, A., et al. (2014). Transcriptional landscape of the prenatal human brain. *Nature* 508, 199–206. doi: 10.1038/nature13185

Molnár, G., Oláh, S., Komlósi, G., Füle, M., Szabadics, J., Varga, C., et al. (2008). Complex events initiated by individual spikes in the human cerebral cortex. *PLoS Biol.* 6:e222. doi: 10.1371/journal.pbio.0060222

Molnár, G., Rózsa, M., Baka, J., Holderith, N., Barzó, P., Nusser, Z., et al. (2016). Human pyramidal to interneuron synapses are mediated by multi-vesicular release and multiple docked vesicles. *Elife* 5:e18167. doi: 10.7554/eLife.18167

Muddashetty, R. S., Nalavadi, V. C., Gross, C., Yao, X., Xing, L., Laur, O., et al. (2011). Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation and mGluR signaling. *Mol. Cell* 42, 673–688. doi: 10.1016/j.molcel.2011.05.006

Murai, K., Sun, G., Ye, P., Tian, E., Yang, S., Cui, Q., et al. (2016). The TLX-miR-219 cascade regulates neural stem cell proliferation in neurodevelopment and schizophrenia iPSC model. *Nat. Commun.* 7:10965. doi: 10.1038/ncomms10965

Nilsen, T. W., and Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature* 463, 457–463. doi: 10.1038/nature08909

Nowakowski, T. J., Bhaduri, A., Pollen, A. A., Alvarado, B., Mostajo-Radji, M. A., Di Lullo, E., et al. (2017). Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. *Science* 358, 1318–1323. doi: 10.1126/science.aap8809

Nowakowski, T. J., Fotaki, V., Pollock, A., Sun, T., Pratt, T., and Price, D. J. (2013). MicroRNA-92b regulates the development of intermediate cortical progenitors in embryonic mouse brain. *Proc. Natl. Acad. Sci. U S A* 110, 7056–7061. doi: 10.1073/pnas.1219385110

Nowakowski, T. J., Rani, N., Golkaram, M., Zhou, H. R., Alvarado, B., Huch, K., et al. (2018). Regulation of cell-type-specific transcriptomes by microRNA networks during human brain development. *Nat. Neurosci.* 21, 1784–1792. doi: 10.1038/s41593-018-0265-3

Nudelman, A. S., DiRocco, D. P., Lambert, T. J., Garelick, M. G., Le, J., Nathanson, N. M., et al. (2010). Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, *in vivo*. *Hippocampus* 20, 492–498. doi: 10.1002/hipo.20646

Oberheim, N. A., Goldman, S. A., and Nedergaard, M. (2012). Heterogeneity of astrocytic form and function. *Methods Mol. Biol.* 814, 23–45. doi: 10.1007/978-1-61779-452-0_3

Otani, T., Marchetto, M. C., Gage, F. H., Simons, B. D., and Livesey, F. J. (2016). 2D and 3D stem cell models of primate cortical development identify species-specific differences in progenitor behavior contributing to brain size. *Cell Stem Cell* 18, 467–480. doi: 10.1016/j.stem.2016.03.003

Paşca, A. M., Sloan, S. A., Clarke, L. E., Tian, Y., Makinson, C. D., Huber, N., et al. (2015). Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat. Methods* 12, 671–678. doi: 10.1038/nmeth.3415

Paul, A., Crow, M., Raudales, R., He, M., Gillis, J., and Huang, Z. J. (2017). Transcriptional architecture of synaptic communication delineates GABAergic neuron identity. *Cell* 171, 522.e20–539.e20. doi: 10.1016/j.cell.2017.08.032

Pletikos, M., Sousa, A. M., Sedmak, G., Meyer, K. A., Zhu, Y., Cheng, F., et al. (2014). Temporal specification and bilaterality of human neocortical topographic gene expression. *Neuron* 81, 321–332. doi: 10.1016/j.neuron.2013.11.018

Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., et al. (2016). Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell* 165, 1238–1254. doi: 10.1016/j.cell.2016.04.032

Quadrato, G., Nguyen, T., Macosko, E. Z., Sherwood, J. L., Min Yang, S., Berger, D. R., et al. (2017). Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* 545, 48–53. doi: 10.1038/nature22047

Raghanti, M. A., Edler, M. K., Stephenson, A. R., Wilson, L. J., Hopkins, W. D., Ely, J. J., et al. (2016). Human-specific increase of dopaminergic innervation in a striatal region associated with speech and language: a comparative analysis of the primate basal ganglia. *J. Comp. Neurol.* 524, 2117–2129. doi: 10.1002/cne.23937

Raghanti, M. A., Stimpson, C. D., Marcinkiewicz, J. L., Erwin, J. M., Hof, P. R., and Sherwood, C. C. (2008). Differences in cortical serotonergic innervation among humans, chimpanzees and macaque monkeys: a comparative study. *Cereb. Cortex* 18, 584–597. doi: 10.1093/cercor/bhm089

Raj, A., and van Oudenaarden, A. (2008). Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* 135, 216–226. doi: 10.1016/j.cell.2008.09.050

Rockel, A. J., Hiorns, R. W., and Powell, T. P. (1980). The basic uniformity in structure of the neocortex. *Brain* 103, 221–244. doi: 10.1093/brain/103.2.221

Rogers, J., Kochunov, P., Zilles, K., Shelledy, W., Lancaster, J., Thompson, P., et al. (2010). On the genetic architecture of cortical folding and brain volume in primates. *Neuroimage* 53, 1103–1108. doi: 10.1016/j.neuroimage.2010.02.020

Rupaimoole, R., and Slack, F. J. (2017). MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat. Rev. Drug Discov.* 16, 203–222. doi: 10.1038/nrd.2016.246

Saliminejad, K., Khorram Khorshid, H. R., Soleymani Fard, S., and Ghaffari, S. H. (2019). An overview of microRNAs: biology, functions, therapeutics and analysis methods. *J. Cell. Physiol.* 234, 5451–5465. doi: 10.1002/jcp.27486

Salmén, F., Ståhl, P. L., Mollbrink, A., Navarro, J. F., Vickovic, S., Frisen, J., et al. (2018). Barcoded solid-phase RNA capture for spatial transcriptomics profiling in mammalian tissue sections. *Nat. Protoc.* 13, 2501–2534. doi: 10.1038/s41596-018-0045-2

Satija, R., Farrell, J. A., Gennert, D., Schier, A. F., and Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nat. Biotechnol.* 33, 495–502. doi: 10.1038/nbt.3192

Schratt, G. (2009). Fine-tuning neural gene expression with microRNAs. *Curr. Opin. Neurobiol.* 19, 213–219. doi: 10.1016/j.conb.2009.05.015

Sherwood, C. C., Holloway, R. L., Erwin, J. M., and Hof, P. R. (2004). Cortical orofacial motor representation in Old World monkeys, great apes and humans: II. Stereologic analysis of chemoarchitecture. *Brain Behav. Evol.* 63, 82–106. doi: 10.1159/000075673

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R. T., et al. (2004). Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature* 427, 78–83. doi: 10.1038/nature02211

Shi, Y., Kirwan, P., Smith, J., Robinson, H. P., and Livesey, F. J. (2012). Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat. Neurosci.* 15, 477–486. doi: 10.1038/nn.3041

Silbereis, J. C., Pochareddy, S., Zhu, Y., Li, M., and Sestan, N. (2016). The cellular and molecular landscapes of the developing human central nervous system. *Neuron* 89, 248–268. doi: 10.1016/j.neuron.2015.12.008

Smart, I. H., Dehay, C., Giroud, P., Berland, M., and Kennedy, H. (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb. Cortex* 12, 37–53. doi: 10.1093/cercor/12.1.37

Søkilde, R., Newie, I., Persson, H., Borg, Å., and Rovira, C. (2015). Passenger strand loading in overexpression experiments using microRNA mimics. *RNA Biol.* 12, 787–791. doi: 10.1080/15476286.2015.1020270

Somel, M., Liu, X., Tang, L., Yan, Z., Hu, H., Guo, S., et al. (2011). MicroRNA-driven developmental remodeling in the brain distinguishes humans from other primates. *PLoS Biol.* 9:e1001214. doi: 10.1371/journal.pbio.1001214

Sosanya, N. M., Huang, P. P., Cacheaux, L. P., Chen, C. J., Nguyen, K., Perrone-Bizzozero, N. I., et al. (2013). Degradation of high affinity HuD targets releases Kv1.1 mRNA from miR-129 repression by mTORC1. *J. Cell Biol.* 202, 53–69. doi: 10.1083/jcb.201212089

Sousa, A. M. M., Meyer, K. A., Santpere, G., Gulden, F. O., and Sestan, N. (2017a). Evolution of the human nervous system function, structure and development. *Cell* 170, 226–247. doi: 10.1016/j.cell.2017.06.036

Sousa, A. M. M., Zhu, Y., Raghanti, M. A., Kitchen, R. R., Onorati, M., Tebbenkamp, A. T. N., et al. (2017b). Molecular and cellular reorganization of neural circuits in the human lineage. *Science* 358, 1027–1032. doi: 10.1126/science.aan3456

Stuart, T., and Satija, R. (2019). Integrative single-cell analysis. *Nat. Rev. Genet.* 20, 257–272. doi: 10.1038/s41576-019-0093-7

Szegedi, V., Paizs, M., Csakvari, E., Molnar, G., Barzo, P., Tamas, G., et al. (2016). Plasticity in single axon glutamatergic connection to GABAergic interneurons regulates complex events in the human neocortex. *PLoS Biol.* 14:e2000237. doi: 10.1371/journal.pbio.2000237

Tasic, B., Menon, V., Nguyen, T. N., Kim, T. K., Jarsky, T., Yao, Z., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat. Neurosci.* 19, 335–346. doi: 10.1038/nn.4216

Tasic, B., Yao, Z., Graybuck, L. T., Smith, K. A., Nguyen, T. N., Bertagnolli, D., et al. (2018). Shared and distinct transcriptomic cell types across neocortical areas. *Nature* 563, 72–78. doi: 10.1038/s41586-018-0654-5

Taverna, E., Götz, M., and Huttner, W. B. (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu. Rev. Cell Dev. Biol.* 30, 465–502. doi: 10.1146/annurev-cellbio-101011-155801

Testa-Silva, G., Verhoog, M. B., Goriounova, N. A., Loebel, A., Hjorth, J., Baayen, J. C., et al. (2010). Human synapses show a wide temporal window for spike-timing-dependent plasticity. *Front. Synaptic Neurosci.* 2:12. doi: 10.3389/fnsyn.2010.00012

Thiebes, K. P., Nam, H., Cambronne, X. A., Shen, R., Glasgow, S. M., Cho, H. H., et al. (2015). miR-218 is essential to establish motor neuron fate as a downstream effector of Isl1-Lhx3. *Nat. Commun.* 6:7718. doi: 10.1038/ncomms8718

Tu, J., Cao, D., Li, L., Cheung, H. H., and Chan, W. Y. (2018). MicroRNA profiling during directed differentiation of cortical interneurons from human-induced pluripotent stem cells. *FEBS Open Bio* 8, 502–512. doi: 10.1002/2211-5463.12377

van Rooij, E., Purcell, A. L., and Levin, A. A. (2012). Developing microRNA therapeutics. *Circ. Res.* 110, 496–507. doi: 10.1161/CIRCRESAHA.111.247916

Verhoog, M. B., Goriounova, N. A., Obermayer, J., Stroeder, J., Hjorth, J. J., Testa-Silva, G., et al. (2013). Mechanisms underlying the rules for associative plasticity at adult human neocortical synapses. *J. Neurosci.* 33, 17197–17208. doi: 10.1523/jneurosci.3158-13.2013

Visvanathan, J., Lee, S., Lee, B., Lee, J. W., and Lee, S. K. (2007). The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev.* 21, 744–749. doi: 10.1101/gad.1519107

Wang, B., Yin, L., Zou, X., Ye, M., Liu, Y., He, T., et al. (2015). A subtype of inhibitory interneuron with intrinsic persistent activity in human and monkey neocortex. *Cell Rep.* 10, 1450–1458. doi: 10.1016/j.celrep.2015.02.018

Winter, J., and Diederichs, S. (2011). Argonaute proteins regulate microRNA stability: increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. *RNA Biol.* 8, 1149–1157. doi: 10.4161/rna.8.6.17665

Winter, J., Jung, S., Keller, S., Gregory, R. I., and Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* 11, 228–234. doi: 10.1038/ncb0309-228

Wood, H. (2018). FDA approves patisiran to treat hereditary transthyretin amyloidosis. *Nat. Rev. Neurol.* 14:570. doi: 10.1038/s41582-018-0065-0

Xiang, Y., Tanaka, Y., Cakir, B., Patterson, B., Kim, K. Y., Sun, P., et al. (2019). hESC-derived thalamic organoids form reciprocal projections when fused with cortical organoids. *Cell Stem Cell* 24, 487.e7–497.e7. doi: 10.1016/j.stem.2018.12.015

Xiang, Y., Tanaka, Y., Patterson, B., Kang, Y. J., Govindaiah, G., Roselaar, N., et al. (2017). Fusion of regionally specified hPSC-derived organoids models human brain development and interneuron migration. *Cell Stem Cell* 21, 383.e7–398.e7. doi: 10.1016/j.stem.2017.07.007

Yang, L., Yang, Y., Yuan, J., Sun, Y., Dai, J., and Su, B. (2019). Transcriptomic landscape of von economo neurons in human anterior cingulate cortex revealed by microdissected-cell RNA sequencing. *Cereb. Cortex* 29, 838–851. doi: 10.1093/cercor/bhy286

Yoo, A. S., Staahl, B. T., Chen, L., and Crabtree, G. R. (2009). MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. *Nature* 460, 642–646. doi: 10.1038/nature08139

Zhang, C., Lu, J., Liu, B., Cui, Q., and Wang, Y. (2016). Primate-specific miR-603 is implicated in the risk and pathogenesis of Alzheimer's disease. *Aging* 8, 272–290. doi: 10.18632/aging.100887

Zhang, C. L., Zou, Y., He, W., Gage, F. H., and Evans, R. M. (2008). A role for adult TLX-positive neural stem cells in learning and behaviour. *Nature* 451, 1004–1007. doi: 10.1038/nature06562

Zhao, C., Sun, G., Li, S., and Shi, Y. (2009). A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat. Struct. Mol. Biol.* 16, 365–371. doi: 10.1038/nsmb.1576

Zhao, X., Wu, J., Zheng, M., Gao, F., and Ju, G. (2012). Specification and maintenance of oligodendrocyte precursor cells from neural progenitor cells: involvement of microRNA-7a. *Mol. Biol. Cell* 23, 2867–2878. doi: 10.1091/mbc.e12-04-0270

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J., et al. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature* 555, 524–528. doi: 10.1038/nature25980

Zhu, Y., Sousa, A. M. M., Gao, T., Skarica, M., Li, M., Santpere, G., et al. (2018). Spatiotemporal transcriptomic divergence across human and macaque brain development. *Science* 362:eaat8077. doi: 10.1126/science.aat8077

Ziats, M. N., and Rennert, O. M. (2014). Identification of differentially expressed microRNAs across the developing human brain. *Mol. Psychiatry* 19, 848–852. doi: 10.1038/mp.2013.93

# L1CAM Is a Marker for Enriching Corticospinal Motor Neurons in the Developing Brain

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The cerebral cortical tissue of murine embryo and pluripotent stem cell-derived neurons can survive in the adult brain and extend axons to the spinal cord. These features suggest that cell transplantation can be a strategy to reconstruct the corticospinal tract (CST). It is unknown, however, which cell population makes for safe and effective donor cells. To address this issue, we grafted the cerebral cortex of E14.5 mouse to the brain of adult mice and found that the cells in the graft extending axons along the CST expressed CTIP2. By using CTIP2:GFP knock-in mouse embryonic stem cells (mESCs), we identified L1CAM as a cell surface marker to enrich CTIP2+ cells. We sorted L1CAM+ cells from E14.5 mouse brain and confirmed that they extended a larger number of axons along the CST compared to L1CAM− cells. Our results suggest that sorting L1CAM+ cells from the embryonic cerebral cortex enriches subcortical projection neurons to reconstruct the CST.

Keywords: L1 cell adhesion molecule, corticospinal motor neurons, transplantation, corticospinal tract, cell sorting

## INTRODUCTION

Reconstruction of the corticospinal tract (CST) by cell transplantation is one of the main strategies to treat brain injury and stroke. Previous studies have reported that behavioral improvement was observed after transplantation of the embryonic cerebral cortex in rat models with brain injury or middle cerebral artery occlusion (Plumet et al., 1993; Grabowski et al., 1995; Mattsson et al., 1997; Riolobos et al., 2001). Furthermore, retrograde labeling of the CST revealed that the grafted cortical tissue contributed to the reconstruction of the adult brain (Gaillard et al., 2007). Recently, cerebral neurons have been induced from both mouse embryonic stem cells (mESCs) and human-induced pluripotent stem cells (hiPSCs) by recapitulating corticogenesis (Gaspard et al., 2008; Espuny-Camacho et al., 2013). When grafted into the frontal cortex, the induced neurons extend their axons to the corresponding targets and integrate into the host brain (Espuny-Camacho et al., 2013). Thus, reconstruction of the CST by PSC-derived neurons can be a novel therapy for brain injury and stroke.

The developing cerebral cortex is a complex structure consisting of various neurons and neural progenitors. When fetal cortical tissue is grafted into the frontal cortex, most surviving neurons extend axons to other cortical areas of the brain (callosal projections), with only a small population extending axons beyond the cortex (subcerebral projections; Ballout et al., 2016).

Furthermore, there is a risk of tumor formation by the progenitor cells, especially in the case of PSCs. Thus, for successful cell-based therapy, it is important to enrich subcerebral projection neurons, especially corticospinal motor neurons (CSMNs), as the donor cell population.

The sorting of dopaminergic progenitors has been shown to be useful for cell-based Parkinson's disease therapies in terms of safety and efficiency (Doi et al., 2014; Samata et al., 2016). However, there are no reports about a similar method for the sorting of CSMN progenitor cells. Here, we identified a cell surface marker for such progenitor cells and show sorting with this marker enhances the survival of donor cells in the brain and extension of axons along the CST after transplantation.

## MATERIALS AND METHODS

### Animals

All animal experiments were performed according to the guidelines for Animal Experiment of Kyoto University, the guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (ILAR; Washington, DC, USA) and the guideline for the Animals in Research: Reporting *in vivo* Experiments (ARRIVE). Sixteen week-*old* female nude rats (*F344/NJcl-rnu/rnu*) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Nine week-*old* male mice (*C57BL/6NCrSlc*) and pregnant female mice (*C57BL/6NCrSlc* and *C57BL/6 6-Tg*) were obtained from Shimizu Laboratory Supplies Company Limited (Kyoto, Japan). All animals were housed under diurnal lighting conditions (12 h light/12 h dark) and given standard food and water *ad libitum*.

### Transplantation Into Adult Animals

We anesthetized the animals with isoflurane (Intervet Inc., Tokyo, Japan), and the motor cortex was aspirated from 0.5 to 2.0 mm lateral to the midline and from 0.5 to 2.0 mm rostral to the Bregma on the corpus callosum of the left hemisphere of each donor's brain. Seven days after the aspiration, we isolated E14.5 mouse frontal cortex from GFP Tg mice and enzymatically digested the cortices using Accumax (Innovative Cell Technologies, Inc., San Diego, CA, USA) for 10 min at 37°C. The cell density was adjusted to $10^5$ cells/µl in DMEM/F12 [Fujifilm Wako Pure Chemical Corporation (Fujifilm)., Osaka, Japan] supplemented with 2 mM L-glutamine (L-Gln; Merck Ltd., Tokyo, Japan), 0.1 mM 2-mercaptoethanol (2-ME; Fujifilm), 1% (vol/vol) N2 supplement (Thermo Fisher Scientific Inc., Tokyo, Japan), 2% (vol/vol) B-27 supplement (Thermo Fisher Scientific), 1% (vol/vol) Penicillin-Streptomycin (PS; Merck) and 10 µM Y-27632 (Fujifilm). The cell suspension (2 µl/site) was injected into the host brain. Before the sorted cell aggregates were injected into the brain, they were replated in a prime surface 96-well plate (Sumitomo Bakelite Company Limited, Tokyo, Japan) at a density of $3 \times 10^4$ cells per well in 200 µl of DMEM/F12 containing 2 mM L-Gln, 0.1 mM 2-ME, 1% N2 supplement, 2% B-27 supplement, 1% PS and 10 µM Y-27632. Two days later, the cell aggregates were adjusted to $2 \times 10^5$ cells (approximately 7–11 cell aggregates) and were

placed into the host brain. After the transplantation, the animals were sacrificed and prepared for immunofluorescence studies.

### Retrograde Labeling

Fast blue (FB; Polysciences Inc., Warrington, PA, USA) was used for the retrograde labeling studies. To label cells that extend axons along the CST, FB solution containing 4% (vol/wt) FB, 4% (vol/vol) dimethyl sulfoxide (Merck) and artificial cerebrospinal fluid (Harvard Apparatus Inc., Holliston, MA, USA) was injected into the pyramidal decussation 7 days before sacrifice.

### Immunostaining

To stain the brain slices, animals transplanted with mouse cortex or sorted cells were sacrificed with pentobarbital (Tokyo Chemical Industry Company Limited, Tokyo, Japan) and perfused with 4% paraformaldehyde (PFA; Fujifilm) overnight, and then replaced with 10% (wt/vol) and 20% (wt/vol) sucrose (Nacalai Tesque Inc., Kyoto, Japan) in PBS overnight, respectively. The fixed brains were embedded in OCT compound (Sakura Finetec Japan Company Limited, Tokyo, Japan) and cut with a cryostat (CM-3050; Leica Inc.) at 16–30 µm thickness. The brain slices were placed into distilled water (DW; Thermo Fisher Scientific) containing 30% (vol/vol) ethylene glycol (Fujifilm), 30% (vol/vol) glycerol (Nacalai Tesque), 0.243% (wt/vol) $NaH_2PO_4$, 0.874% (wt/vol) $Na_2HPO_4$ and 0.34% (wt/vol) NaCl at −20°C until use. To stain the cell aggregates, the cell aggregates were perfused with 4% PFA for 15 min and then replaced with 10% (wt/vol) sucrose in PBS overnight. The fixed cell aggregates were embedded in OCT compound and cut with a cryostat at 16 µm thickness. The slices were attached on the surface of a MAS-coated slide glass (Matsunami Glass Inc Ltd., Osaka, Japan) and stocked at −20°C until use. To stain the cultured cells, the cells were perfused with 4% PFA for 15 min and washed with PBS. The fixed cells were stocked at 4°C until use.

The samples were permeabilized in PBS containing 2% (vol/vol) Triton X-100 (PBST; Nacalai Tesque) for 30 min. Then the samples were blocked with PBS containing 4% (wt/vol) Block Ace (Sumitomo Dainippon Pharma Company Limited, Tokyo, Japan) and 0.1% (vol/vol) Triton X-100 for 30 min. The primary antibodies were diluted in 0.1% (vol/vol) PBST containing 4% (wt/vol) Block Ace and incubated overnight at 4°C. The samples were washed with 0.1% (vol/vol) PBST and incubated with secondary antibodies in 0.1% (vol/vol) PBST containing 4% (wt/vol) Block Ace for 60 min at room temperature (RT), followed by washing with 0.1% PBST and incubating with 4′-6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for 5 min at RT. Finally, the samples were mounted with DW containing 44% (vol/vol) 200 mM Tris-HCl (Nacalai Tesque), 22% (vol/vol) glycerol (Fujifilm), 0.02% (wt/vol) 1,4-diazabicyclo (2,2,2) octane (DABCO; Fujifilm) and 0.09% (wt/vol) Mowiol 4–88 reagent (Merck).

The primary antibodies used are as follows: anti-GFP (1:1,000; #598, Medical and Biological Laboratories Company Limited, Nagoya, Japan and 1:1,000; #04404-26, Nacalai Tesque), anti-CTIP2 (1:200; #12120S, Cell Signaling Technology Inc., Tokyo, Japan and 1:1,000; #ab18465, Abcam Inc., Cambridge, MA, USA), anti-FOXG1 (1:500; #ab18259, Abcam),

anti-GSH2 (1:5,000; a kind gift from Dr. Mototsugu Eiraku, RIKEN, CDB), anti-M2 [1:100; #AB531785, Developmental Studies Hybridoma Bank (DSHB)], anti-M6 (1:100; AB2149607, DSHB), anti-L1CAM (1:1,000; #MAB5674, R&D Systems, Inc., Minneapolis, MN, USA; 1:500; #554273, BD Biosciences, San Jose, CA, USA), anti-NRP1 (1:500; #NP2111, ECM Biosciences, Versailles, KY, USA), anti-TBR2 (1:500; #ab23345, Abcam), anti-PAX6 (1:1,000; PRB-278P, BioLegend, San Diego, CA, USA; 1:500; #561462, BD Biosciences) and anti-CUX1 (1:200; #sc-13024, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alexa fluorescent-conjugated antibodies (1:500; Thermo Fisher Scientific) were used as secondary antibodies.

## Production of CTIP2:GFP Knockin (KI) mESC Line

A CTIP2:GFP KI targeting vector was assembled using pBS-IRES-GFP-polyA-neo-DTA. A 4-kb 5′-arm genomic fragment containing an ORF region of the exon 4 of the Ctip2 gene and a 3.5-kb 3′-arm fragment just downstream of the stop codon of Ctip2 were amplified by PCR using 129SV genomic DNA as a template and separately cloned into the NotI/XhoI and SalI/SpeI sites of pBS-IRES-GFP-polyA-neo-DTA to generate the targeting vector. CTIP2:GFP KI mESCs were generated by homologous recombination in the 129SVEV ESC line according to standard procedures and genotyped by PCR. Because the presence of the Neo cassette did not affect reporter gene expression in a similar Bcl11b-YFP KI mouse (Kueh et al., 2016), we used CTIP2:GFP KI ESCs containing the Neo cassette.

## Cell Culture

mESCs (EB5; passages 35–45) and CTIP2:GFP KI mESCs (passages 11–21) were maintained on a mitotically inactivated mouse embryonic fibroblast feeder layer in KnockOut DMEM (Thermo Fisher Scientific) supplemented with 20% (vol/vol) Fetal Bovine Serum (FBS; Merck), 1% PS, 0.1 mM 2-ME, 2 mM L-Gln, 2,000 U ml$^{-1}$ Leukocyte Inhibitory Factor (LIF; Merck) and 1% (vol/vol) Nucleosides (Merck). The culture medium was replaced with fresh medium every day. To induce cortical neurons, the mESCs were replated in prime surface 96-well plates (Sumitomo Bakelite) at a density of 9,000 cells per well in differentiation medium containing GMEM (Thermo Fisher Scientific) supplemented with 10% (vol/vol) KnockOut Serum Replacement (KSR; Thermo Fisher Scientific), 0.1 mM MEM non-essential amino acids solution (NEAA; Thermo Fisher Scientific), 0.1 mM 2-ME, 1 mM sodium pyruvate solution (Pyruvate; Merck) and 2 mM L-Gln. 10 μM SB-431542 (Merck), which is a TGF-β receptor inhibitor, and 20 nM WNT-C59 (Collagen Technology, San Diego, CA, USA), which is a WNT inhibitor, were added by day 6. On day 7, we switched the medium from GMEM to DMEM/F12 (Fujifilm) supplemented with 0.1 mM 2-ME, 2 mM L-Gln, 1% N2 supplement and 2% B-27 supplement. Half of the medium was replaced with fresh medium every 3 days.

## Quantitative PCR (qPCR)

Total RNA was extracted from mouse embryos and cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) or RNeasy Micro Kit (Qiagen). The cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The quantitative PCR (qPCR) was performed with SYBR Premix Ex Taq (Takara Bio) and with the Thermal Cycler Dice Real-Time System (Takara Bio). The data were analyzed using the delta-delta Ct method and normalized to Gapdh levels. Primers were designed by using prime3 plus, and the sequences were as follows: mGapdh, forward 5′-TGTT CCTACCCCCAATGTGTC-3′, reverse 5′-TAGCCCAAGATG CCCTTCAG-3′; mFoxg1, forward 5′-ACCCTGCCCTGTGA GTCTTT-3′, reverse 5′-GACCCCTGATTTTGATGTGTG-3′; mReelin, forward 5′-GCCACTGCTTACTCGCACCT-3′, reverse 5′-GCCACACTGCTCTCCCATCT-3′; mCtip2, forward 5′-TT GGATGCCAGTGTGAGTTG-3′, reverse 5′-ATGTGTGTTCTG TGCGTGCT-3′; mCux1, forward 5′-TCCTGGAACAAGCC AAGAGG-3′, reverse 5′-CTGTAGGATGGAGCGGATGG-3′; mNrp1, forward 5′-CCGCCTGAACTACCCTGAAA-3′, reverse 5′-CACCCTGTGTCCCTACAGCA-3′; mTbr2, forward 5′-TG TGACGGCCTACCAAAACA-3′, reverse 5′-GTACCGACCTCC AGGGACAA-3′; and mPax6, forward 5′-GTGCCCTTCCATCT TTGCTT-3′, reverse 5′-CGCCCATCTGTTGCTTTTC-3′.

## Cell Sorting

Cell suspensions were prepared by using Accumax, and then the cells were resuspended in PBS containing 2% FBS, 20 mM D(+)-glucose (Fujifilm) and 1% PS. The samples were stained with anti-L1CAM antibody for 20 min. After primary antibody reactions, the samples were stained with Alexa fluorescent-conjugated antibodies (1:400) for 20 min. Dead cells were labeled with 7-amino-actinomycin D (7AAD; BD Biosciences). Cell sorting was performed using a FACS Aria II (BD Biosciences), and the data were analyzed with FACS Diva software (BD Biosciences).

After sorting, mouse cells were cultured in DMEM/F12 containing 2 mM L-Gln, 1% N2 supplement, 2% B-27 supplement, 1% PS, 20 ng ml$^{-1}$ BDNF (Fujifilm), 10 μg ml$^{-1}$ GDNF (Fujifilm) and 30 μM Y-27632. For in vitro studies, the sorted cells were cultured on chambered cell culture slides (Thermo Fisher Scientific) coated with poly-L-ornithine (50 μg ml$^{-1}$, Merck), laminin (5 μg ml$^{-1}$, Thermo Fisher Scientific) and fibronectin (5 μg ml$^{-1}$, Merck). For in vivo studies, we cultured the sorted cells for 2 days before transplantation, because a lot of cells were dead or dying immediately after sorting and the efficiency was low and unstable. The sorted cells were replated in low cell adhesion 96-well plates at a density of $3 \times 10^4$ cells per well. Half of the culture medium was replaced with fresh medium every 3 days.

## Microarray Analysis

Total RNA was extracted using the RNeasy Mini Kit. The samples were subjected to microarray analysis using GeneChip Mouse Gene 1.0 ST Arrays (Thermo Fisher Scientific). The arrays were scanned using the Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA). The data were analyzed using the GeneSpring software program (Agilent Technologies). The expression signals of the probe sets were calculated using

RMA16. The microarray data are available from the Gene Expression Omnibus (GEO database) with the accession number GSE132362.

## EdU Incorporation Assay

Ten microgram EdU (Thermo Fisher Scientific) was added into the culture medium at 2 h before fixation. The detection of EdU incorporation into the DNA was performed with the Click-iT Plus Alexa Fluor 647 Cell Proliferation Assay Kit (Thermo Fisher Scientific). Fixed cells were incubated with 0.3% PBST for 30 min at RT. The Click-iT reaction cocktail was prepared according to the manufacturer's instruction. The samples were incubated with the Click-iT reaction cocktail for 30 min at RT. After washing, the samples were subjected to immunostaining procedure.

## RNA Fluorescence *in situ* Hybridization (FISH)

Mouse embryos were fixed in PBS containing 4% PFA overnight at 4°C. Fixed samples were dehydrated in PBS containing 15% sucrose overnight at 4°C. Subsequently, the samples were sectioned with a cryostat at 16 μm thickness and attached to a MAS-coated slide glass. RNA FISH was performed using the RNAscope Multiplex Fluorescent v2 Kit (Advanced Cell Diagnostics Inc., Hayward, CA, USA). Sample slides were boiled with target retrieval buffer for 3 min, rinsed in 99.5% ethanol (Fujifilm) for 3 min, and then air-dried. The sample slides were subjected to protease digestion for 15 min at 40°C and incubated with RNAscope oligonucleotide probes (*Ctip2*, NM_021399.2) for 2 h at 40°C. After hybridization, the sample slides were incubated with AMP1 and AMP2 sequentially for 30 min each at 40°C. Subsequently, the sample slides were incubated with AMP3 for 15 min at 40°C. Finally, the sample slides were labeled with OPAL 590 (Perkin Elmer Japan Company Limited, Yokohama, Japan) for 30 min at 40°C. The reaction was stopped with an HRP blocker for 30 min at 40°C. After washing, the sample slides were subjected to immunostaining.

## Imaging and Data Analysis

Images were visualized using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), In Cell Analyzer 6000 (GE Healthcare) and confocal laser microscope (Fluoview FV1000D; Olympus). To measure cell counts, immunopositive cells were manually counted for at least three independent samples to calculate the age of positive cells for each marker. The number of immunopositive cells in the graft was quantified in every 6 sections and corrected. To measure the graft size, low magnified GFP images were imported into the BZ-II Analyzer software (Keyence), and the graft areas were quantified every six sections. The estimated graft volumes were calculated based on the thickness of the brain slices. The number of axons derived from a graft was counted in the coronal section at the internal capsule and the cerebral peduncle. The site of interest in each animal was labeled using an anti-GFP antibody, and the mean number of axons was recorded.

## Statistical Analysis

Statistical analysis was performed using a software package (GraphPad Prism 7; GraphPad). Data from the *in vitro* and *in vivo* experiments were analyzed by Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison tests. The data were considered statistically significant for $p < 0.05$ and are shown as the mean ± standard error of the mean (SEM). All data were acquired from at least three independent experiments.

# RESULTS

## The Frontal Cortex of E14.5 Mouse Contains CSMNs and Their Progenitors

To identify which cells extend axons along the CST, we isolated the cerebral cortices of GFP transgenic (Tg) mice at embryonic day (E) 14.5 (Okabe et al., 1997) and transplanted the dissociated tissue into the frontal lobe of adult mice (**Figure 1A**). Two months after the transplantation, we performed immunohistological analyses of the brain. GFP+ graft-derived fibers were observed along the CST at the corpus callosum, internal capsule, pons, medulla oblongata and pyramidal decussation (**Figures 1B,C**). Seven days prior to sacrifice, we injected a retrograde axonal tracer, FB, into the pyramidal decussation and found it labeled cells in layer V of the frontal lobe (**Figure 1D**). This observation is consistent with CSMNs residing in cortical layer V. A subpopulation of FB+ cells expressed GFP, and all GFP+/FB+ cells expressed CTIP2, which is a marker for layer V neurons and plays a critical role in the development of CSMN axonal projections to the spinal cord (Arlotta et al., 2005; **Figure 1E**). These results indicate that the frontal cortex of E14.5 mouse contains cells that extend their axons along the CST and that these cells express CTIP2.

## Mouse ESC-Derived CTIP2:GFP+ Cells Have Characteristics of CSMNs

To investigate the characteristics of CTIP2+ cells, we generated CTIP2:GFP knock-in (KI) mESCs and differentiated them into neural lineage by inhibiting WNT and TGF/Activin/Nodal signaling in a floating culture of cell aggregates (**Figure 2A**; Motono et al., 2016). The sphere size gradually increased, and GFP expression became detectable by around day 12 (**Figure 2B**). Temporal gene expression analyses revealed that the mRNA levels of *Foxg1* (telencephalic progenitors), *Reelin* (layer I), *Ctip2* (deep layer) and *Cux1* (upper layer) were gradually increased along with the differentiation (**Figure 2C**). Immunofluorescence studies revealed that >95% of the GFP expression of day-12 spheres was colocalized with the expressions of CTIP2 and FOXG1 (**Figures 2D,E**). In the telencephalon of the mouse embryo, CTIP2 is expressed not only in the cerebral cortex but also in the basal ganglia (Leid et al., 2004). However, we did not find cells that expressed GSH, a marker for the lateral ganglionic eminence (**Figure 2F**), thus confirming CTIP2+ cells are cortical cells.

To determine whether mESC-derived CTIP2:GFP+ cells have the characteristics of CSMNs, we isolated CTIP2:GFP+ cells by fluorescence-activated cell sorting (FACS) on day 12. After incubation for 2 days, we injected the CTIP2:GFP+ cell aggregates into the frontal lobe of adult nude rats. Three months after transplantation, we injected FB into the pyramidal decussation,
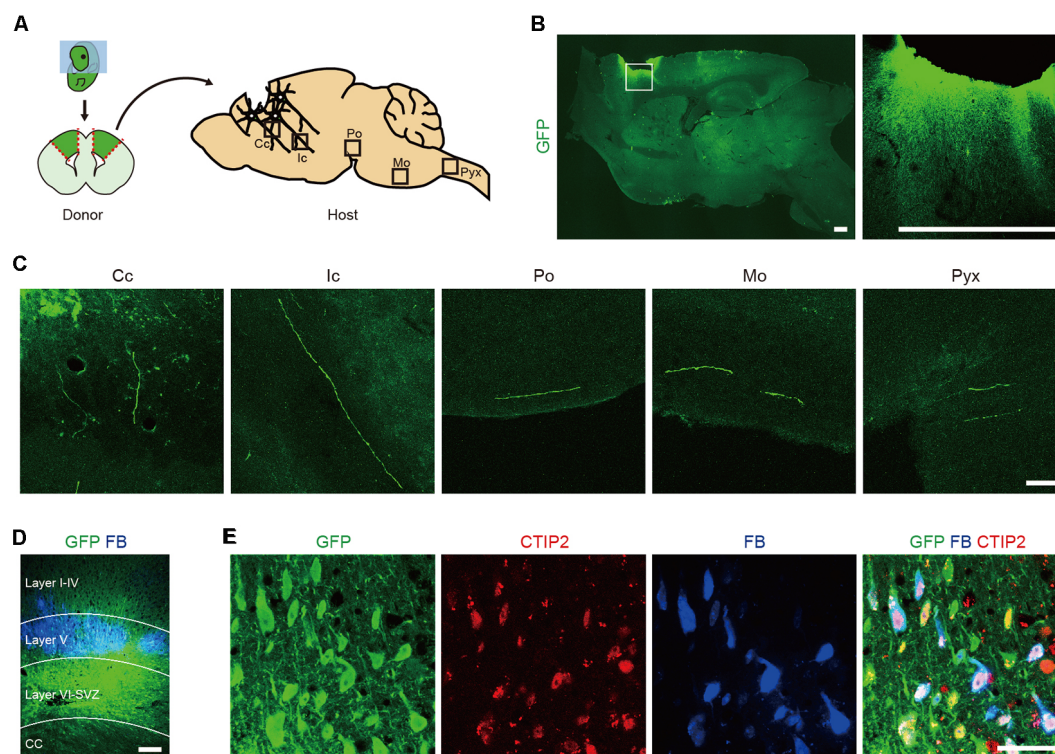
**FIGURE 1 |** The frontal cortex of E14.5 mouse contains Corticospinal motor neurons (CSMNs) and their progenitors. **(A)** Schematic of the transplantation of fetal cortical tissue from E14.5 GFP Tg mice into the lesion cavity of adult mice. **(B)** After 2 months, the transplanted cells survive and extend axons into the host brain, as shown by GFP+ fiber innervations. Scale bars represent 500 μm. **(C)** GFP+ fibers were found in the corpus callosum (Cc), internal capsule (Ic), pons (Po), medulla oblongata (Mo) and pyramidal decussation (Pyx). Scale bar represents 50 μm. **(D)** FB was injected into the pyramidal decussation at 7 days before sacrifice. GFP+/FB+ cells were found in the cortical layer V. Scale bar represents 200 μm. **(E)** Immunofluorescence images of a graft stained with anti-GFP (green) and anti-CTIP2 (red) antibodies. GFP+/FB+ cells expressed CTIP2. Scale bar represents 50 μm.

and 7 days later, the rats were subjected to an immunohistological study. Grafted cells were identified by the expression of M2/M6, a specific marker for mouse cell membrane (Lagenaur and Schachner, 1981; Lagenaur et al., 1992), and the FB signal was observed in the M2+/M6+ cells, suggesting that the sorted cells show characteristics of CSMNs (**Figure 2G**).

## mESC-Derived CTIP2:GFP+ Cells Express L1CAM

To identify a cell surface marker for CTIP2:GFP+ cells, the differentiated cells were divided into GFP+ and GFP− cells by FACS on day 11 to day 13 (see **Supplementary Figure S1**) and were subjected to microarray analysis. A gene expression profile revealed that 324 genes were up-regulated more than 2-fold in the CTIP2:GFP+ population (**Figure 3A**). Classification of the up-regulated genes by a Gene Ontology (GO) analysis showed that 25 genes encoded plasma membrane-related proteins. Among them, we chose six candidates (Erbb4, Grin2b, Robo2, Erc2, Dlg2, L1cam) for which antibodies are commercially available. Then, we excluded three candidates (Grin2b, Erc2, Dlg2) because they are mainly expressed in synapses and axons (Moriyoshi et al., 1991; Ohtsuka et al., 2002; Tao et al., 2003). We finally selected three genes that code proteins expressed on

the surface of the cell body: Roundabout guidance receptor 2 (*Robo2*), Erb-b2 receptor tyrosine kinase 4 (*Erbb4*) and L1 cell adhesion molecule (*L1cam*; **Figure 3B**).

Robo2 is a member of the immunoglobulin superfamily of cell adhesion molecules and acts as a guidance receptor by binding secreted SLIT ligands (Holmes et al., 1998; Long et al., 2004). In the developing cerebral cortex, Robo2 is distinctly expressed in the intermediate zone (IZ), where prospective interneurons and projection neurons migrate tangentially from the ventricular zone (VZ) and the subventricular zone (SVZ) to the cortical plate (CP; Andrews et al., 2007; López-Bendito et al., 2007). Erbb4 is a member of the type I receptor tyrosine kinase subfamily and is involved in cell proliferation, migration and differentiation (Burden and Yarden, 1997; Adlkofer and Lai, 2000; Buonanno and Fischbach, 2001). In the developing cerebral cortex, Erbb4 is expressed in the IZ (Yau et al., 2003). The protein is preferentially expressed in parvalbumin+ interneurons and subsets of other GABAergic interneurons in the adult cerebral cortex. L1CAM is a transmembrane glycoprotein composed of six immunoglobulin domains and five fibronectin type III repeats (Schachner, 1991). In mammals, L1CAM is expressed throughout the nervous system and is involved in axon growth and guidance during development, interactions between Schwann cells and axons,
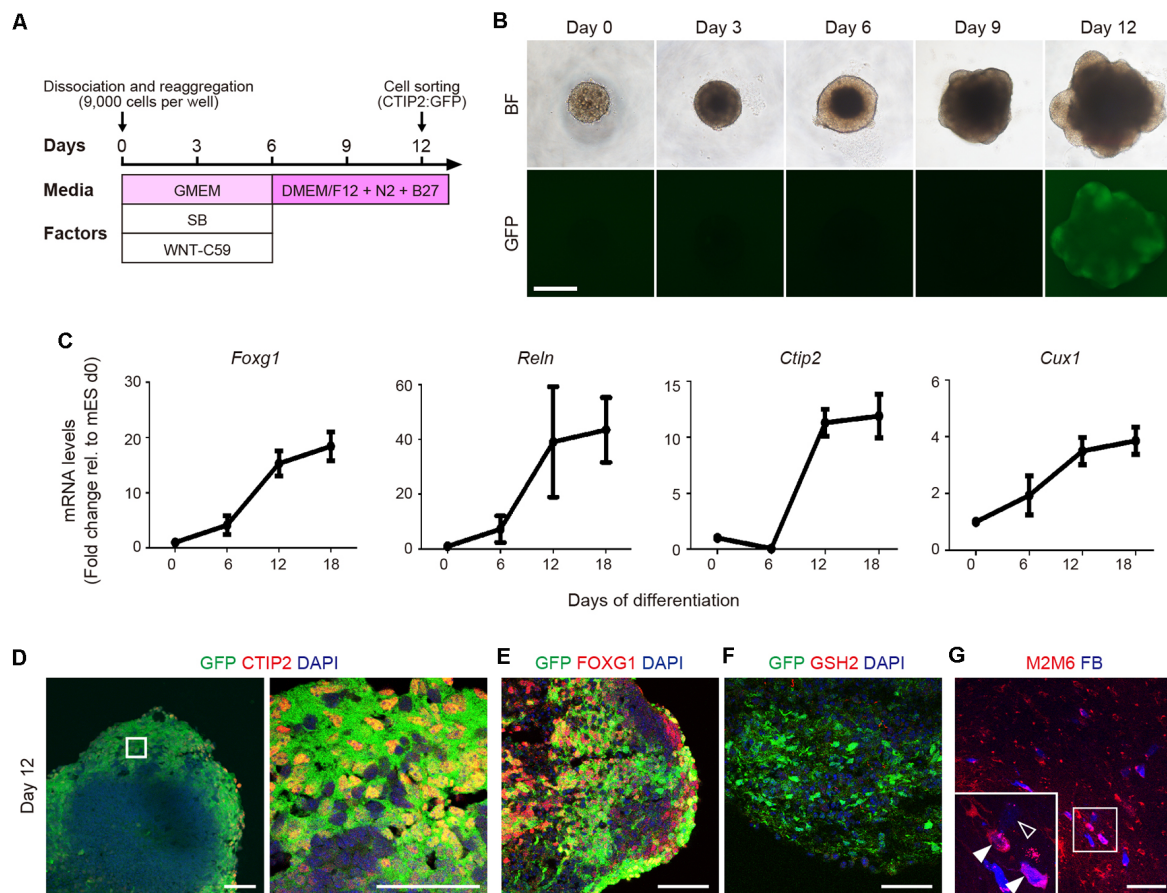
**FIGURE 2** | Mouse ESC-derived CTIP2:GFP$^+$ cells show characteristics of CSMNs. **(A)** Schematic diagram of the cortical differentiation protocol from mouse embryonic stem cells (mESCs). **(B)** Bright-field and CTIP2:GFP images of the floating culture of cell aggregates from day 0 to day 12. The GFP signal is detected from day 12 onwards. Scale bar represents 500 μm. **(C)** Gene expression analysis for Foxg1, Reln, Ctip2 and Cux1 by qRT-PCR ($n = 4$). All values are displayed as means ± SEM. **(D–F)** Immunofluorescence images of CTIP2:GFP KI mESC aggregates for GFP (green), CTIP2 (red), FOXG1 (red), GSH2 (red) and DAPI (blue) on day 12. Scale bars represent 50 μm. **(G)** Immunofluorescence image of a graft stained with anti-M2/M6 (red) antibodies. FB (blue) shows host cells (M2M6$^-$/FB$^+$, open arrowhead) and graft-derived cells (M2M6$^+$/FB$^+$, filled arrowhead). Scale bar represents 100 μm.

neuronal cell migration, neuronal survival, synaptogenesis and myelination (Lindner et al., 1983; Bixby et al., 1988; Chen et al., 1999). In the adult cerebral cortex, L1CAM is specifically localized in cortical layer V (Munakata et al., 2003). Based on these characteristic features, we decided to focus on L1CAM as a candidate surface marker of CTIP2:GFP$^+$ cells.

## Sorting of L1CAM$^+$ Cells Enriches Cortical and Migrating Neurons

Next, we examined the expression pattern of L1CAM in the brain of E14.5 mice (**Figure 3C**). An immunofluorescence study revealed that L1CAM was expressed in the CP (CTIP2$^+$), IZ (NRP1$^+$), and upper part of the SVZ (TBR2$^+$), but not in the VZ (PAX6$^+$; **Figure 3D**). Moreover, we found that CTIP2 is expressed not only in the CP but also in the IZ. To confirm this observation, we performed fluorescence *in situ* hybridization (FISH) using the *Ctip2* mRNA probe combined with immunostaining for L1CAM. As expected, an intense

Ctip2 signal was observed in the CP, and a weaker but clear signal was observed in the IZ (**Figure 3E**).

Next, we dissociated the cortical tissue of E14.5 mice and divided it into L1CAM$^+$ and L1CAM$^-$ cells by FACS. An immunofluorescent study revealed that CTIP2$^+$ cells were more frequently observed in the L1CAM$^+$ population than the unsorted or L1CAM$^-$ population (75.8 ± 5.0% vs. 36.4 ± 2.9% or 13.9 ± 2.7%, respectively; $n = 6$; **Figures 4A,B**). A quantitative reverse transcriptase-polymerase chain reaction (qPCR) analysis revealed that L1CAM$^+$ cells expressed higher levels of Ctip2 and Nrp1 mRNAs compared to L1CAM$^-$ cells (see **Supplementary Figure S2**). On the other hand, the mRNA expression levels of Tbr2 and Pax6 were lower in L1CAM$^+$ cells (see **Supplementary Figure S2**). When we continued the culture as spheres for 2 days after sorting, CTIP2$^+$ cells were again more frequently observed in the L1CAM$^+$ population than the unsorted or L1CAM$^-$ populations (47.2 ± 2.0% vs. 32.9 ± 4.0% or 24.5 ± 2.1%, $n = 9$, 9 and 8, respectively; **Figures 4C,D**). On the other hand, the L1CAM$^+$ population contained fewer EdU$^+$/PAX6$^+$
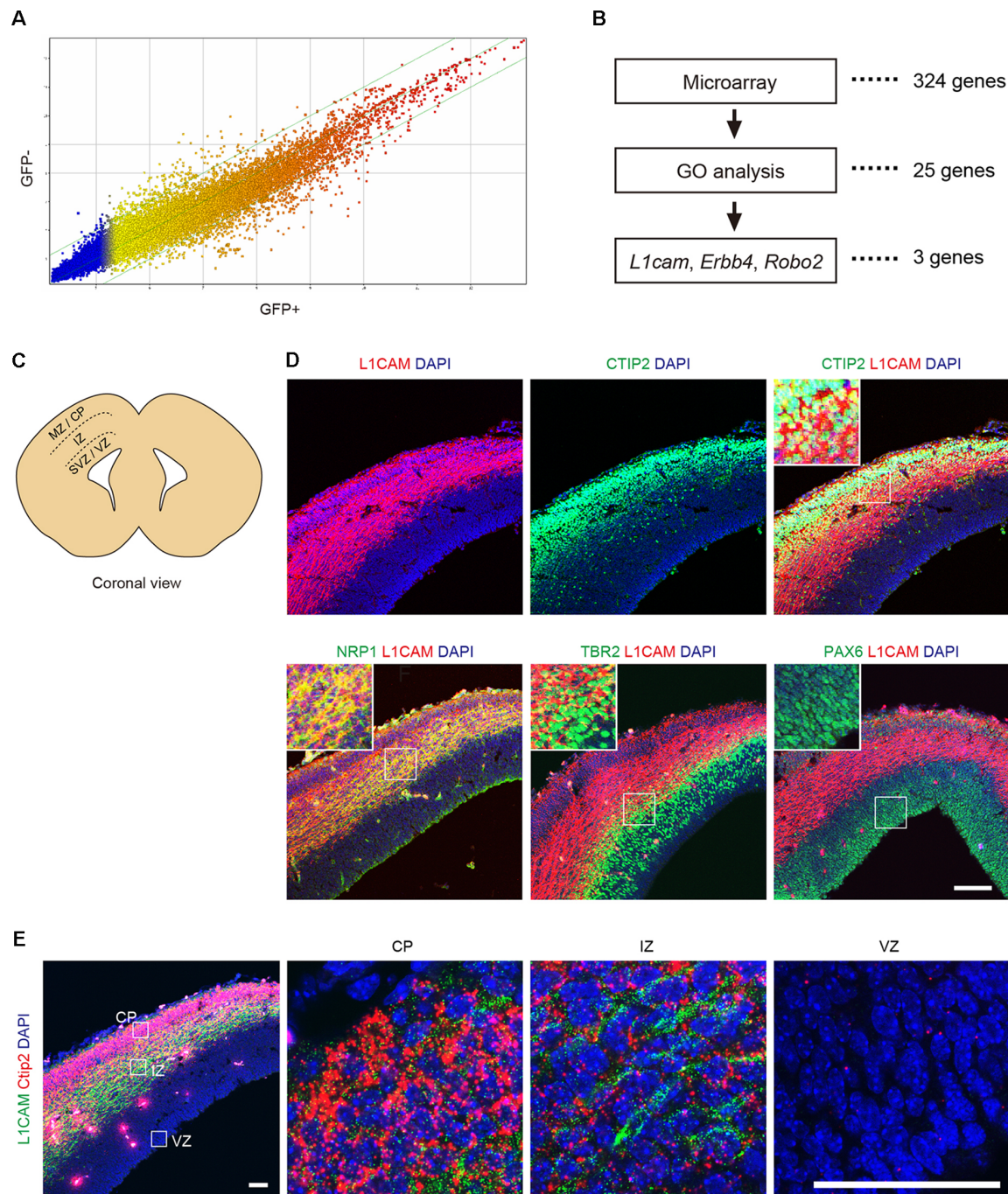
**FIGURE 3 |** L1CAM is a marker for CTIP2 of E14.5 mouse. **(A)** Comparison of the gene expression profiles between GFP⁺ and GFP⁻ cells in CTIP2:GFP KI mESCs on day 11 to day 13. **(B)** Screening of the candidate molecules by microarray analysis, gene ontology (GO) analysis and literature. First, the microarray analysis revealed 324 genes as 2-fold upregulated in CTIP2:GFP⁺ cells. Next, 25 genes were identified by GO analysis as the plasma membrane. Finally, three genes were selected from the literature as expressed in the cell body. **(C)** Diagram of the brain slices of the E14.5 mouse frontal cortex. **(D)** Immunofluorescence images for CTIP2 (green), NRP1 (green), TBR2 (green), PAX6 (green), L1CAM (red) and DAPI (blue). Scale bar represents 100 μm. **(E)** Immunofluorescence image for L1CAM (green) and RNA FISH image for *Ctip2* (red) and DAPI (blue). Scale bars represent 50 μm.

proliferating cells compared to the unsorted or L1CAM⁻ populations (6.4 ± 1.0% vs. 15.6 ± 1.6% or 21.1 ± 1.0%, *n* = 9, 9 and 8, respectively; **Figures 4E,F**). These results suggested that

sorting for L1CAM+ cells can be a strategy for efficient and safe cell therapy by enriching CSMNs and eliminating proliferating progenitor cells.
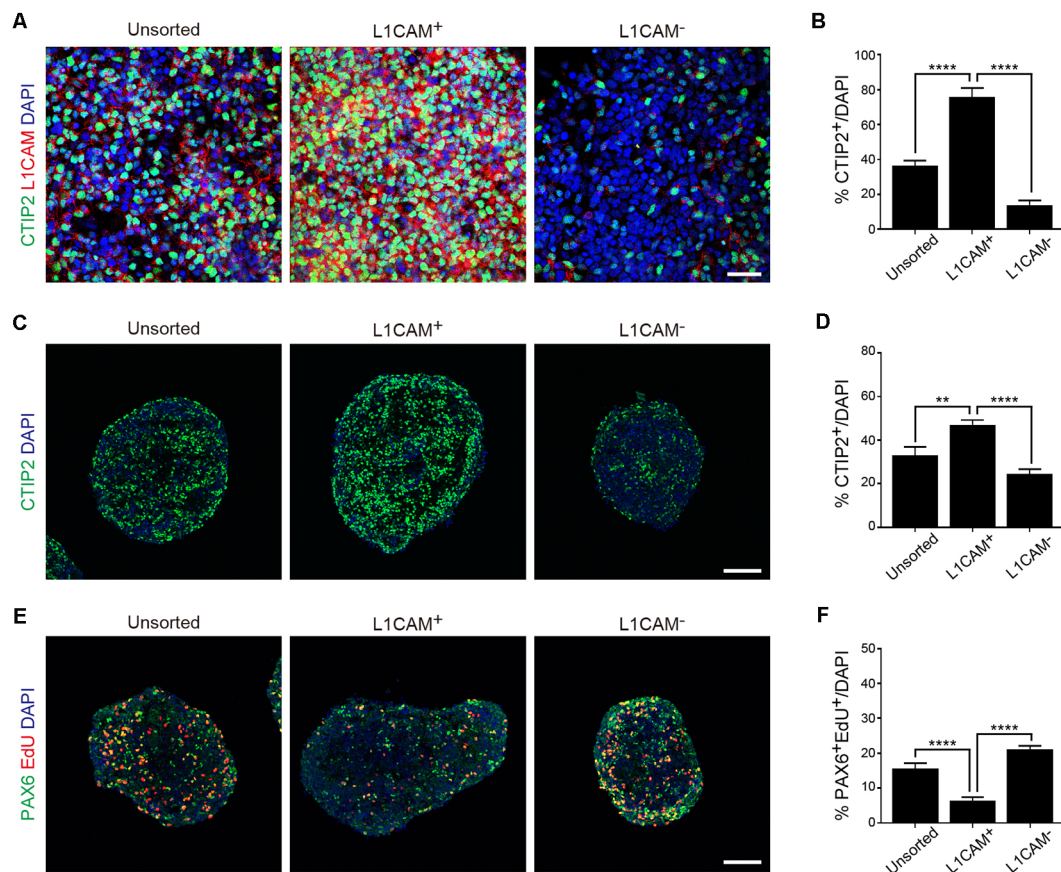
**FIGURE 4 |** Sorting of L1CAM$^+$ cells enriches cortical and migrating neurons. The frontal cortex was taken from E14.5 mice and used for *in vitro* studies.
**(A)** Immunofluorescence images of the cells from unsorted, L1CAM$^+$ and L1CAM$^-$ cells for CTIP2 (green), L1CAM (red) and DAPI (blue) several hours after sorting.
Scale bar represents 30 μm. **(B)** Percentage of CTIP2$^+$ cells in total cells stained with DAPI (unsorted: $n = 6$; L1CAM$^+$: $n = 6$; L1CAM$^-$: $n = 6$).
**(C)** Immunofluorescence images of unsorted, L1CAM$^+$ and L1CAM$^-$ cells for CTIP2 (green) and DAPI (blue) 2 days after sorting. Scale bar represents 100 μm.
**(D)** Percentage of CTIP2$^+$ cells in total cells stained with DAPI (unsorted: $n = 9$; L1CAM$^+$: $n = 9$; L1CAM$^-$: $n = 8$). **(E)** Immunofluorescence images of unsorted,
L1CAM$^+$ and L1CAM$^-$ cells for PAX6 (green), EdU (red) and DAPI (blue) 2 days after sorting. 10 μM EdU was added in the culture medium 2 h before fixation. Scale
bar represents 100 μm. **(F)** Percentage of PAX6$^+$/EdU$^+$ cells in total cells stained with DAPI (unsorted: $n = 9$; L1CAM$^+$: $n = 9$; L1CAM$^-$: $n = 8$). All values are
displayed as means ± SEM. One-way ANOVA with Bonferroni's multiple comparison test, $^{**}P < 0.01$ and $^{****}P < 0.0001$.

## L1CAM$^+$ Cells Preferentially Extend Axons Along the CST

To investigate the survival and neurite extension of transplanted L1CAM$^+$ cells *in vivo*, we dissected cortical tissues from E14.5 GFP Tg mice and divided them into L1CAM$^+$ and L1CAM$^-$ cells by using FACS. At 2 days after sorting, we transplanted the cell aggregates into the frontal cortex of adult mice. FB was injected into the pyramidal decussation at 2 months after transplantation, and 7 days later, these mice were subjected to immunohistochemical analyses.

Immunostaining for GFP revealed that the size of the L1CAM$^+$ grafts was significantly smaller than that of the L1CAM$^-$ grafts ($0.09 \pm 0.02$ mm$^3$ vs. $0.18 \pm 0.03$ mm$^3$, respectively; $n = 6$; **Figures 5A,B**). The axons from the L1CAM$^+$ grafts were observed along the CST including the internal capsule and cerebral peduncle (**Figures 5C,D**). On the other hand, those from the L1CAM$^-$ grafts were observed in the ipsilateral

cortex or restricted within the striatum. The total number and percentage of CTIP2$^+$ cells were higher in the L1CAM$^+$ grafts than in the L1CAM$^-$ grafts (**Figures 6A–C**). Furthermore, FB$^+$ cells were more frequently observed in L1CAM$^+$ grafts than L1CAM$^-$ grafts ($125 \pm 48$ cells vs. $16 \pm 8$ cells, respectively; $n = 6$; **Figures 6D,E**). On the other hand, CUX1$^+$ cells (upper neurons) were more frequently observed in L1CAM$^-$ grafts (see **Supplementary Figure S3**), but the cell density was not significantly different between the two grafts (see **Supplementary Figure S3**). These results indicate that L1CAM$^+$ grafts contained more CSMNs and preferentially extended axons along the CST.

## DISCUSSION

In this study, we grafted fetal brain tissue into the mouse brain and found that the cells in the graft extending axons along
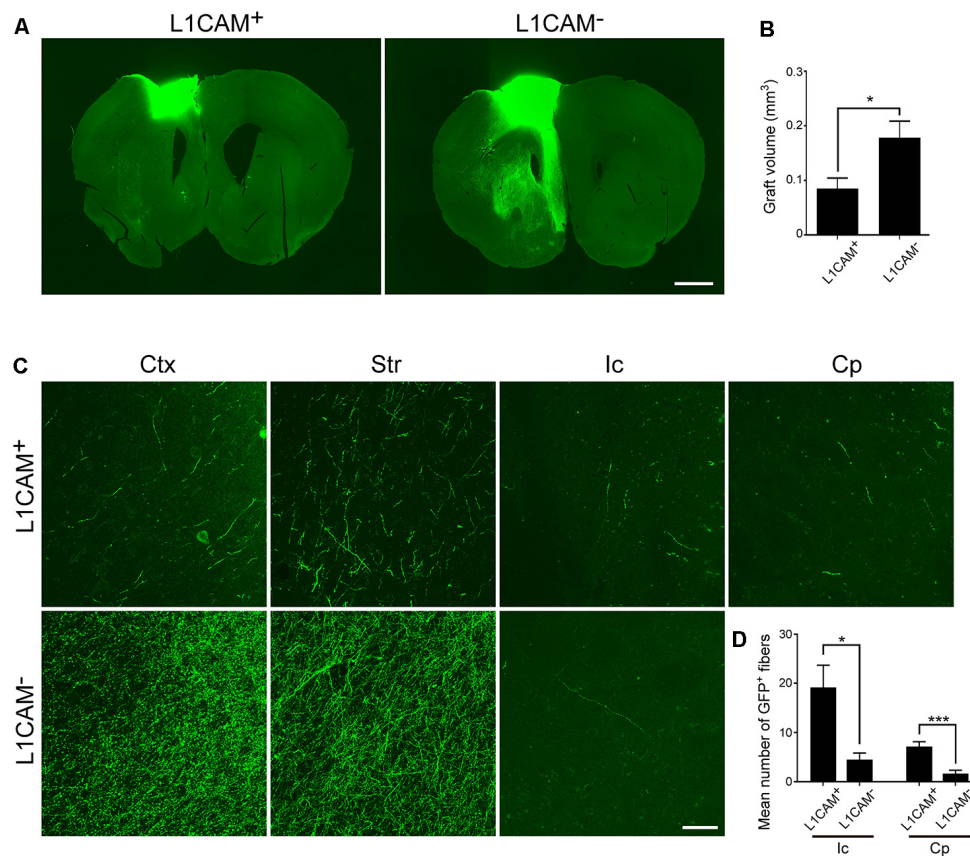
**FIGURE 5 |** L1CAM+ cells survive and extend axons along the corticospinal tract (CST). L1CAM+ cells and L1CAM− cells were isolated from E14.5 mouse frontal cortex, and 2 days later they were injected into the adult brain for 2 months. **(A)** Immunofluorescence images of the graft for GFP (green). Scale bar represents 1 mm. **(B)** Quantification of the volume of L1CAM+ cells and L1CAM− cells in the grafts (L1CAM+: $n = 6$ and L1CAM−: $n = 6$). **(C)** Immunofluorescence images of the graft fibers for GFP (green). GFP+ fibers were found in the cerebral cortex (Ctx), striatum (Str), internal capsule (Ic) and cerebral peduncle (Cp). Scale bar represents 50 μm. **(D)** Quantification of GFP+ fibers from L1CAM+ cells and L1CAM− cells (L1CAM+: $n = 6$ and L1CAM−: $n = 6$) at the Ic and Cp. All values are displayed as means ± SEM. Student's $t$-test, $*P < 0.05$ and $***P < 0.001$.

the CST expressed CTIP2. By using CTIP2:GFP KI mESCs, we identified L1CAM as a cell surface marker for cortical CTIP2+ cells. Finally, we sorted L1CAM+ cells of the fetal brain and confirmed that these cells more efficiently extended axons along the CST compared to L1CAM− cells.

As mentioned above, L1CAM is a transmembrane glycoprotein composed of six immunoglobulin domains and five fibronectin type III repeats (Schachner, 1991). In mammals, L1CAM is expressed throughout the nervous system and is involved in axonal growth and guidance during development (Lindner et al., 1983; Bixby et al., 1988; Chen et al., 1999).

In a developing mouse brain, cortical neurons are differentiated from progenitor cells that line the dorsal aspect of the lateral ventricles in the forebrain (Nieto et al., 2004; Leone et al., 2008). Proliferating progenitor cells that express PAX6 are found in the VZ, which is immediately adjacent to the ventricles, and at later stages in the SVZ, which forms between the VZ and the overlying IZ. The immature neurons exit the cell cycle at around E10, migrate out of the VZ along radial glia and reach the CP. Within the CP, neurons of the deep layers

(VI and V) are generated at around E14, and neurons of the upper layers (IV, III and II) are generated at around E16. The deep layers are mainly composed of subcerebral projection neurons, which express CTIP2 and extend axons beyond the cortex such as the thalamus and spinal cord. In contrast, the upper layers are mainly composed of callosal projection neurons, which express SATB2 and CUX1 and extend axons to the other cortical area.

This complexity of the developing brain has made it difficult to identify which cell populations contribute to the reconstruction of the CST after transplantation. A previous transplantation study of E15.5 mouse brain revealed that early postmitotic neurons, which are fate-restricted for deep-layer neurons, can extend axons as CST and establish functional connectivity after transplantation (Wuttke et al., 2018). These neurons underwent final mitosis during E11.5-13.5 and were postmitotic at the time of transplantation. In contrast, the cells dividing at E14.5 did not differentiate into CTIP2+ cells in the deep layers. In the present study, we found that L1CAM+ cells of E14.5 mouse brain more efficiently extended axons along the
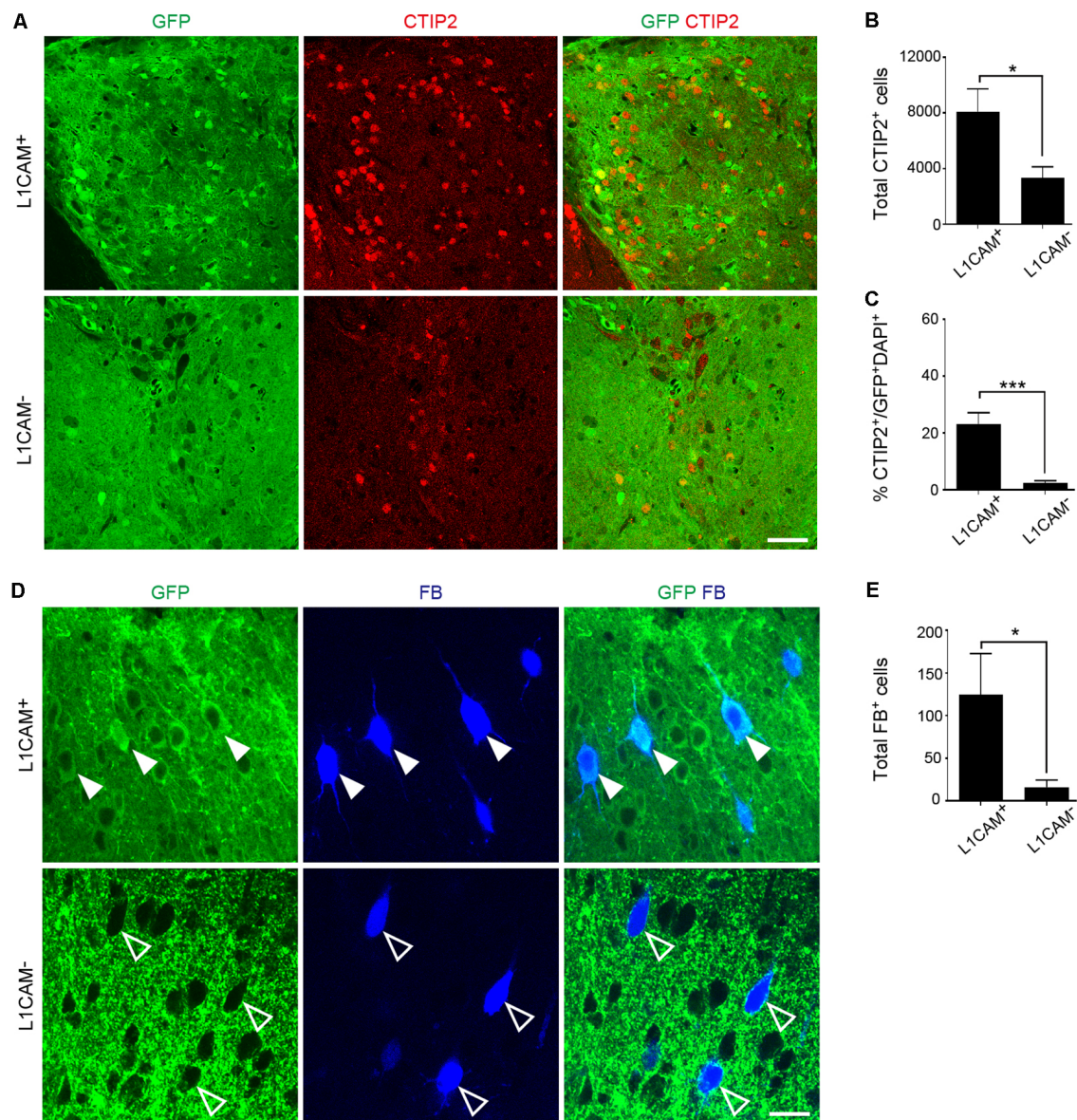
**FIGURE 6 |** L1CAM$^+$ grafts have a character consistent with CSMNs. L1CAM$^+$ cells and L1CAM$^-$ cells were isolated from E14.5 mouse frontal cortex, and 2 days later they were injected into the adult brain for 2 months. **(A)** Immunofluorescence images of the graft for GFP (green) and CTIP2 (red). Scale bar represents 50 μm. **(B)** Total number of CTIP2$^+$ cells in the L1CAM$^+$ grafts and L1CAM$^-$ grafts (L1CAM$^+$: $n = 6$ and L1CAM$^-$: $n = 6$). **(C)** Percentage of CTIP2$^+$ cells in total cells stained with DAPI and GFP (L1CAM$^+$: $n = 6$ and L1CAM$^-$: $n = 6$). **(D)** Immunofluorescence images of graft for GFP (green) and FB (blue). FB was injected into the pyramidal decussation 7 days before sacrifice. Solid white arrowheads represent graft-derived cells. Open arrowheads represent host-derived cells. Scale bar represents 25 μm. **(E)** Total number of FB$^+$ cells in L1CAM$^+$ grafts and L1CAM$^-$ grafts (L1CAM$^+$: $n = 6$ and L1CAM$^-$: $n = 6$). All values are displayed as means ± SEM. Student's $t$-test, *$P < 0.05$ and ***$P < 0.001$.

CST compared to L1CAM$^-$ cells. Intriguingly, the former cell population contained more postmitotic CTIP2$^+$ cells, while the latter contained more proliferating PAX6$^+$ progenitor cells. This distinction suggests sorting for L1CAM$^+$ cells of the E14.5 mouse brain could enrich cells that contribute to the reconstruction of the CST. In this context, the timing of sorting during neuronal development is critical. To enrich CSMNs, L1CAM$^+$ cells need to be sorted at the early cortical development when only deep layer neurons emerged.

We previously reported that neuropilin-1 (NRP1)$^+$ cells in the frontal cortex of E14.5 mice survive and extend axons to the spinal cord of neonatal brain (Sano et al., 2017). NRP1 is a Sema 3 receptor, is essential for the initial stage of axonal sprouting (Bagnard et al., 1998; Fujisawa, 2004), and is distributed in the IZ of the cerebral cortex of E13.5-15.5 mouse (Kawakami et al., 1996; Hatanaka et al., 2009). NRP1$^+$ cells are mainly migrating neurons in the IZ, but they are also subcortical projection neurons with axonal

extensions. In this study, we found L1CAM by microarray analysis using CTIP2:GFP KI mESCs as a cell surface marker to enrich cells that extend axons along the CST. In addition, L1CAM and NRP1 form a complex as a Sema 3A receptor for transducing signaling pathways within the growth cone (Castellani et al., 2000). These facts suggest that L1CAM+/NRP1+ cells can efficiently contribute to the reconstruction of the CST, but it remains unknown whether deep layer neurons in the CP or migrating neurons in the IZ play a more important role.

Another advantage of sorting L1CAM+ cells is that one can eliminate proliferating progenitor cells that are unlikely to become subcerebral projection neurons or have tumorigenicity. Tumorigenicity is especially a concern in the case of transplantation using PSC-derived cells.

In contrast to L1CAM+ grafts, L1CAM− grafts extended axonal fibers mainly to the ipsilateral cortex and striatum. Additionally, only a few fibers were observed in other areas including the thalamus and superior colliculus in both cases. For a complete analysis of fiber extension in the brain, we need to employ the brain tissue-clearing and 3D-imaging technique, which is the next challenge in the near future.

In conclusion, we demonstrated that sorting L1CAM+ cells from the cerebral cortex of E14.5 mouse is advantageous for enriching cells that can extend axons along the CST after transplantation. However, it remains to be explored whether these cells have a functional effect on mouse behavior after transplantation. In addition, it is unknown whether the same strategy can be applied to human PSC-derived neurons. Recently, we have published a article about human PSC-derived cerebral organoids (Sakaguchi et al., 2019). For clinical application, we need to determine the optimal timing for sorting in the induction of human cerebral organoids and examine if human L1CAM+ cells contribute to safe and efficient transplantation. An investigation into these questions will advance cell-based therapies to treat CST damaged by stroke or brain injury.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the microarray data available from the Gene Expression Omnibus (GEO database) with the accession number GSE132362.

## ETHICS STATEMENT

The animal study was reviewed and approved by The animal experimentation committee of Center for iPS Cell Research and Application (CiRA), Kyoto University.

## AUTHOR CONTRIBUTIONS

BS and JT designed the study and wrote the manuscript. BS, RT, YI, and KF performed the experiments and analyzed the data. HN and YO generated the transgenic mESC clones.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00031/full#supplementary-material.

## REFERENCES

Adlkofer, K., and Lai, C. (2000). Role of neuregulins in glial cell development. Glia 29, 104–111. doi: 10.1002/(sici)1098-1136(20000115)29:2<104::aid-glia2>3.0. co;2-2

Andrews, W. D., Barber, M., and Parnavelas, J. G. (2007). Slit-Robo interactions during cortical development. J. Anat. 211, 188–198. doi: 10.1111/j.1469-7580. 2007.00750.x

Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R., and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron 45, 207–221. doi: 10.1016/j.neuron.2004. 12.036

Bagnard, D., Lohrum, M., Uziel, D., Puschel, A. W., and Bolz, J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development 125, 5043–5053.

Ballout, N., Frappe, I., Peron, S., Jaber, M., Zibara, K., and Gaillard, A. (2016). Development and maturation of embryonic cortical neurons grafted into the damaged adult motor cortex. Front. Neural Circuits 10:55. doi: 10.3389/fncir. 2016.00055

Bixby, J. L., Lilien, J., and Reichardt, L. F. (1988). Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. J. Cell. Biol. 107, 353–361. doi: 10.1083/jcb.107.1.353

Buonanno, A., and Fischbach, G. D. (2001). Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr. Opin. Neurobiol. 11, 287–296. doi: 10.1016/s0959-4388(00)00210-5

Burden, S., and Yarden, Y. (1997). Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. Neuron 18, 847–855. doi: 10.1016/s0896-6273(00)80324-4

Castellani, V., Chedotal, A., Schachner, M., Faivre-Sarrailh, C., and Rougon, G. (2000). Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. Neuron 27, 237–249. doi: 10.1016/s0896-6273(00)00033-7

Chen, S., Mantei, N., Dong, L., and Schachner, M. (1999). Prevention of neuronal cell death by neural adhesion molecules L1 and CHL1. J. Neurobiol. 38, 428–439. doi: 10.1002/(sici)1097-4695(19990215)38:3<428::aid-neu10>3.0. co;2-6

Doi, D., Samata, B., Katsukawa, M., Kikuchi, T., Morizane, A., Ono, Y., et al. (2014). Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation. Stem Cell Reports 2, 337–350. doi: 10.1016/j.stemcr.2014.01.013

Espuny-Camacho, I., Michelsen, K. A., Gall, D., Linaro, D., Hasche, A., Bonnefont, J., et al. (2013). Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo. Neuron 77, 440–456. doi: 10.1016/j.neuron.2012.12.011

Fujisawa, H. (2004). Discovery of semaphorin receptors, neuropilin and plexin and their functions in neural development. *J. Neurobiol.* 59, 24–33. doi: 10.1002/neu.10337

Gaillard, A., Prestoz, L., Dumartin, B., Cantereau, A., Morel, F., Roger, M., et al. (2007). Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons. *Nat. Neurosci.* 10, 1294–1299. doi: 10.1038/nn1970

Gaspard, N., Bouschet, T., Hourez, R., Dimidschstein, J., Naeije, G., van den Ameele, J., et al. (2008). An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* 455, 351–357. doi: 10.1038/nature07287

Grabowski, M., Sorensen, J. C., Mattsson, B., Zimmer, J., and Johansson, B. B. (1995). Influence of an enriched environment and cortical grafting on functional outcome in brain infarcts of adult rats. *Exp. Neurol.* 133, 96–102. doi: 10.1006/exnr.1995.1011

Hatanaka, Y., Matsumoto, T., Yanagawa, Y., Fujisawa, H., Murakami, F., and Masu, M. (2009). Distinct roles of neuropilin 1 signaling for radial and tangential extension of callosal axons. *J. Comp. Neurol.* 514, 215–225. doi: 10.1002/cne.22021

Holmes, G. P., Negus, K., Burridge, L., Raman, S., Algar, E., Yamada, T., et al. (1998). Distinct but overlapping expression patterns of two vertebrate slit homologs implies functional roles in CNS development and organogenesis. *Mech. Dev.* 79, 57–72. doi: 10.1016/s0925-4773(98)00174-9

Kawakami, A., Kitsukawa, T., Takagi, S., and Fujisawa, H. (1996). Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *J. Neurobiol.* 29, 1–17. doi: 10.1002/(SICI)1097-4695(199601)29:1<1::AID-NEU1>3.0.CO;2-F

Kueh, H. Y., Yui, M. A., Ng, K. K. H., Pease, S. S., Zhang, J. A., Damle, S. S., et al. (2016). Asynchronous combinatorial action of four regulatory factors activates Bcl11b for T cell commitment. *Nat. Immunol.* 17, 956–965. doi: 10.1038/ni.3514

Lagenaur, C., Kunemund, V., Fischer, G., Fushiki, S., and Schachner, M. (1992). Monoclonal M6 antibody interferes with neurite extension of cultured neurons. *J. Neurobiol.* 23, 71–88. doi: 10.1002/neu.480230108

Lagenaur, C., and Schachner, M. (1981). Monoclonal antibody (M2) to glial and neuronal cell surfaces. *J. Supramol. Struct. Cell. Biochem.* 15, 335–346. doi: 10.1002/jsscb.1981.380150404

Leid, M., Ishmael, J. E., Avram, D., Shepherd, D., Fraulob, V., and Dolle, P. (2004). CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis. *Gene Expr. Patterns* 4, 733–739. doi: 10.1016/j.modgep.2004.03.009

Leone, D. P., Srinivasan, K., Chen, B., Alcamo, E., and McConnell, S. K. (2008). The determination of projection neuron identity in the developing cerebral cortex. *Curr. Opin. Neurobiol.* 18, 28–35. doi: 10.1016/j.conb.2008.05.006

Lindner, J., Rathjen, F. G., and Schachner, M. (1983). L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. *Nature* 305, 427–430. doi: 10.1038/305427a0

Long, H., Sabatier, C., Ma, L., Plump, A., Yuan, W., Ornitz, D. M., et al. (2004). Conserved roles for Slit and Robo proteins in midline commissural axon guidance. *Neuron* 42, 213–223. doi: 10.1016/s0896-6273(04)00179-5

López-Bendito, G., Flames, N., Ma, L., Fouquet, C., Di Meglio, T., Chedotal, A., et al. (2007). Robo1 and Robo2 cooperate to control the guidance of major axonal tracts in the mammalian forebrain. *J. Neurosci.* 27, 3395–3407. doi: 10.1523/JNEUROSCI.4605-06.2007

Mattsson, B., Sørensen, J. C., Zimmer, J., and Johansson, B. B. (1997). Neural grafting to experimental neocortical infarcts improves behavioral outcome and reduces thalamic atrophy in rats housed in enriched but not in standard environments. *Stroke* 28, 1225–1231; discussion 1231–1232. doi: 10.1161/01.str.28.6.1225

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991). Molecular cloning and characterization of the rat NMDA receptor. *Nature* 354, 31–37. doi: 10.1038/354031a0

Motono, M., Ioroi, Y., Ogura, T., and Takahashi, J. (2016). WNT-C59, a small-molecule WNT Inhibitor, efficiently induces anterior cortex that includes cortical motor neurons from human pluripotent stem cells. *Stem Cells Transl. Med.* 5, 552–560. doi: 10.5966/sctm.2015-0261

Munakata, H., Nakamura, Y., Matsumoto-Miyai, K., Itoh, K., Yamasaki, H., and Shiosaka, S. (2003). Distribution and densitometry mapping of L1-CAM immunoreactivity in the adult mouse brain--light microscopic observation. *BMC Neurosci.* 4:7. doi: 10.1186/1471-2202-4-7

Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., et al. (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II–IV of the cerebral cortex. *J. Comp. Neurol.* 479, 168–180. doi: 10.1002/cne.20322

Ohtsuka, T., Takao-Rikitsu, E., Inoue, M., Inoue, M., Takeuchi, M., Matsubara, K., et al. (2002). Cast: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and munc13–1. *J. Cell. Biol.* 158, 577–590. doi: 10.1083/jcb.200202083

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* 407, 313–319. doi: 10.1016/s0014-5793(97)00313-x

Plumet, J., Ebrahimi, A., Guitet, J., and Roger, M. (1993). Partial recovery of skilled forelimb reaching after transplantation of fetal cortical tissue in adult rats with motor cortex lesion-anatomical and functional aspects. *Restor. Neurol. Neurosci.* 6, 9–27. doi: 10.3233/rnn-1993-6102

Riolobos, A. S., Heredia, M., de la Fuente, J. A., Criado, J. M., Yajeya, J., Campos, J., et al. (2001). Functional recovery of skilled forelimb use in rats obliged to use the impaired limb after grafting of the frontal cortex lesion with homotopic fetal cortex. *Neurobiol. Learn. Mem.* 75, 274–292. doi: 10.1006/nlme.2000.3979

Sakaguchi, H., Ozaki, Y., Ashida, T., Matsubara, T., Oishi, N., Kihara, S., et al. (2019). Self-organized synchronous calcium transients in a cultured human neural network derived from cerebral organoids. *Stem Cell Reports* 13, 458–473. doi: 10.1016/j.stemcr.2019.05.029

Samata, B., Doi, D., Nishimura, K., Kikuchi, T., Watanabe, A., Sakamoto, Y., et al. (2016). Purification of functional human ES and iPSC-derived midbrain dopaminergic progenitors using LRTM1. *Nat. Commun.* 7:13097. doi: 10.1038/ncomms13097

Sano, N., Shimogawa, T., Sakaguchi, H., Ioroi, Y., Miyawaki, Y., Morizane, A., et al. (2017). Enhanced axonal extension of subcortical projection neurons isolated from murine embryonic cortex using neuropilin-1. *Front. Cell. Neurosci.* 11:123. doi: 10.3389/fncel.2017.00123

Schachner, M. (1991). Cell surface recognition and neuron-glia interactions. *Ann. N Y Acad. Sci.* 633, 105–112. doi: 10.1111/j.1749-6632.1991.tb15601.x

Tao, Y. X., Rumbaugh, G., Wang, G. D., Petralia, R. S., Zhao, C., Kauer, F. W., et al. (2003). Impaired NMDA receptor-mediated postsynaptic function and blunted NMDA receptor-dependent persistent pain in mice lacking postsynaptic density-93 protein. *J. Neurosci.* 23, 6703–6712. doi: 10.1523/JNEUROSCI.23-17-06703.2003

Wuttke, T. V., Markopoulos, F., Padmanabhan, H., Wheeler, A. P., Murthy, V. N., and Macklis, J. D. (2018). Developmentally primed cortical neurons maintain fidelity of differentiation and establish appropriate functional connectivity after transplantation. *Nat. Neurosci.* 21, 517–529. doi: 10.1038/s41593-018-0098-0

Yau, H. J., Wang, H. F., Lai, C., and Liu, F. C. (2003). Neural development of the neuregulin receptor ErbB4 in the cerebral cortex and the hippocampus: preferential expression by interneurons tangentially migrating from the ganglionic eminences. *Cereb. Cortex* 13, 252–264. doi: 10.1093/cercor/13.3.252

# Filling the Gaps – A Call for Comprehensive Analysis of Extracellular Matrix of the Glial Scar in Region- and Injury-Specific Contexts

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Central nervous system (CNS) injury results in chronic scar formation that interferes with function and inhibits repair. Extracellular matrix (ECM) is prominent in the scar and potently regulates cell behavior. However, comprehensive information about the ECM proteome is largely lacking, and region- as well as injury-specific differences are often not taken into account. These aspects are the focus of our perspective on injury and scar formation. To highlight the importance of such comprehensive proteome analysis we include data obtained with novel analysis tools of the ECM composition in the scar and show the contribution of monocytes to the ECM composition after traumatic brain injury (TBI). Monocyte invasion was reduced using the CCR2-/- mouse line and step-wise de-cellularization and proteomics allowed determining monocyte-dependent ECM composition and architecture of the glial scar. We find significant reduction in the ECM proteins Tgm1, Itih (1,2, and 3), and Ftl in the absence of monocyte invasion. We also describe the scar ECM comprising zones with distinctive composition and show a subacute signature upon comparison to proteome obtained at earlier times after TBI. These results are discussed in light of injury-, region- and time-specific regulation of scar formation highlighting the urgent need to differentiate injury conditions and CNS-regions using comprehensive ECM analysis.

Keywords: brain injury, extracellular matrix, proteomics, glial scar, monocytes, macrophages

## THE GLIAL SCAR AND ECM

Upon trauma adult mammalian tissue typically scar causing tissue and organ dysfunction. In the central nervous system (CNS) scars affect information processing by several means (Robel and Sontheimer, 2016) including the formation of barriers for re-establishing connectivity (Cregg et al., 2014). Hence scars act as permanent barriers for self-repair and remain an obstacle for therapies enhancing plasticity or neuronal regeneration (Barker et al., 2018).

Scars are typically composed of a cell mixture comprising tissue resident cells, such as different glial cells in the CNS (Adams and Gallo, 2018), and invading cells such as inflammatory cells derived

from the immune system, e.g., monocytes (Orr and Gensel, 2018). In addition, in some injury paradigms and CNS regions (e.g., after spinal cord injury) fibroblasts and/or pericytes accumulate in the core of the injury site (Göritz et al., 2011; Soderblom et al., 2013). Notably, this cell mixture differs profoundly depending on the injury type (TBI, stroke, amyloid deposition, autoimmune-reaction) and the CNS region. For example, in spinal cord injury fibroblast-like cells settle in the lesion core that is shielded/surrounded by reactive glial cells, such as astrocytes (Kjell and Olson, 2016). This is can be very different in stab wound brain injury with prominent reactive gliosis and little to no detectable fibrosis (Frik et al., 2018). This is also the case, when the brain injury reaches into the White Matter (WM) (Mattugini et al., 2018) that is often less affected in brain injury as it is buried deep in the brain below the Gray Matter (GM). After spinal cord injury WM is always affected first as it is located at the surface. Effects on WM are thus one of the many major differences upon injury inflicted to these very distinct regions. Notably, monocyte invasion continues into much later stages after the injury when WM is affected, while newly invading monocytes can no longer be detected 5–7 days after injury of GM only (Mattugini et al., 2018.). Thus, the cellular composition of the wound and scar differs profoundly in a region-specific manner in the CNS.

Given that all of these different cells communicate and interact by a plethora of cell surface signaling pathways and secretion of specific proteins it is essential to unravel this complex proteome, not the least to also understand how injury- and region-specific extracellular matrix (ECM) composition contributes to scar formation. A suitable approach is to deplete one population and then examine relevant changes. For invading monocytes, this can be done by blocking or deleting the CCR2 – a receptor absolutely essential for invasion of monocytes into the brain (Saederup et al., 2010). Intriguingly, lack of monocyte invasion shows profoundly different outcomes in different injury conditions and distinct CNS regions. Preventing monocyte invasion after ischemic stroke has been reported to worsen the hemorrhagic consequences and reduce the long-term recovery (Gliem et al., 2012; Wattananit et al., 2016). However, in TBI models the prevention of monocyte invasion has reduced the volume of the injury-affected region and improved cognitive function (Hsieh et al., 2014; Morganti et al., 2015). These data may well reflect, the differences in ECM composition in different regions and injury conditions.

Extracellular matrix changes have mostly been examined after spinal cord injury aiming to understand how they affect the restoration of ascending and descending axonal connections (Didangelos et al., 2016; Bradbury and Burnside, 2019). Several ECM components have been found to be inhibitory for repair, especially chondroitin sulfate glycosaminoglycan-chains (GAG-chains) on proteoglycans at the injury site. These sugar-chains have proven inhibitory to axon growth and digesting them enzymatically or keeping them from stable growth cone interactions improves regeneration (Bradbury et al., 2002; Bartus et al., 2014; Lang et al., 2015). The Tenascin glycoproteins have been found to be upregulated in the same region as the CSPG, where Tenascin-R is a component normally part of the Perineuronal nets (PNN) (Deepa et al., 2006; Carulli et al., 2010) and Tenacin-C (Tn-C) is an inflammation-associated ECM

protein upregulated following CNS injury (Roll and Faissner, 2019). Other commonly reported scar components are those of the fibrotic scar that are of similar composition to the basal membrane, containing e.g., collagen, laminins, and fibronectin. Moreover, injury is associated with the degradation of such core ECM proteins (Roll and Faissner, 2014). Responsible for this are peptidases, include the elastases and matrix metallopeptidases. In spinal cord, another catalytic ECM-associated protein group called cathepsins has recently been highlighted using proteomics and transcriptomics as potential ECM regulators (Tica et al., 2018), providing an example of the multitude of unexplored avenues for ECM and its associated proteins in CNS injury.

## MONOCYTE-DEPENDENT ECM IN THE GLIAL SCAR

Inflammation is part of the cascade of events that follows traumatic injury and has also been found to regulate the extent of the scarring after TBI in cerebral cortex GM (Frik et al., 2018). Of the multiple invading immune cells, monocyte comprises the largest group, and surprisingly little is known about the role of macrophages in affecting the ECM composition at the scar. With scarring reduced after stab wound injury in the CCR2-/- cerebral cortex (Frik et al., 2018) we investigated which ECM components may be affected by the virtual absence of macrophages in the brain parenchyma after injury in this mouse line. We used state-of the-art proteomics (Cox and Mann, 2008; Mann et al., 2013; Cox et al., 2014; Kulak et al., 2014; Tyanova et al., 2016) and the quantitative detergent solubility profile (QDSP) method, in order to also investigate the architecture and composition of the ECM. The QDSP method analyses all the tissue lysates fractions after the tissue has been sequentially processed in increasing strength of detergent (see **Supplementary Material** or Kjell et al., 2020), with the most detergent-insoluble proteins in a separate fraction (fraction 4 – see **Figure 1A**). Basically, it is a tissue decellularization that also provides information regarding the intermediates solubilities as proteins are identified and quantified in all (four) fractions using label-free mass spectrometry. The ECM proteins are identified in the data-set by the currently most comprehensive annotation for the ECM proteins – the matrisome annotation that uses a combination of proteomic measurements from decellularized tissue and *in silico* prediction to identify the ECM proteins (Naba et al., 2012). The advantage of analyzing all detergent-fractions in the QDSP method is that it allows determining how the total abundance of a protein is distributed in the different fractions and how this solubility profile shifts under different conditions. This provides crucial information regarding changes in ECM protein distribution, e.g., from basal lamina (highly insoluble) to interstitial space (soluble) after trauma. Indeed, Transglutaminase 1 (Tgm1) changes its solubility profile reducing the insoluble fraction after injury with a peak in fraction 2, i.e., becoming more soluble. Conversely, the inter-alpha-trypsin inhibitors 1,2,3 (Itih1,2,3) became rather less soluble at the scar stage (28 days post injury; dpi) after TBI (**Figure 1B**).
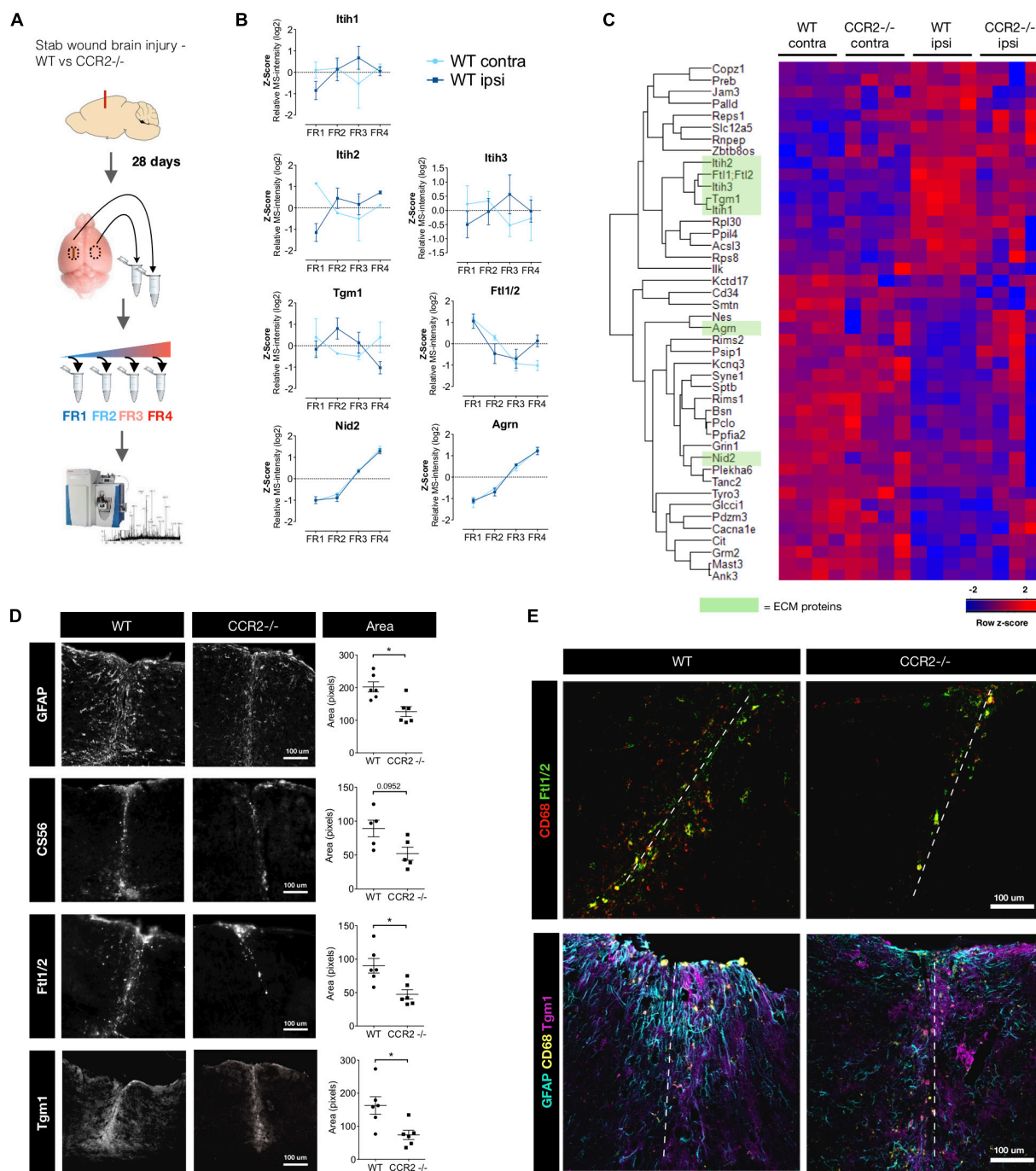
**FIGURE 1 |** Macrophages contribute to the ECM-component of the scar after stab wound injury. **(A)** Schematic of the proteome comparison 28 days after brain stab wound injury in wild-type mice compared to CCR2-/- mice using step-wise detergent-decellularization protocol, named quantitative detergent solubility profiling (QDSP). Mouse brain picture courtesy: National Science Foundation. **(B)** The QDSP method subjects the tissues to increasing strength of detergent lysis and results in four individual fractions. Fraction measurements have are here compared for ECM and secreted proteins in the contralateral and the ipsilateral side of the injured wild-type brains, which returned to more normal levels in the CCR2-/- mouse. Overall the solubility changes trend toward a more insoluble nature, although not exclusively insoluble. Notably, while the total abundance of Nid2 and Agrn normalized (got more abundant) in the CCR2 -/- mice, there was no difference in their solubility profiles. **(C)** Combined fraction analysis reveal that the overall protein changes are normalized in the CCR2 -/- mice compared to wild-type mice ($n$ = 4 per group). Heatmap displays proteins that had similar abundance in the contralateral samples ($t$-test, $p \geq 0.05$), while being significantly different when comparing contralateral to ipsilateral side of the injured wild-type mouse brain ($t$-test, $p \leq 0.01$). ECM and secreted proteins are highlighted in green. **(D)** Quantifications of the immunoreactive tissue-area at the injury site confirm that Ftl1/2 and Tgm1 are reduced in the CCR2-/- mice closer to levels contralateral to the injury. This is in line with the reduced spread of other glial scar markers such as GFAP and CS56. * $p \leq 0.05$. **(E)** Ftl1/2 primarily co-localizes with the macrophage marker CD68, while Tgm1 instead is predominantly found in the area of reactive astrocytes (GFAP + cells).

In our investigation of the adult neurogenic niche (Kjell et al., 2020), also using the QDSP protocol, we found that ECM proteins, such as Tn-C, are more soluble compared to the brain parenchyma. Tn-C is also highly soluble in the scar region analyzed by QDSP at 28 dpi here, which is opposite to its insoluble nature in lung injury and atherosclerotic plaques (Schiller et al., 2015; Wierer et al., 2018). These are examples of how any quantification and information regarding ECM architecture would be lost without adopting a protocol that allows composition-dependent sample analysis.

Next, we examined combined fraction analysis to determine total abundance comparisons for any protein (**Figure 1C**). To identify proteins regulated by invading monocytes and their influence in scar formation, we compared proteins of similar magnitude in the non-injured contralateral side of the WT and CCR2-/- mice brains (two-tailed $t$-test, $p \geq 0.05$) that were significantly changed following injury in the WT mice 28 days after stab wound injury (two-tailed $t$-test, $p \leq 0.01$) (**Figure 1C**). We found four ECM proteins and one secreted protein that were elevated with injury, but reduced in the CCR2-/- mice (green bar in **Figure 1C**). These were the above mentioned Tgm1, Itih1,2,3 and the ferritin light chain proteins 1 or 2 (here referred to as Ftl1/2). Notably, all of these have previously received little if any attention with regard to brain injury and glial scarring. Tgm1 is part of the cross-linking enzyme family of transglutaminases that have many roles, including the regulation of tissue stiffness when crosslinking ECM (Gundemir et al., 2012; Majkut et al., 2013). Immunostaining for Tgm1 was found to be rather diffuse around the injury in the entire area dominated by astrogliosis (**Figures 1D,E**). Taken together, this may suggest that the multi-function enzyme Tgm1 crosslinks cell surface proteins and/or less detergent resistant ECM proteins and/or prevails in the cytoplasm where it has intracellular functions (Eckert et al., 2014).

Itih 1,2,3 are hyaluronic acid binding proteins that can act as protease inhibitor and are often present with inflammation. Ftl1/2 binds ferric ions that would otherwise be toxic to the cells. These proteins could originate from the blood (Geyer et al., 2016). However, bioinformatic comparison from our previous publications with proteomes from the stab wound at 3 and 5 days after injury, hinted that this was unlikely (Frik et al., 2018; Mattugini et al., 2018). In these proteomes, blood proteins are highly abundant at 3 days after stab wound injury, but are instead reduced at 5 days. At 5 days, we find Tgm1, Itih3, and Ftl1/2 to be more abundant, while the overall blood-related proteins have decreased. Furthermore, this highlights that much of the ECM changes remain from, or have similar composition to, a subacute stage after injury.

We confirm the presence of Ftl1/2 and Tgm1 in the tissue with immunohistochemistry. Our area-coverage analysis suggests the reduced abundance of these proteins in the CCR2-/- mouse stab wound injury site at 28 days is due to being restricted to a smaller area (**Figure 1D**). Most of the Ftl1/2 could be attributed to CD68+ macrophages or activated microglia and was present in a similar area to the chondroitin sulfate GAG-chains at the injury site (**Figures 1D,E**). Given that Ftl1/2 is secreted and became more insoluble at the scar (**Figure 1B**), we propose this protein

to be a matrisome-associated protein. An interesting possibility is that it may be bound to the ECM of the vasculature to capture Fe ions prior to entering the brain parenchyma to prevent the induction of toxic phospholipid oxidation products that lead to ferroptosis of cells at the injury site (Stockwell et al., 2017; Conrad and Pratt, 2019). Neurons – also in direct reprogramming from glial cells – can be particularly vulnerable to ferroptosis as recently shown (Gascón et al., 2016).

Interestingly, the block of proteins that are reduced ipsilateral compared to contralateral by injury are often associated to synapses (**Figure 1C**, e.g., Agrin, Cacna1e, Kcnq3, Mast3) indicative of the synapse loss persisting in the scar region of the injury site. Intriguingly, this loss is alleviated in the injury site of the CCR2-/- mice consistent with the notion that the scar is indeed reduced and the absence of monocyte invasion is beneficial for neuronal network recovery (Dimitrijevic et al., 2007; Hsieh et al., 2014; Morganti et al., 2015; Frik et al., 2018). We also see changes in the solubility of synaptic proteins (e.g., Gria2-3, Olfm3, Glgap1, and Vamp1), while we did not see an obvious change in the solubility of PNN proteins in the scar stage between the genotypes. Our previous QDSP analysis of PNN proteins in the uninjured cerebral cortex had revealed that they are typically not insoluble (in fraction 4), but rather belong to a less detergent resistant category (fractions 2–3; Kjell et al., 2020). Taken together, lack of monocyte invasion affects ECM proteins in a long-term manner toward a state closer to the un-injured contralateral site, thus normalizing the scar ECM.

## ECM ORIGIN AND SCARRING ZONES

Comparing previous proteomes obtained at the acute stages 3 and 5 dpi (Frik et al., 2018; Mattugini et al., 2018), we find scar-related ECM proteins peak at 5 days. At this time, we find Ftl1/2 to be present on and around CD68+ macrophages, although it seems to only be a subset that is responsible for the Ftl1/2 secretion (**Figure 2A**). Hence, we suggest that reduced levels of Ftl1/2 are a direct consequence of preventing the invasion of monocytes. Changes in Tgm1 on the other hand, rather seem to be an indirect consequence, since Tgm1 is not produced by macrophages, but rather astrocytes, as seen by immunostaining (**Figures 2A,B**). Indeed, Tgm1 has been proposed as a marker for neuroprotective astrocytes (Liddelow et al., 2017). Interestingly, Tgm1 spreads much further than the area densely populated with macrophages/activated microglia, giving credit to the idea that Tgm1 would be part of a neuroprotective response, possibly by maintaining the tissue integrity. The tissue of the glial scar is softer at the area affected by macrophages (Moeendarbary et al., 2017) and perhaps tissue more peripheral to the injury would also succumb to a similarly soft mechanical signature if Tgm1 was not present to counteract this by crosslinking ECM proteins.

Comparing the localization of Tgm1 with Tn-C and CSPG at 5 dpi, we find the increased levels of Tn-C and chondroitin sulfate GAG-chains (CS56) to be more associated to the core area of the injury with dense macrophage infiltrates, rather than the astrogliosis (**Figure 2B**). Hence, our results suggest that there are zones with different ECM composition in the scar-forming
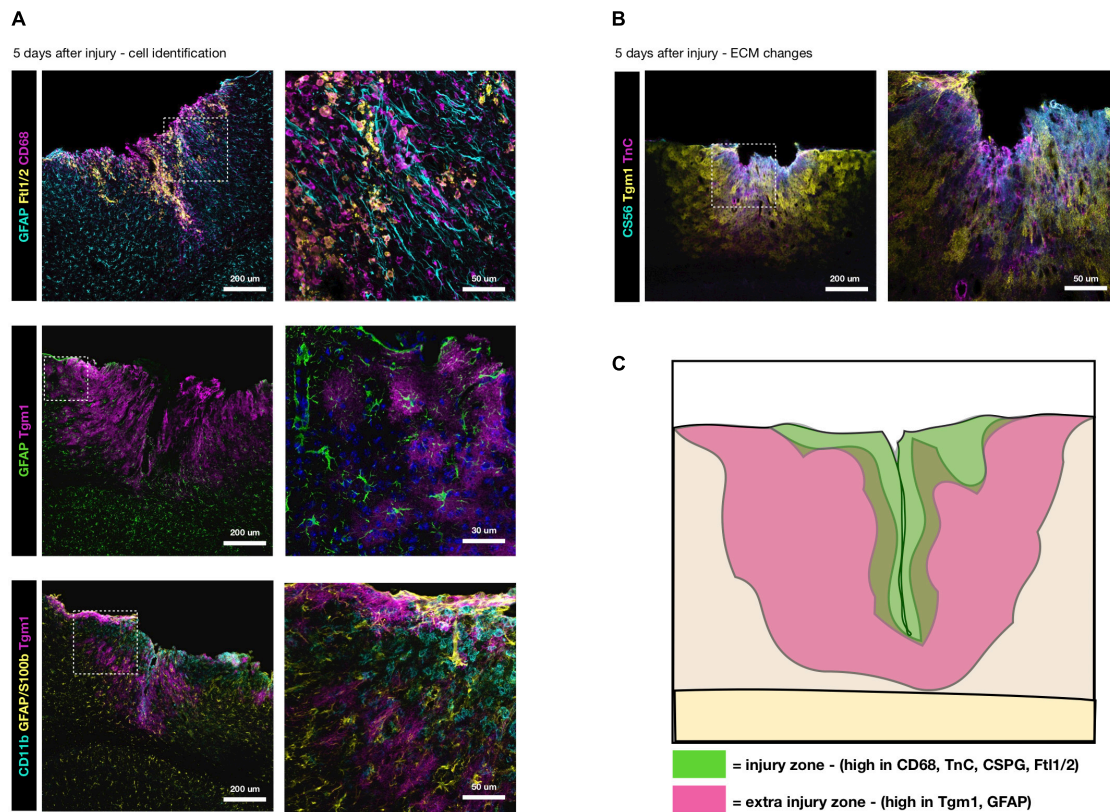
**FIGURE 2 |** Distinctive ECM zones at 5 days after TBI. **(A)** Tgm1 and Ftl1/2 immunostaining at 5 dpi, the peak of their deposition (Frik et al., 2018). Ftl1/2 is mostly found around CD68+ macrophages or activated microglia at the center of the injury site. Instead Tgm1 is present in a wider area overlapping with the region of GFAP+ reactive astrocytes, suggesting astrocytes may be a likely candidate for much of the Tgm1 expression/deposition. Interestingly, Tgm1 seems to surround the macrophage/activated microglia dense area suggestive of regionalization. **(B)** Tn-C and CS56, two ECM markers typical for the reactive glia, are localized to the center of the forming scar, while Tgm1 is more peripheral to it. **(C)** Here, in a conceptual summary of the subacute wound after stab wound injury of the brain, we suggest there are two primary zones (at the injury site and peripheral to the injury site) with different changes in the extracellular microenvironment that are fundamental to the final composition of the glial scar.

region (**Figure 2C**). The ECM and cell markers also suggest these zones may represent neurotoxic (core) and neuroprotective (surround) areas. Clearly, invasion of monocytes increases the neurotoxic core of the forming scar and its specific ECM, both directly and indirectly.

## COMPLEXITY IN INVESTING THE GLIAL SCAR

In any organ, scarring is a process that renders the respective part of an organ chronically non-functional. Scars are typically different from normal tissue in their ECM composition and hence a lot can be read from the changes in ECM in different tissue under abnormal conditions. However, in most cases only few ECM proteins are monitored as "representative" of scar formation and a comprehensive analysis is missing. Proteomics now offer a robust way to detect abundances of a large set of ECM, even in small tissue samples with maintained depth of detection and identification. Here we combined the proteomics with a protocol that gives a good indication on the cellular compartment

of all proteins. However, there may be further aspects of the ECM to be resolved with other sample-separation protocols, including investigations concerning the sugar-chain composition with glycomics or glycoproteomics. Comparative proteome-to-transcriptome analysis may yield further insights (Schiller et al., 2015; Angelidis et al., 2019; Kjell et al., 2020), specifically with single-cell RNA sequencing data that may allow identification of the cellular source of specific ECM proteins. For example, our analysis of the adult neurogenic niche shows that quiescent NSCs are by large the main contributor to the ECM composition of this niche (Kjell et al., 2020).

Moreover, proteome analysis at different times after stab wound injury unraveled that scar formation is determined at a subacute stages. When we compared the proteome of the injury site at 5 and 28 dpi between WT and CCR2-/- mice, we observed that many scar-related factors (such as enzymes for gycosaminoglycanes, but also many scar-resident proteins, such as Ftl1/2, Itih) are already present at 5 dpi and higher in WT. At this time the injury site and reactive gliosis region surrounding the injury is not yet obviously different between the genotypes, but the

proteomic composition including ECM proteins is already profoundly changed in the absence of monocyte invasion (Frik et al., 2018).

Given the potent effects of monocyte invasion on scar formation, it is important to note the differences in monocyte invasion in GM and WM regions. This can best be demonstrated when GM and WM injury are directly compared in the same brain region, as was recently done for the cerebral cortex using the GM injury paradigm described above and extending it into the WM (Mattugini et al., 2018). This revealed a much prolonged and bi-phasic monocyte invasion up to 2–3 weeks after injury in the WM, very much reminiscent of data obtained after spinal cord injury (Blight, 1992; Popovich et al., 1997; Beck et al., 2010). As WM sits at the surface of the spinal cord, it is always affected upon mechanical injury in this region, while brain injury is often restricted to GM given its location at the brain surface. Along with different patterns of monocyte invasion, many other aspects of gliosis, such as NG2 glia proliferation, were also different in brain TBI comprising the WM compared to GM only injury conditions (Mattugini et al., 2018). These data highlight that results obtained in one CNS region, such as the spinal cord, can not simply be extended to other regions, such as the cerebral cortex. Likewise, results obtained in one injury paradigm can not simply be extended to others as highlighted by the diversity of effects obtained by deletion or blocking of CCR2 in different injury paradigms. Unfortunately, this obvious message is all too often ignored.

These profound region- and injury-specific differences can also be observed when considering the zonation at the subacute injury site and the aspects of it that persist as part of the scar. Here we described two partly overlapping zones consisting of different cell types and ECM proteins in an injury largely lacking fibrosis. For other injury types with a fibrotic core it will be important to understand the different ECM composition of the fibrotic ECM and the surrounding gliotic one. This could also be done by using proteomic techniques analyzing the proteins directly from tissue sections such as MALDI-TOF (Lahiri et al., 2016; Quanico et al., 2018). Although fresh frozen tissue is preferred for proteomics, analyzing fixed tissue is now feasible, while maintaining a reasonable dept (Coscia et al., 2018) allowing exploration of ECM in patient samples. For example, such investigations have recently elucidated the role of the ECM-rich stromal compartments for cancer progression (Eckert et al., 2019). In addition to extending to human samples, it will be important to extend ECM analysis to samples of vertebrates with scar-less wound healing also after brain injury, such as the zebrafish (Baumgart et al., 2012; Kizil et al., 2015). Such data could teach us the composition of an extracellular environment that mediates wound healing without scar formation and allows neurogenesis and the integration of the new neurons. Characterizing such ECM changes may then help to steer ECM composition in mammalian brains toward scar-less wound healing supporting also neuronal replacement therapies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available via ProteomeXchange (Vizcaino et al., 2013) with identifier PXD017478.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Government of Upper Bavaria (Regierung von Oberbayern).

## AUTHOR CONTRIBUTIONS

MG and JK wrote the perspective. MG conceived the project. JK and MG conceptualized and planned the project. JK performed all experiments and analysis.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00032/full#supplementary-material

## REFERENCES

Adams, K. L., and Gallo, V. (2018). The diversity and disparity of the glial scar. *Nat. Neurosci.* 21, 9–15. doi: 10.1038/s41593-017-0033-9

Angelidis, I., Simon, L. M., Fernandez, I. E., Strunz, M., Mayr, C. H., Greiffo, F. R., et al. (2019). An atlas of the aging lung mapped by single cell transcriptomics and deep tissue proteomics. *Nat. Commun.* 10:963. doi: 10.1038/s41467-019-08831-9

Barker, R. A., Götz, M., and Parmar, M. (2018). New approaches for brain repair-from rescue to reprogramming. *Nature* 557, 329–334. doi: 10.1038/s41586-018-0087-1

Bartus, K., James, N. D., Didangelos, A., Bosch, K. D., Verhaagen, J., Yáñez-Muñoz, R. J., et al. (2014). Large-scale chondroitin sulfate proteoglycan digestion with chondroitinase gene therapy leads to reduced pathology and modulates macrophage phenotype following spinal cord contusion injury. *J. Neurosci.* 34, 4822–4836. doi: 10.1523/JNEUROSCI.4369-13.2014

Baumgart, E. V., Barbosa, J. S., Bally-Cuif, L., Götz, M., and Ninkovic, J. (2012). Stab wound injury of the zebrafish telencephalon: a model for comparative analysis of reactive gliosis. *Glia.* 60, 343–357. doi: 10.1002/glia.22269

Beck, K. D., Nguyen, H. X., Galvan, M. D., Salazar, D. L., Woodruff, T. M., and Anderson, A. J. (2010). Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* 133, 433–447. doi: 10.1093/brain/awp322

Blight, A. R. (1992). Macrophages and inflammatory damage in spinal cord injury. *J. Neurotrauma* 1, 83–91.

Bradbury, E. J., and Burnside, E. R. (2019). Moving beyond the glial scar for spinal cord repair. *Nat. Commun.* 10:3879. doi: 10.1038/s41467-019-11707-7

Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., et al. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416, 636–640.

Carulli, D., Pizzorusso, T., Kwok, J. C., Putignano, E., Poli, A., Forostyak, S., et al. (2010). Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. *Brain* 133, 2331–2347. doi: 10.1093/brain/awq145

Conrad, M., and Pratt, D. A. (2019). The chemical basis of ferroptosis. *Nat. Chem. Biol.* 15, 1137–1147. doi: 10.1038/s41589-019-0408-1

Coscia, F., Lengyel, E., Duraiswamy, J., Ashcroft, B., Bassani-Sternberg, M., and Wierer, M. (2018). Multi-level proteomics identifies CT45 as a chemosensitivity mediator and immunotherapy target in ovarian cancer. *Cell* 175, 159.e16–170.e16. doi: 10.1016/j.cell.2018.08.065

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., and Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* 13, 2513–2526. doi: 10.1074/mcp.m113.031591

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi: 10.1038/nbt.1511

Cregg, J. M., DePaul, M. A., Filous, A. R., Lang, B. T., Tran, A., and Silver, J. (2014). Functional regeneration beyond the glial scar. *Exp. Neurol.* 253, 197–207. doi: 10.1016/j.expneurol.2013.12.024

Deepa, S. S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., et al. (2006). Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans. *J Biol. Chem.* 281, 17789–17800. doi: 10.1074/jbc.m600544200

Didangelos, A., Puglia, M., Iberl, M., Sanchez-Bellot, C., Roschitzki, B., and Bradbury, E. J. (2016). High-throughput proteomics reveal alarmins as amplifiers of tissue pathology and inflammation after spinal cord injury. *Sci. Rep.* 6:21607. doi: 10.1038/srep21607

Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., and Andjelkovic, A. V. (2007). Absence of the chemokine receptor CCR2 protects against cerebral ischemia/reperfusion injury in mice. *Stroke* 38, 1345–1353. doi: 10.1161/01.str.0000259709.16654.8f

Eckert, M. A., Coscia, F., Chryplewicz, A., Chang, J. W., Hernandez, K. M., and Pan, S. (2019). Proteomics reveals NNMT as a master metabolic regulator of cancer-associated fibroblasts. *Nature* 569, 723–728. doi: 10.1038/s41586-019-1173-8

Eckert, R. L., Kaartinen, M. T., Nurminskaya, M., Belkin, A. M., Colak, G., Johnson, G. V., et al. (2014). Transglutaminase regulation of cell function. *Physiol. Rev.* 94, 383–417. doi: 10.1152/physrev.00019.2013

Frik, J., Merl-Pham, J., Plesnila, N., Mattugini, N., Kjell, J., Kraska, J., et al. (2018). Cross-talk between monocyte invasion and astrocyte proliferation regulates scarring in brain injury. *EMBO Rep.* 19:e45294. doi: 10.15252/embr.201745294

Gascón, S., Murenu, E., Masserdotti, G., Ortega, F., Russo, G. L., Petrik, D., et al. (2016). Identification and successful negotiation of a metabolic checkpoint in direct neuronal reprogramming. *Cell Stem Cell* 18, 396–409. doi: 10.1016/j.stem.2015.12.003

Geyer, P. E., Kulak, N. A., Pichler, G., Holdt, L. M., Teupser, D., and Mann, M. (2016). Plasma proteome profiling to assess human health and disease. *Cell Syst.* 2, 185–195. doi: 10.1016/j.cels.2016.02.015

Gliem, M., Mausberg, A. K., Lee, J. I., Simiantonakis, I., van Rooijen, N., Hartung, H. P., et al. (2012). Macrophages prevent hemorrhagic infarct transformation in murine stroke models. *Ann. Neurol.* 71, 743–752. doi: 10.1002/ana.23529

Göritz, C., Dias, D. O., Tomilin, N., Barbacid, M., Shupliakov, O., and Frisén, J. (2011). A pericyte origin of spinal cord scar tissue. *Science* 333, 238–242. doi: 10.1126/science.1203165

Gundemir, S., Colak, G., Tucholski, J., and Johnson, G. V. (2012). Transglutaminase 2: a molecular Swiss army knife. *Biochim. Biophys. Acta* 1823, 406–419. doi: 10.1016/j.bbamcr.2011.09.012

Hsieh, C. L., Niemi, E. C., Wang, S. H., Lee, C. C., Bingham, D., Zhang, J., et al. (2014). CCR2 deficiency impairs macrophage infiltration and improves cognitive function after traumatic brain injury. *J. Neurotrauma* 31, 1677–1688. doi: 10.1089/neu.2013.3252

Kizil, C., Kyritsis, N., and Brand, M. (2015). Effects of inflammation on stem cells: together they strive? *EMBO Rep.* 16, 416–426. doi: 10.15252/embr.201439702

Kjell, J., Fischer-Sternjak, J., Thompson, A. J., Friess, C., Sticco, M. J., Salinas, F., et al. (2020). Defining the adult neural stem cell niche proteome identifies key regulators of adult neurogenesis. *Cell Stem Cell* 26, 277–293. doi: 10.1016/j.stem.2020.01.002

Kjell, J., and Olson, L. (2016). Rat models of spinal cord injury: from pathology to potential therapies. *Dis Model Mech.* 9, 1125–1137. doi: 10.1242/dmm.025833

Kulak, N. A., Pichler, G., Paron, I., Nagaraj, N., and Mann, M. (2014). Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. *Nature Methods* 11, 319–324. doi: 10.1038/nmeth.2834

Lahiri, S., Sun, N., Solis-Mezarino, V., Fedisch, A., Ninkovic, J., Feuchtinger, A., et al. (2016). In situ detection of histone variants and modifications in mouse brain using imaging mass spectrometry. *Proteomics* 16, 437–447. doi: 10.1002/pmic.201500345

Lang, B. T., Cregg, J. M., DePaul, M. A., Tran, A. P., Xu, K., and Dyck, S. M. (2015). Modulation of the proteoglycan receptor PTPσ promotes recovery after spinal cord injury. *Nature* 518, 404–408. doi: 10.1038/nature13974

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., and Schirmer, L. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481–487. doi: 10.1038/nature21029

Majkut, S., Idema, T., Swift, J., Krieger, C., Liu, A., and Discher, D. E. (2013). Heart-specific stiffening in early embryos parallels matrix and myosin expression to optimize beating. *Curr. Bio.* 23, 2434–2439. doi: 10.1016/j.cub.2013.10.057

Mann, M., Kulak, N. A., Nagaraj, N., and Cox, J. (2013). The coming age of complete, accurate, and ubiquitous proteomes. *Mol. Cell* 49, 583–590. doi: 10.1016/j.molcel.2013.01.029

Mattugini, N., Merl-Pham, J., Petrozziello, E., Schindler, L., Bernhagen, J., Hauck, S. M., et al. (2018). Influence of white matter injury on gray matter reactive gliosis upon stab wound in the adult murine cerebral cortex. *Glia* 66, 1644–1662. doi: 10.1002/glia.23329

Moeendarbary, E., Weber, I. P., Sheridan, G. K., Koser, D. E., Soleman, S., Haenzi, B., et al. (2017). The soft mechanical signature of glial scars in the central nervous system. *Nat. Commun.* 8:14787. doi: 10.1038/ncomms14787

Morganti, J. M., Jopson, T. D., Liu, S., Riparip, L. K., Guandique, C. K., Gupta, N., et al. (2015). CCR2 antagonism alters brain macrophage polarization and ameliorates cognitive dysfunction induced by traumatic brain injury. *J. Neurosci.* 35, 748–760. doi: 10.1523/JNEUROSCI.2405-14.2015

Naba, A., Clauser, K. R., Hoersch, S., Liu, H., Carr, S. A., and Hynes, R. O. (2012). The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. *Mol. Cell. Proteom.* 11:M111.014647. doi: 10.1074/mcp.M111.014647

Orr, M. B., and Gensel, J. C. (2018). Spinal cord injury scarring and inflammation: therapies targeting glial and inflammatory responses. *Neurotherapeutics* 15, 541–553. doi: 10.1007/s13311-018-0631-6

Popovich, P. G., Wei, P., and Stokes, B. T. (1997). Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats. *J. Comp. Neurol.* 377, 443–464. doi: 10.1002/(sici)1096-9861(19970120)377:3<443::aid-cne10>3.0.co;2-s

Quanico, J., Hauberg-Lotte, L., Devaux, S., Laouby, Z., Meriaux, C., and Raffo-Romero, A. (2018). 3D MALDI mass spectrometry imaging reveals specific localization of long-chain acylcarnitines within a 10-day time window of spinal cord injury. *Sci. Rep.* 8:16083. doi: 10.1038/s41598-018-34518-0

Robel, S., and Sontheimer, H. (2016). Glia as drivers of abnormal neuronal activity. *Nat. Neurosci.* 19, 28–33. doi: 10.1038/nn.4184

Roll, L., and Faissner, A. (2014). Influence of the extracellular matrix on endogenous and transplanted stem cells after brain damage. *Front. Cell Neurosci.* 8:219. doi: 10.3389/fncel.2014.00219

Roll, L., and Faissner, A. (2019). Tenascins in CNS lesions. *Semin. Cell Dev. Biol.* 89, 118–124. doi: 10.1016/j.semcdb.2018.09.012

Saederup, N., Cardona, A. E., Croft, K., Mizutani, M., Cotleur, A. C., Tsou, C. L., et al. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS One* 5:e13693. doi: 10.1371/journal.pone.0013693

Schiller, H. B., Fernandez, I. E., Burgstaller, G., Schaab, C., Scheltema, R. A., and Schwarzmayr, T. (2015). Time- and compartment-resolved proteome profiling of the extracellular niche in lung injury and repair. *Mol. Syst. Biol.* 11, 819–819. doi: 10.15252/msb.20156123

Soderblom, C., Luo, X., Blumenthal, E., Bray, E., Lyapichev, K., and Ramos, J. (2013). Perivascular fibroblasts form the fibrotic scar after contusive spinal cord injury. *J. Neurosci.* 33, 13882–13887. doi: 10.1523/JNEUROSCI.2524-13.2013

Stockwell, B. R., Friedmann Angeli, J. P., Bayir, H., Bush, A. I., Conrad, M., and Dixon, S. J. (2017). Ferroptosis: a regulated cell death nexus linking metabolism. *Redox Biol.Dis. Cell* 171, 273–285. doi: 10.1016/j.cell.2017.09.021

Tica, J., Bradbury, E. J., and Didangelos, A. (2018). Combined transcriptomics, proteomics and bioinformatics identify drug targets in spinal cord injury. *Int. J. Mol. Sci.* 19, E1461. doi: 10.3390/ijms19051461

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., et al. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods* 13, 731–740. doi: 10.1038/nmeth. 3901

Vizcaino, J. A., Cote, R. G., Csordas, A., Dianes, J. A., Fabregat, A., Foster, J. M., et al. (2013). The Proteomics identifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* 41, D1063–D1069. doi: 10.1093/nar/gks1262

Wattananit, S., Tornero, D., Graubardt, N., Memanishvili, T., Monni, E., and Tatarishvili, J. (2016). Monocyte-derived macrophages contribute to spontaneous long-term functional recovery after stroke in mice. *J. Neurosci.* 36, 4182–4195. doi: 10.1523/JNEUROSCI.4317-15.2016

Wierer, M., Prestel, M., Schiller, H. B., Yan, G., Schaab, C., and Azghandi, S. (2018). Compartment-resolved proteomic analysis of mouse aorta during atherosclerotic plaque formation reveals osteoclast-specific protein expression. *Mol. Cell. Proteomics* 17, 321–334. doi: 10.1074/mcp.RA117.000315

# Advantages and Recent Developments of Autologous Cell Therapy for Parkinson's Disease Patients

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Parkinson's Disease (PD) is a progressive degenerative disease characterized by tremor, bradykinesia, rigidity and postural instability. There are approximately 7–10 million PD patients worldwide. Currently, there are no biomarkers available or pharmaceuticals that can halt the dopaminergic neuron degeneration. At the time of diagnosis about 60% of the midbrain dopamine (mDA) neurons have already degenerated, resulting in a depletion of roughly 70% of striatal dopamine (DA) levels and synapses. Symptomatic treatment (e.g., with L-dopa) can initially restore DA levels and motor function, but with time often lead to side-effects like dyskinesia. Deep-brain-stimulation can alleviate these side-effects and some of the motor symptoms but requires repeat procedures and adds limitations for the patients. Restoration of dopaminergic synapses using neuronal cell replacement therapy has shown benefit in clinical studies using cells from fetal ventral midbrain. This approach, if done correctly, increases DA levels and restores synapses, allowing biofeedback regulation between the grafted cells and the host brain. Drawbacks are that it is not scalable for a large patient population and the patients require immunosuppression. Stem cells differentiated *in vitro* to mDA neurons or progenitors have shown promise in animal studies and is a scalable approach that allows for cryopreservation of transplantable cells and rigorous quality control prior to transplantation. However, all allogeneic grafts require immunosuppression. HLA-donor-matching, reduces, but does not completely eliminate, the need for immunosuppression, and is currently investigated in a clinical trial for PD in Japan. Since immune compatibility is very important in all areas of transplantation, these approaches may ultimately be of less benefit to the patients than an autologous approach. By using the patient's own somatic cells, reprogrammed to induced pluripotent stem cells (iPSCs) and differentiated to mDA neurons immunosuppression is not required, and may also present with several biological and functional advantages in the patients, as described in this article. The proof-of-principle of autologous iPSC mDA restoration of function has been shown in parkinsonian non-human primates (NHPs), and this can now be investigated in clinical trials in addition to the allogeneic and HLA-matched approaches. In this review, we focus on the autologous approach of cell therapy for PD.

**Keywords: Parkinson's disease, autologous, transplantation, dopamine neurons, cell therapy**

# OVERVIEW OF DOPAMINE NEURON CELL THERAPY IN PARKINSON'S DISEASE AND ADVANTAGES OVER CURRENT AVAILABLE THERAPIES

## Current Therapies

The clinical movement disorder syndrome of Parkinson's Disease (PD) occurs due to a progressive loss of midbrain dopamine (mDA) neurons. In fact, most patients remain free of clinical motor symptoms until the PD pathology has already reached an advanced stage with most (∼60%) of the selectively vulnerable dopamine (DA) neurons dysfunctional or dead, and with a consequent depletion of roughly 70% of striatal DA levels and synapses (Engelender and Isacson, 2017). For this reason, and since any retardation of degeneration is unlikely to be absolute, it is reasonable to develop cell replacement for the lost neurons. Such "live cell" replacement therapies are conceptually different from classical pharmacology. The current mainstay treatment for PD related motor symptoms is based on a pharmacological approach from 1957 using levodopa (L-dopa) (Carlsson et al., 1957) or dopaminergic agonists that elevate DA levels or stimulate DA receptors (Radad et al., 2005; **Figure 1**). Although this treatment can be effective for many years, its long-term and chronic use can result in the development of "motor complications," including wearing-off, on-off fluctuations (Eriksson et al., 1984) and abnormal movements termed L-dopa-induced dyskinesias (Fahn, 2003). L-dopa crosses the blood-brain-barrier where it is converted to DA by dopa-decarboxylase containing cells; these include the remaining striatal dopaminergic terminals themselves, and also non-dopaminergic cells including cells in the blood-brain-barrier wall and serotoninergic neurons. Conversion of L-dopa to DA in non-dopaminergic cells following oral (non-continuous) administration of L-dopa, results in a pulsatile, non-physiological release of DA, which may act on supersensitive DA receptors in the striatum and contribute to the development of dyskinesias (Fahn, 2003). Newer dopaminergic agonists can activate DA receptors but they are not as effective as L-dopa, and in fact create substantial side effects on their own, including dyskinesia, albeit at a slower rate (Borovac, 2016). Deep brain stimulation (DBS) is another therapeutic modality but does not treat the DA neuron generation itself. DBS disrupts the circuits and functions as a depolarization blocker, which allows patients to take the same or higher L-dopa dose with less side effects, including dyskinesia, and dystonia (Herrington et al., 2016). It includes a surgical insertion of a medical device containing electrodes extending to the target region (subthalamic nucleus) and a pulse generator (Herrington et al., 2016) and carries the risk of major surgery. Generally, DBS works well initially but with time the circuitry readapts and over time is less effective in symptomatic relief (Buttner et al., 2019). In addition to the high initial surgical cost, patients require battery replacements every 3–5 years (Dang et al., 2019) and anticipating battery failure is also a critical clinical issue since it can result in a subacute worsening of symptoms (Montuno et al., 2013). Additionally, there is a risk that the DBS can result in cognitive side-effects if not implanted properly (Fields and Troster, 2000; Cernera et al., 2019).

## New Modalities

Other new modalities being explored for PD include gene therapy and cell therapy (Isacson and Kordower, 2008; **Figure 1**). Gene therapy creates enzymes to make L-dopa and DA non-synaptically (Mandel et al., 1999, 2008), similar to the use of a pump, without any cellular specificity or feedback control. From our and previous studies, gene therapy for neurotransmission defects are not likely to be helpful to the circuitry in patients as gene additions to striatal neurons will not control DA release.

Due to a lack of biofeedback and synaptic control in all current pharmacological therapies, they eventually lead to dyskinesias or other side-effects (see **Figure 1**). Therefore, there are efforts toward re-creating a synaptic DA release through the use of neural transplantation (Strecker et al., 1987; Clarke et al., 1988; Bjorklund et al., 2002; Isacson et al., 2003; Vinuela et al., 2008; Tsui and Isacson, 2011). To date, mDA cell preparations from aborted fetuses have been clinically tested and shown efficacy in PD patients (Lindvall et al., 1990; Freed et al., 1992; Kordower et al., 1998; Piccini et al., 1999; Hagell et al., 2000; Mendez et al., 2005; Redmond et al., 2008; Hallett et al., 2014; Kefalopoulou et al., 2014; Li et al., 2016). From a meta-analysis it was clear that if the fetal mDA cells are prepared and surgically injected appropriately, a 10–15 years of benefit is obtained with reductions in dyskinesia and off-time, and no additional side-effects appear (Barker et al., 2013). This improved function is because cell replacement using mDA neurons restores lost synapses. These new synapses functions with biofeedback regulation of DA locally in the synaptic microenvironment, resulting in physiological DA release and uptake, reducing the number of supersensitive DA receptors and providing long-term benefits for the patients with fewer side-effects (**Figure 1**, Vinuela et al., 2008; Tsui and Isacson, 2011). For these reasons and in a future perspective, cell therapy for PD when tested clinically to be safe and efficacious in moderate to severe patients, may potentially be used as a first-line of treatment to obviate the use of pharmacological DA therapies. When clinical trials using autologous or allogeneic midbrain neuron transplantation to PD patients are successful then cell therapy for PD would be a highly competitive treatment compared to currently available modalities.

The cell-based therapy approaches in PD aim to replace nigrostriatal DA terminals lost by the disease process, with fetal or stem cell derived DA neurons placed directly into the caudate-putamen, and potentially also in substantia nigra. Cell replacement therapy with mDA neurons in PD addresses both the motor symptoms of PD, as well as L-dopa-induced dyskinesias. In the most successful cases (Mendez et al., 2005; Kefalopoulou et al., 2014), the requirement for L-dopa medication has been negated or substantially reduced. When new mDA neurons (which are autonomous pace-maker neurons, not needing afferent input to regulate transmitter release) are engrafted into the normal target regions of nigrostriatal DA neurons, they establish synapses with mature host striatal neurons and provide physiologically appropriate DA release and synaptic feedback
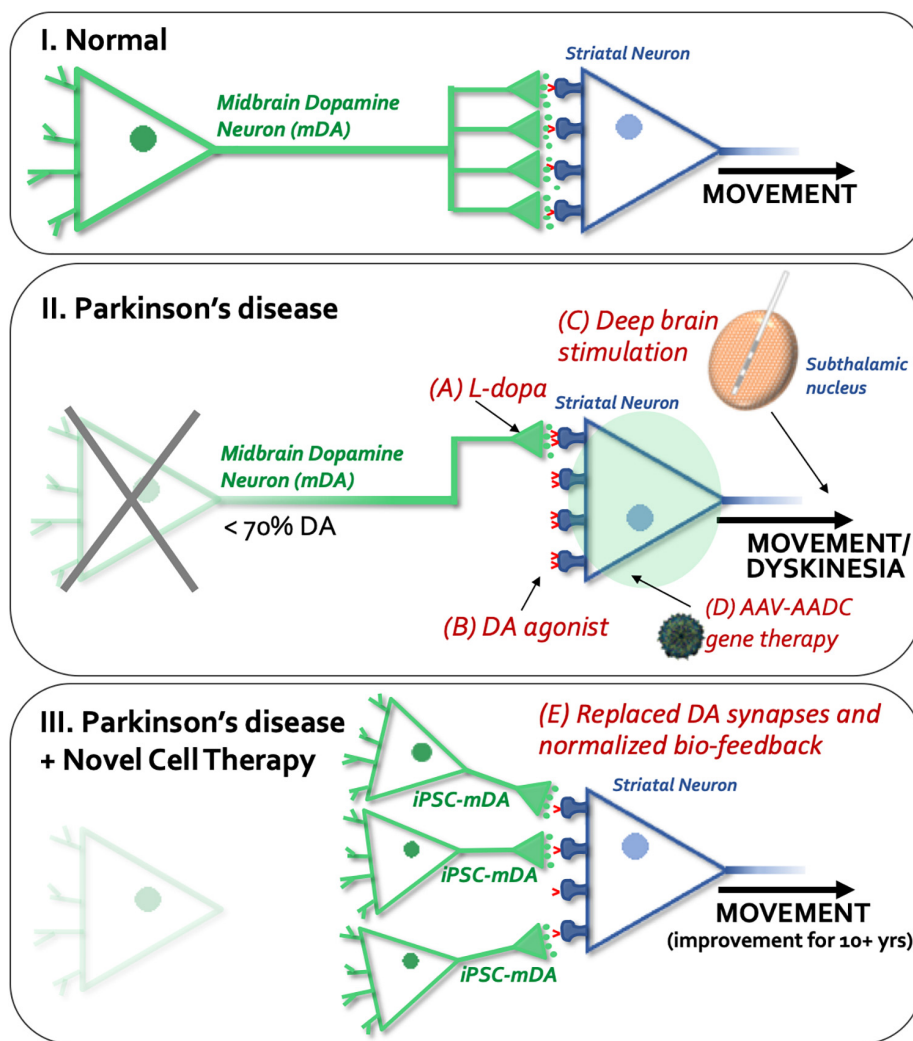
**FIGURE 1 |** Schematic overview of available or experimental treatments and procedures for Parkinson's disease. **(I)** Normal dopaminergic (shown in green) and striatal (shown in blue) synapses, and regulated dopamine (DA) release and reuptake (dopamine transporters shown in red) in the normal brain. **(II)** Shows current and experimental treatments: A, L-dopa; B, dopamine agonists; C) Deep brain stimulation (DBS), and D, gene therapy. The unique aspect of cell therapy (see **III**, E) is that it restores physiological dopamine release by synapses provided through new neurons implanted into the striatum.

control in the host brain (Zetterstrom et al., 1986; Vinuela et al., 2008). As discussed above, replacement of DA terminals in this manner may be far more effective in ameliorating the motor symptoms of PD than a pump-like pharmacological delivery of DA into the striatum that lacks physiological release and reuptake mechanisms (**Figure 1**). Long-term remarkable and neurologically clear benefits [approximately 50–60% reduction in Unified Parkinson's Disease Rating Scale (UPDRS) scores off DA drug therapy] to PD patients following fetal DA neuron transplantation has been reported for over 18 years, including our own work (Mendez et al., 2005; Politis et al., 2010; Hallett et al., 2014; Kefalopoulou et al., 2014), and this outcompetes any current treatment for PD. Moreover, implanted fetal DA neurons can prevent progressive worsening of PD motor scores over at least 14 years (Kefalopoulou et al., 2014; Ivar Mendez, unpublished data). These clinical benefits are associated with

evidence of physiological changes using PET and functional MRI neuroimaging (see **Figure 2**; Mendez et al., 2005). Our data also shows that transplanted fetal ventral mDA neurons remain healthy long-term (up to 14 years post-transplantation, the longest time-point we have studied to date) following transplantation into the putamen of PD patients, and despite ongoing disease processes in the host brain (Hallett et al., 2014).

# EMERGING OPTIONS FOR MIDBRAIN DOPAMINE NEURON CELL SOURCES

## Fetal Cells

Cell replacement therapy using DA neurons for PD in the clinic, has so far utilized ventral mDA neurons derived from fetal sources. More recently, attempts to replace missing DA neurons
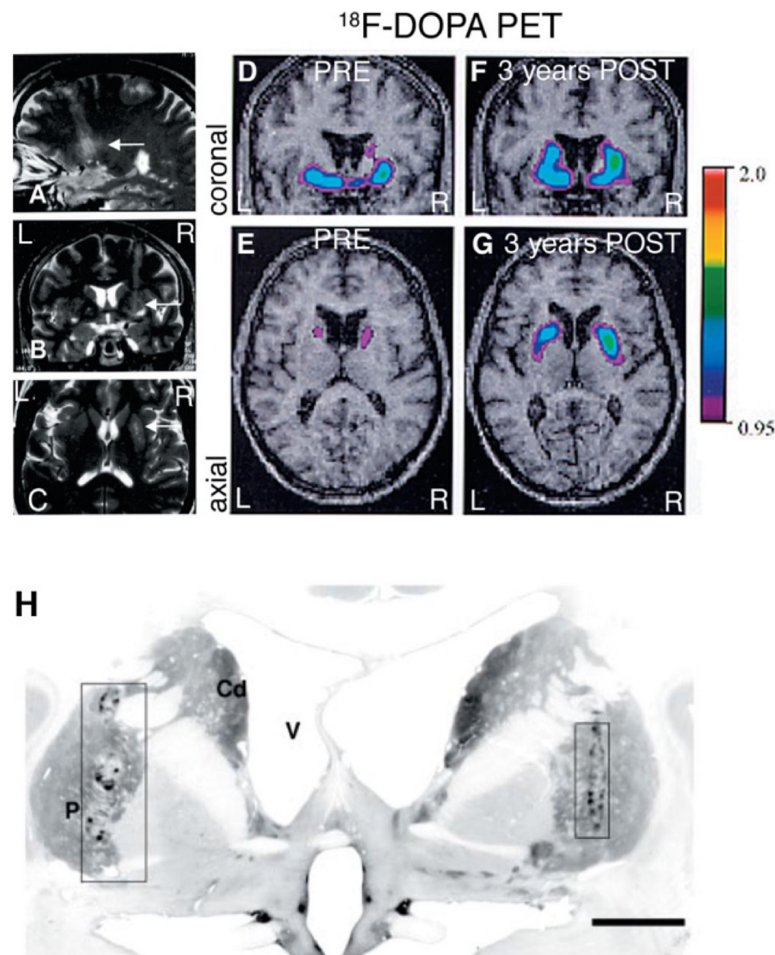
**FIGURE 2 |** Fetal VM grafts. **(A–C)** MRI 24 h after fetal VM transplantation surgery in the right putamen showing the 4 needle tracks. **(D,E)** Preoperative PET scan showed a marked, asymmetrical decrease in putaminal18F-DOPA uptake, consistent with the diagnosis of idiopathic PD. **(F,G)** 3 years after transplantation the PETs show a significant increase in 18F-DOPA uptake. **(H)** TH immunostaining of the grafts 4 years after transplantation. (P = putamen; Cd = caudate nucleus; V = lateral ventricle. Scale bar: **H**, 1 cm). Figure originally published in Mendez et al. (2005). Reused by permission of Oxford University Press.

in PD preclinical models have evolved from this innovative and complex fetal DA cell transplantation method toward a potential scalable method that depends on stem cell-derived DA neurons (Hargus et al., 2010; Kriks et al., 2011; Sundberg et al., 2013; Grealish et al., 2014). The replacement using fetal neurons depended on the events of elective abortions providing fetal tissue of the midbrain including the dopaminergic neurons. This method was not scalable and, in most cases, only a few dozen patients were appropriately transplanted worldwide in monitored clinical trials (Barker et al., 2013). However, these conceptual advances of cell therapy for PD have helped accelerate the realization of regenerative medicine in PD.

## Induced Pluripotent Stem Cells and Embryonic Stem Cells

Induced pluripotent stem cells (iPSCs) provide several advantages over embryonic stem cells (ESCs) as a cell source for cell replacement in PD and other disorders, including the ability

to use patient's own cells, or HLA-matched cells and thus reduce (HLA-matched) or eliminate (autologous iPSCs) the need for any immunosuppression. Immune compatibility is universally known to be very important in all fields of transplantation. Immune suppression is not a trivial matter and may underlie some of the variability previously reported in clinical trials of fetal derived DA neuron transplantation (Barker et al., 2013). Indeed, it was previously reported that following the cessation of immunotherapy 6 months after fetal cell transplantation, PD patients lost the benefits of the transplantation (Olanow et al., 2003), and thus, a delayed immune or inflammatory response could have affected the long-term survival, growth, and function of the transplanted DA neurons. Currently there are plans to transplant dopaminergic neurons obtained from general human ES cells (allogeneic) into PD patients in clinical trials (Studer, 2017; Parmar et al., 2020). In addition, there has been an initiation of a clinical trial in Japan based on the work of Takahashi's laboratory (Takahashi, 2017), where PD patients will receive HLA matched iPSC-derived dopamine

neurons. The authors of this review will elect to test the fully autologous patient derived mDA neurons in clinical trials. The results of all of these studies will help guide the future approach for most benefit for the patients. Given how far along the PD field is in the steps toward developing sustainable cell therapy for PD, it is likely that what is learnt from these studies will also serve as a major milestone for cell and regenerative therapy for other parts of the brain and nervous system. Beyond PD, cell therapy as a modality can be used for other neuronal and glial disorders associated with the brain and the periphery (Freeman et al., 2000; Goldman et al., 2012; Cunningham et al., 2014).

## Universal Donor Cells

Another approach to avoid the need of immune suppression is the generation of a universal donor cell that will evade the immune system either by HLA engineering or immune cloaking strategies (Lanza et al., 2019). These approaches are developed from knowledge of how malignant and transmissible cancer cells evade the immune system or understandings of how pathogens and parasites have evolved to escape immune recognition (Lanza et al., 2019), and it is not fully known what potential safety issues the introduction of such changes in the cells might lead to. The specific risk with using HLA-negative cells is malignancies. Reduced HLA expression is a known mechanism with which cancer cells can evade the immune system and any transplanted cells turned cancerous could therefore be likely to go undetected by the immune system. Introduction of suicide genes that can be activated if cells turn malignant might be a safety strategy in the future (Lanza et al., 2019). However, autologous cells already provide a great system for naturally recognizing a dying or dysfunctional cell.

## Direct Conversion of Astrocytes

Another approach that would also eliminate the need of immune suppression is the direct conversion of somatic cells to DA neurons *in vivo* using virus technology. The current approaches for PD aim to convert astrocytes to DA neurons (Rivetti di Val Cervo et al., 2017). This could be a potentially interesting approach but is still in early exploratory stages. A potential pitfall of this strategy is the local loss of the astrocytes that are reprogrammed to neurons and the potential associated problems with this local astrocyte loss in a human brain. Astrocytes have numerous important functions, and many of these functions are essential for brain homeostasis and neuronal health. For example, they provide neurotrophic and metabolic support, regulate synaptogenesis and synaptic function, contribute to the blood-brain-barrier and play an important role in limiting the spread of local immune response initiated my microglia, preventing cell damage to surrounding tissue. There is also a cellular and molecular diversity among astrocytes, thus understanding what cells and functions are lost would be important to predict how a conversion of local astrocytes to DA neurons might affect the function of the brain in a PD patient (Khakh and Deneen, 2019).

## EARLY EFFORTS TOWARD STEM CELL-BASED CELL REPLACEMENT THERAPY FOR PARKINSON'S DISEASE

As described in **Figure 3**, our research team started an original stem cell-based cell therapy program for PD in 1998 (Deacon et al., 1998) and had by 2002 (Bjorklund et al., 2002) reached a point when mouse midbrain DA neurons could be derived from ES cells and work functionally in rodent models of PD. This work continued with the use of iPSCs, and in 2008 our team and collaborators published work on the first mDA neurons differentiated from mouse iPSCs and their function in PD animal models (Wernig et al., 2008), followed by mDA neurons differentiated from human iPSCs from healthy donors and sporadic PD patients in 2009, which also demonstrated functional effect in rodent PD animal models (Hargus et al., 2010).

## PROOF-OF-CONCEPT FOR AUTOLOGOUS TRANSPLANTATION OF CYNOMOLGUS MONKEY IPSC-DERIVED MIDBRAIN DOPAMINE NEURONS

In 2015, our team published the first proof-of-concept (POC) data in non-human primates (NHPs) showing functional recovery and long-term survival of autologous transplanted iPSC-derived DA neurons. However, in this parkinsonian NHP model, unilateral autologous transplantation provided POC data for the long-term functional recovery of PD-like motor symptoms (increased daytime activity and reduction of time taken to complete a skilled motor task) for up to at least 2 years (Hallett et al., 2015). Behavioral improvement was accompanied by increased (compared to pre-transplantation values) DA transporter binding sites using PET neuroimaging, survival of engrafted DA neurons (>13,000), no inflammatory response, and no proliferating cells. Notably, no immunosuppression was administered to the recovered primate included in this study at any time, thus validating the use of autologous transplantation for use in clinical studies. In a recent study (Osborn et al., 2020) we have demonstrated functional restoration in two additional parkinsonian NHPs receiving autologous transplantation of iPSC-derived mDA cells (see **Figure 4**). In these animals, *even after 8 years of chronic PD without any spontaneous recovery, the parkinsonian NHPs improved functionally by the implanted iPSC-derived dopaminergic cells.* The degree of survival of the transplanted mDA neurons was consistent with our previously published work in primates (Hallett et al., 2015). From this work, we conclude that autologous transplantation provides functional recovery (reduced motor dysfunction) and transplanted tyrosine hydroxylase positive DA neurons survived in the putamen for at least 2 years after transplantation (the time of sacrifice). The additional confirmative data (see **Figure 4**) showed that functional recovery was observed with ~20,000 surviving tyrosine hydroxylase positive neurons in the graft. There was no observable immune response present, as assessed by staining for microglia in the graft and in the neighboring host

**FIGURE 3 |** Progression of autologous cell therapy for Parkinson's disease. In green are the discoveries and publications that have contributed to this timeline by the authors and their collaborators (Schmidt et al., 1981; Lindvall et al., 1988; Widner et al., 1992; Dinsmore et al., 1996; Deacon et al., 1997, 1998; Fink et al., 2000; Schumacher et al., 2000; Bjorklund et al., 2002; Mendez et al., 2005; Takahashi et al., 2007; Wernig et al., 2008; Cooper et al., 2010; Hargus et al., 2010; Hallett et al., 2014, 2015).

putamen. Consistent with these data, a recent study using xeno-grafting of human iPSCs into parkinsonian NHPs, demonstrated that a similar number of surviving DA neurons (~16,000) resulted in functional improvement of the immunosuppressed primates, validating our present and previous reports of the effectiveness of this cell-dose in NHPs (Kikuchi et al., 2017).

## METHODS RELEVANT FOR GENERATING MIDBRAIN DOPAMINE NEURONS FROM INDUCED PLURIPOTENT CELLS FOR AUTOLOGOUS TRANSPLANTATION

### mDA Neuron Differentiation

iPSCs, when exposed to a mixture of small molecules, can be differentiated toward mDA neuron fate (**Figure 5**; Cooper et al., 2010). Protocols published prior to 2010 (Roy et al., 2006; Sonntag et al., 2007; Chiba et al., 2008; Yang et al., 2008) resulted in a very low fraction of the A9 mDA neuron subpopulation that originates in the substantia nigra pars compacta and is lost in PD patients. A comprehensive study was published in 2010 (Cooper et al., 2010) that discerned factors important for the generation of this DA neuron subpopulation. Careful titrations of retinoic acid levels show that 10nM retinoic acid significantly improved the expression of the transcription factor (TF) Engrailed-1, a TF important for mDA development and survival (Cooper et al., 2010). Additional changes to previously published protocols (Sonntag et al., 2007; Cai et al., 2009)

included a more potent form of sonic hedgehog recombinant protein, specification of the subtype of FGF-8 used (FGF-8a rather than FGF-8b) and the use of recombinant Wnt-1 for canonical Wnt pathway (Wnt/β-catenin pathway) activation. These changes generated a human neural progenitor cell population that exhibited a transcriptional profile (Muhr et al., 1999; Nordstrom et al., 2002, 2006) consistent with midbrain regionalization (Cooper et al., 2010). Several protocols are now available worldwide for midbrain differentiation that includes DA neurons (Cooper et al., 2010; Kriks et al., 2011; Sundberg et al., 2013; Kikuchi et al., 2017; Nolbrant et al., 2017) and protocols are being refined by use of xeno-free procedures and highest grade available reagents (Nolbrant et al., 2017; Osborn et al., 2017) in order to improve reproducibility of differentiations and eliminate components incompatible with human transplantations (Cooper et al., 2012). These protocols differ in a few specific aspects (Cooper et al., 2010; Kriks et al., 2011; Sundberg et al., 2013; Kikuchi et al., 2017; Nolbrant et al., 2017) and as described below some teams chose to use progenitor cells whereas others prefer the post mitotic equivalence of fetal neurons.

### Maturity of Transplantable Cells

The decision of whether to transplant the mDA patterned cells at a progenitor stage, as post-mitotic neurons or a mix thereof determines what cell type markers are used in the quality control process. Furthermore, in order to allow for quality control testing of cell batches for transplantation and allow flexibility as to when and where a patient undergoes the transplantation surgery the cells need to be cryopreserved.
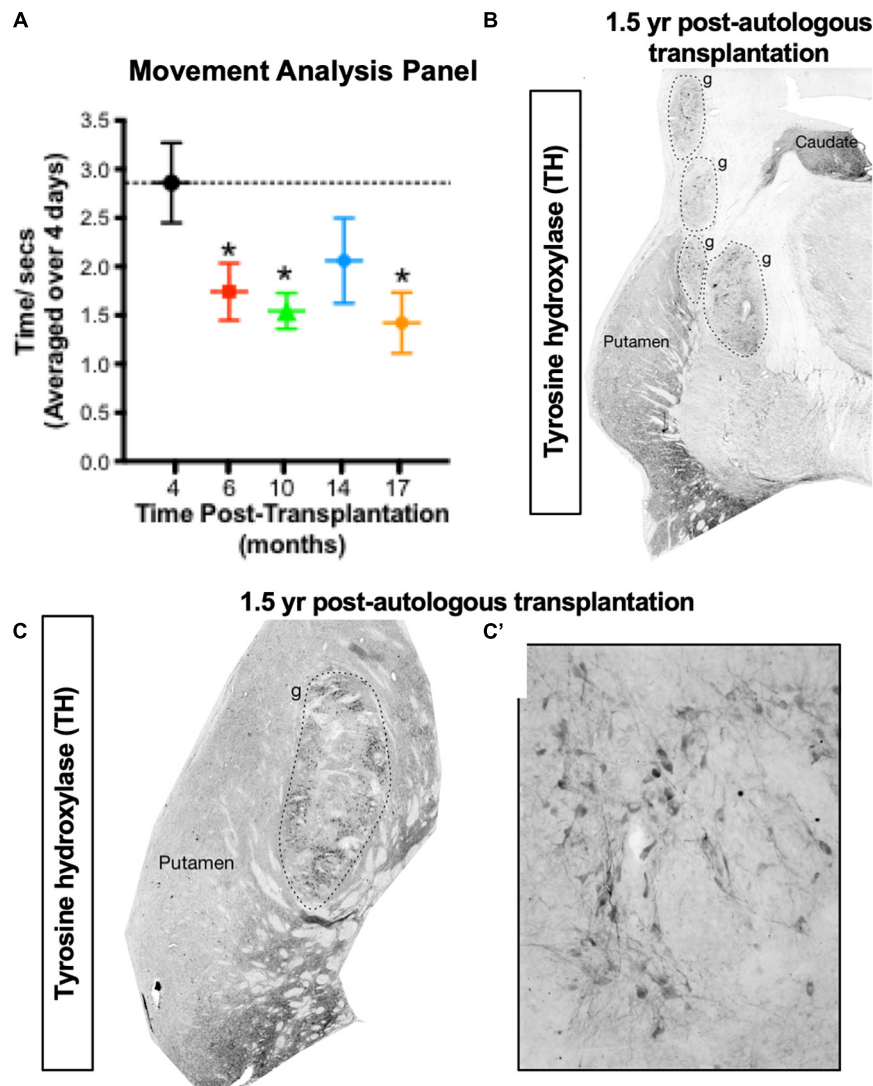
**FIGURE 4 |** Proof of concept for the autologous transplantation approach in parkinsonian NHPs. **(A,B)** Autologous transplantation of iPSC-mDA cells into the left putamen of an MPTP-lesioned primate provides functional improvement in the right (contralateral) forelimb in an automated Movement Analysis Panel **(A)**, and survival of dopamine (TH+) neurons (>20,000) in the transplanted putamen **(B)**. **(C,C')** Robust survival of TH + neurons at 1.5 years following autologous transplantation of iPSC-mDA cells into the left putamen of an additional MPTP-lesioned NHP (Osborn et al., 2020). These recent NHP data are supportive of our previous NHP findings (Hallett et al., 2015).

The maturity and timing selected for cryopreservation is also important as it can impact the survival of the dopaminergic neurons. If the cells are frozen at too mature of a stage, the survival of the DA neurons after transplantation is less than if frozen and transplanted at an early post-mitotic stage or progenitor stage (Osborn et al., 2015). Different groups are using different approaches and timing for freezing (Kriks et al., 2011; Nolbrant et al., 2017; Osborn et al., 2017), which in turn leads to different markers being relevant for use in pre-transplantation criteria (Kee et al., 2017; Kirkeby et al., 2017; Osborn and Hallett, 2017). Tyrosine hydroxylase (TH) staining is an indicator of post-mitotic dopaminergic neurons that in recent protocols start to be expressed at about day *in vitro* 17. Therefore, it

can be used together with FoxA2 as an indicator of midbrain dopaminergic neurons and the percentual co-positivity of the two can be set as a positive cellular marker criterion for mDA cell transplantations. In cases where only progenitor cells are transplanted one must rely on additional markers to determine dopaminergic progenitors and predict future dopaminergic cell content (Kee et al., 2017; Kirkeby et al., 2017; Osborn and Hallett, 2017). Given that several different preclinical studies have been successful in transplanting at various time-points (DIV16-49) (Hargus et al., 2010; Kriks et al., 2011; Sundberg et al., 2013; Doi et al., 2014; Hallett et al., 2015; Kikuchi et al., 2017; Nolbrant et al., 2017; Osborn et al., 2017; Wakeman et al., 2017) and resulting in similar functional grafts containing

**FIGURE 5 |** Phenotypic characterization of FOXA2 + dopaminergic neurons generated by the differentiation protocol described in Cooper et al. (2010) at DIV49. **(A)** Human iPSCs generate FOXA2 + (red) dopaminergic neurons (TH, blue; β-tubulin, green; arrowheads). **(B,C)** Cells co-expressed TH (blue), FOXA2 (red) and calbindin (green, B) or GIRK2 (green, C), indicative of an A10 or A9 DA neuron phenotype, respectively. Scale bar **(A)** = 50 μm, **(B,C)** = 10 μm. Reprinted from Cooper et al. (2010) with permission from Elsevier.

mDA neurons, it is premature to say what is the most optimal protocol and strategy. Of note, the only grafted cells that have shown to generate functional recovery in parkinsonian NHPs have been with cell preparations that contain post-mitotic neurons (Hallett et al., 2015; Kikuchi et al., 2017). Furthermore, although mDA neurons are the cell type that is responsible for the functional effect in grafts, the midbrain cell population produced does not necessarily require cell sorting since all clinical experience to date, using fetal cell transplantation, includes a mixture of midbrain cells. It is in fact possible that removing the other midbrain companion cells may reduce trophic interactions necessary (positive bystander effect) for substantia nigra survival (Hedlund et al., 2008). The autologous approach as planned by this team is summarized in **Figure 6**. The future pre-clinical and clinical studies, on-going and planned will provide a guide of the specifics for the most efficacious and safe cells or cell compositions for transplantation, whether pure mDA neurons or a mixed midbrain cell composition and whether progenitors, post-mitotic neurons or a mixture of both are preferred.

## PROSPECTIVE LONG-TERM HEALTH OF TRANSPLANTED DOPAMINE NEURONS

A misconception associated with using cell replacement therapy in neurodegenerative disorders, is that the transplanted cells will eventually succumb to the same pathological processes and disease that presented in the host brain, resulting in reduced function of the transplanted cells. In the PD field, this is based on popular theory about pathological "spread" of α-synuclein from host to transplant (Kordower et al., 2008; Li et al., 2008), and the observations that a low percentage of transplanted fetal dopaminergic neurons contain α-synuclein

immune reactive inclusions over a decade after transplantation (Kordower et al., 2008; Li et al., 2008, 2016). Discrepancies in success of fetal dopaminergic neurons come from procedural differences in these transplantations (Redmond et al., 2008; Cooper et al., 2009). When the fetal tissue is dissociated to a cell suspension prior to transplantation, the grafts remain healthy 14 years post-transplantation and have none or very few α-synuclein inclusions after 14 years (Mendez et al., 2008; Hallett et al., 2014). However, if transplanting cellular aggregates instead of cells in suspension, the grafts are surrounded by activated microglia (Li et al., 2008; Kurowska et al., 2011) and around 2–12% of the dopaminergic neurons in the grafts have α-synuclein positive inclusions after 12–24 years (Kordower et al., 2008; Li et al., 2008, 2016). Importantly, there is no evidence of any clinical or functional relevance of such limited pathology (Cooper et al., 2009). In fact, transplanted fetal midbrain dopaminergic neurons have been shown to function, as evidenced by improvements in PD motor symptoms, for over a decade after transplantation (Li et al., 2016) with the longest documented functional improvement for at least 18 + years (Hallett et al., 2014; Kefalopoulou et al., 2014). In fact, even at these long time frames, patients have been able to reduce or discontinue pharmacological DA replacement therapy (Kefalopoulou et al., 2014). Another way of looking at this issue in a medical and biological perspective is to raise the simple functional question that if the transplants really were under "attack," this would be contradicted by the clinical facts of striking clinical benefits that cell replacement of mDA neurons can provide. Using cell therapy, it is surprising but true that transplanted neurons can remain functional for at least 10–20 years and show no histological evidence or neuritic pathology. These cell therapy clinical studies have had no gene modifiers, including of α-synuclein, or any or blocking of α-synuclein function (Hallett et al., 2014; Kefalopoulou et al., 2014). Therefore, the planned clinical trials by several groups worldwide are aligned on the scientific and biological view that newly implanted functional DA neurons are not affected significantly or functionally by the underlying disease process at least for several decades (Astradsson et al., 2008). Moreover, our data and others studying human cells transplanted to patients with PD, provides a perspective of the actual development of clinical PD in the patient's own DA cells (Hallett et al., 2014) which do not succumb to dysfunction and detrimental pathology until in the vast majority of cases, at least in the 6th–7th decade of life.

## THE USE OF AUTOLOGOUS CELLS WITH POSSIBLE GENETIC PREDISPOSITION TO DISEASE

The majority of patients with PD (>85%) have sporadic forms, and the genetic forms of PD are relatively rare. However, even in cases where the patients have underlying cellular problems due to genetic variants, they have surviving functional DA neurons for the majority of their life, in some cases up to >60 years of

**FIGURE 6 |** Schematic describing the process for autologous cell therapy for Parkinson's disease. Blood is collected from patients by venipuncture. Peripheral blood mononuclear cells (PBMCs) are isolated and the specific somatic cell population for reprogramming is expanded. Reprogramming factors (Takahashi et al., 2007) are introduced and clonal lines expanded for quality control testing. Clones that pass all quality control (QC) steps are used for mDA differentiations. The mDA neurons are cryopreserved and the batches quality control tested prior to autologous transplantation.

age, before any noticeable degeneration that results in functional impairment occur. Even in severe familial genetic cases (for example, α-synuclein mutation, or copy number variants), PD does not present for 30 years or more. Therefore, it is both logical and reasonable to assume that even the most severe, rare genetic forms would, after transplantation of new young cells, have highly adaptive functional DA neurons for synaptic signaling for at least 20–25 years. In fact, both rodent (Hargus et al., 2010) and NHP (Kikuchi et al., 2017) studies have demonstrated that human iPSC-derived mDA cell preparations from both healthy subjects and PD patients work equally well in restoring synapses and motor function in parkinsonian animal models. On the contrary, long-term restorative function has not yet been shown in parkinsonian NHPs for mDA neuron preparations differentiated from human ES cell lines, despite significant efforts.

## ADVANTAGES OF AUTOLOGOUS CELLS FOR TRANSPLANTATION IN PD OVER ALLOGENEIC OR MHC-MATCHING

There are several advantages of the autologous cell therapy approach over allogeneic or MHC matching (Emborg et al., 2013; Morizane et al., 2013; Hallett et al., 2015; **Table 1**). One obvious advantage, relative to allogeneic cell transplants, is that autologous iPSC can be used in PD patients without the need for immunosuppression (Hallett et al., 2015). The question about the need for immunosuppression is important at many levels:

(1) *Basic biology of cell integration and recognition*: Autologous neural cell transplants potentially are better integrated, have better axonal networks (Emborg et al., 2013; Hallett et al., 2015) and have better functional effects than non-autologous transplants (Hallett et al., 2015). A relatively low number of

autologous dopaminergic midbrain neurons derived from iPSCs ($\sim$13–14,000) can be sufficient to reverse parkinsonism in NHPs (**Figure 4**; Hallett et al., 2015; Osborn et al., 2020).

(2) *Rejection by immunological mechanisms at cellular and synaptic levels*: A primate study demonstrated clear benefits to the use of autologous transplants rather than allografts in both cell survival and immune response (Morizane et al., 2013). A different primate study looking at both the acute and subacute immune response showed that MHC matching improves the engraftment of iPSC-derived mDA neurons in NHPs. But although MHC matching reduced the immune response, it did not completely prevent an immune reaction and the conclusion was that MHC matching still needs to be combined with immunosuppressive drugs but MHC matching might reduce the required dose and duration of such therapy (Morizane et al., 2017). Studies with fetal mDA neurons in the clinic have demonstrated several cases in which the clinical beneficial response is reduced after removal of immune suppression by 6–9 months after cell implantation (Olanow et al., 2003). Decades of studies of allogeneic brain transplantation demonstrates sensitization of the B cell component of the immune system (Kordower et al., 1997), providing increasing antibody titers to such allogeneic transplants. Clearly, the brain immune system is capable of activating both T and B cell responses to interfere with neuronal transplants (Kordower et al., 1997). Should future trials of allogeneic transplants show variable rejections, it will be an enormous obstacle to remove immune suppression or scale to a larger group of patients, as patients would not want to risk transplant rejection and loss of function. Attempts to simplify an allogeneic universal cell source by removing HLA antigens and related MHC systems may occur in the future, but there is almost nothing known about the need for the immune system to eliminate unhealthy or dying cells

- Autologous approach requires no immune suppression in patients (as also demonstrated by the team's POC non-human primate data).
- Competing allogeneic and MHC-matched cell therapy approaches will require long-term (6–12 + months) immune suppression. Systemic immune suppression, as required for MHC-matched and allogeneic approaches is not trivial in older or frail patients, and some patients may not be able to tolerate it and will have significant morbidity.
- There are significant immunological effects of allogeneic transplants.
- Autologous neural cell transplants potentially are better integrated and have axonal networks and better functional effects than non-autologous transplants. Even modest immune activation is expected to be detrimental to graft function and synaptic connectivity.
- The medical transplantation field, hospitals, health care providers, and payors of health care have learned significantly from decades of autologous vs. allogeneic cell therapy in patients requiring bone marrow transplantation. Some of this learning may apply to autologous cell therapy for brain degenerative diseases like Parkinson's disease.

that are genetically manipulated in such a way. The advantage of autologous cell sources is that the natural biology for cell/transplant integration, recognition and function is coupled with an immune competence to eliminate dying or dysfunctional cells, as would occur normally in any brain or biological tissue. Given this, autologous cell therapy may become a gold standard for which the other future cell therapies need to be measured; and

(3) *The health risks to the patient recipients*: Patients with compromised immune system are always considered high risk recipients for systemic immune suppression for any amount of time. Immune responses as observed with allografting or xenografting can be detrimental to transplant function (Soderstrom et al., 2008; Morizane et al., 2013). Data from already well-established medical disciplines demonstrate significant differences in risk profiles between autologous and allogeneic transplants. An illustration is the relevant stem cell bone marrow transplantation therapy, where the morbidity, mortality, length of hospital stays and costs are reduced by autologous approaches (Rowe et al., 1994; Majhail et al., 2013). Taken together, autologous transplantations overcome several limitations as described above, which will likely lead to improved outcomes in many scenarios, including risk for graft-host rejections, local immune responses that clearly reduces functional synaptic transmission, and morbidity risk for patients taking severe immunosuppressive drugs.

## CONSIDERATION OF HEALTHCARE COSTS AND BENEFITS RELATIVE TO NEW CELL THERAPIES

A conventional view, criticism and perception is that autologous cell therapy is always more expensive than allogeneic cell therapy. It is true that an allogeneic or HLA-matched approach would allow for larger batches of cells to be produced and quality control tested for use in multiple patients, whereas an autologous cell transplantation approach requires preparation of cell batches for each patient and quality control testing of each batch, which initially (but less so with increased scale and automation) drives up the cost of the cell production step. However, when looking at the total healthcare cost, this conventional view is not necessarily true (Majhail et al., 2013). An interesting future perspective is that many payors may try to avoid allogeneic transplants given the documented higher current costs due to immune suppression and transplant rejection. In addition, as outlined above, for neural transplantation and maybe other cell types, the functional integration is improved with recognition of autologous antigens. In well-established medical disciplines there are significant differences in risk profiles between autologous and allogeneic transplants. An example is stem cell bone marrow transplantation cell therapy, where the morbidity and costs for failed allogeneic transplants is much higher than for autologous transplants (Majhail et al., 2013). The medical transplantation field, hospitals, health care providers, and payors of health care have learned significantly from decades of autologous vs. allogeneic cell therapy in patients requiring bone marrow transplantation. Some of this learning may apply to autologous cell therapy for brain degenerative diseases like PD. For bone marrow stem cell transplantation, payors and health care providers can estimate over $100,000 per patient for completed *autologous* bone marrow transplantation cell therapy and follow-up. However, the average estimation for payor and health care costs for *allogeneic* transplants and cell therapy can be several-fold higher (Majhail et al., 2013). The reason is that allogeneic transplantation presents a large and significant morbidity risk to the patients due to immunological and prominently immune suppression issues (Rowe et al., 1994). The high risk for patient morbidity creates a significant burden and additional cost to the health care system, where the potential very large cost per patient of allogeneic cell therapy needs to be viewed as a potential loss of benefit to those who could receive an effective autologous transplant. For payors and the healthcare system the average expected cost of allogeneic transplants is therefore in reality higher than autologous cell therapy. Whether these considerations also apply to allogeneic vs. autologous transplantation of brain cells into the CNS will hopefully become evident early in the process of safety/Phase 1 trials in humans. With this future perspective in mind, we believe there are several reasons why overall, the health care systems will likely support technical and medical innovation that support autologous transplants for most applicable medical conditions. In such a perspective, this overall strategic support for autologous transplantation will also apply to autologous cell therapy approaches for PD and related disorders.

## AUTHOR CONTRIBUTIONS

TO, PH, JS, and OI wrote this manuscript.

## REFERENCES

Astradsson, A., Cooper, O., Vinuela, A., and Isacson, O. (2008). Recent advances in cell-based therapy for Parkinson disease. *Neurosurg. Focus* 24:E6. doi: 10.3171/FOC/2008/24/3-4/E5

Barker, R. A., Barrett, J., Mason, S. L., and Bjorklund, A. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. *Lancet Neurol.* 12, 84–91. doi: 10.1016/s1474-4422(12)70295-8

Bjorklund, L. M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., et al. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2344–2349. doi: 10.1073/pnas.022438099

Borovac, J. A. (2016). Side effects of a dopamine agonist therapy for Parkinson's disease: a mini-review of clinical pharmacology. *Yale J. Biol. Med.* 89, 37–47.

Buttner, C., Maack, M., Janitzky, K., and Witt, K. (2019). The evolution of quality of life after subthalamic stimulation for parkinson's disease: a meta-analysis. *Mov. Disord. Clin. Pract.* 6, 521–530. doi: 10.1002/mdc3.12819

Cai, J., Donaldson, A., Yang, M., German, M. S., Enikolopov, G., and Iacovitti, L. (2009). The role of Lmx1a in the differentiation of human embryonic stem cells into midbrain dopamine neurons in culture and after transplantation into a Parkinson's disease model. *Stem Cells* 27, 220–229. doi: 10.1634/stemcells.2008-0734

Carlsson, A., Lindqvist, M., and Magnusson, T. (1957). 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature* 180:1200. doi: 10.1038/1801200a0

Cernera, S., Okun, M. S., and Gunduz, A. (2019). A review of cognitive outcomes across movement disorder patients undergoing deep brain stimulation. *Front. Neurol.* 10:419. doi: 10.3389/fneur.2019.00419

Chiba, S., Lee, Y. M., Zhou, W., and Freed, C. R. (2008). Noggin enhances dopamine neuron production from human embryonic stem cells and improves behavioral outcome after transplantation into Parkinsonian rats. *Stem Cells* 26, 2810–2820. doi: 10.1634/stemcells.2008-0085

Clarke, D. J., Brundin, P., Strecker, R. E., Nilsson, O. G., Björklund, A., and Lindvall, O. (1988). Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: ultrastructural evidence for synapse formation using tyrosine hydroxylase immunocytochemistry. *Exp. Brain Res.* 73, 115–126. doi: 10.1007/bf00279666

Cooper, O., Astradsson, A., Hallett, P., Robertson, H., Mendez, I., and Isacson, O. (2009). Lack of functional relevance of isolated cell damage in transplants of Parkinson's disease patients. *J. Neurol.* 256(Suppl. 3), 310–316. doi: 10.1007/s00415-009-5242-z

Cooper, O., Hargus, G., Deleidi, M., Blak, A., Osborn, T., Marlow, E., et al. (2010). Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol. Cell. Neurosci.* 45, 258–266. doi: 10.1016/j.mcn.2010.06.017

Cooper, O., Parmar, M., and Isacson, O. (2012). Characterization and criteria of embryonic stem and induced pluripotent stem cells for a dopamine replacement therapy. *Prog. Brain Res.* 200, 265–276. doi: 10.1016/b978-0-444-59575-1.00012-0

Cunningham, M., Cho, J. H., Leung, A., Savvidis, G., Ahn, S., Moon, M., et al. (2014). hPSC-derived maturing GABAergic interneurons ameliorate seizures and abnormal behavior in epileptic mice. *Cell Stem Cell* 15, 559–573. doi: 10.1016/j.stem.2014.10.006

Dang, T. T. H., Rowell, D., and Connelly, L. B. (2019). Cost-effectiveness of deep brain stimulation with movement disorders: a systematic review. *Mov. Disord. Clin. Pract.* 6, 348–358. doi: 10.1002/mdc3.12780

Deacon, T., Dinsmore, J., Costantini, L., Ratliff, J., and Isacson, O. (1998). Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. *Exp. Neurol.* 149, 28–41. doi: 10.1006/exnr.1997.6674

Deacon, T., Schumacher, J., Dinsmore, J., Thomas, C., Palmer, P., Kott, S., et al. (1997). Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nat. Med.* 130, 350–353. doi: 10.1038/nm0397-350

Dinsmore, J., Ratliff, J., Deacon, T., Pakzaban, P., Jacoby, D., Galpern, W., et al. (1996). Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. *Cell Transpl.* 5, 131–143. doi: 10.1016/0963-6897(95)02040-3

Doi, D., Samata, B., Katsukawa, M., Kikuchi, T., Morizane, A., Ono, Y., et al. (2014). Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation. *Stem Cell Rep.* 2, 337–350. doi: 10.1016/j.stemcr.2014.01.013

Emborg, M. E., Liu, Y., Xi, J., Zhang, X., Yin, Y., Lu, J., et al. (2013). Induced pluripotent stem cell-derived neural cells survive and mature in the nonhuman primate brain. *Cell Rep.* 3, 646–650. doi: 10.1016/j.celrep.2013.02.016

Engelender, S., and Isacson, O. (2017). The threshold theory for Parkinson's disease. *Trends Neurosci.* 40, 4–14. doi: 10.1016/j.tins.2016.10.008

Eriksson, T., Magnusson, T., Carlsson, A., Linde, A., and Granerus, A. K. (1984). "On-off" phenomenon in Parkinson's disease: correlation to the concentration of dopa in plasma. *J Neural Transm* 59, 229–240. doi: 10.1007/bf01250010

Fahn, S. (2003). Description of Parkinson's disease as a clinical syndrome. *Ann. N. Y. Acad. Sci.* 991, 1–14. doi: 10.1111/j.1749-6632.2003.tb07458.x

Fields, J. A., and Troster, A. I. (2000). Cognitive outcomes after deep brain stimulation for Parkinson's disease: a review of initial studies and recommendations for future research. *Brain Cogn.* 42, 268–293. doi: 10.1006/brcg.1999.1104

Fink, J. S., Schumacher, J. M., Ellias, S. L., Palmer, E. P., Saint-Hilaire, M., Shannon, K., et al. (2000). Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. *Cell Transpl.* 9, 273–278. doi: 10.1177/096368970000900212

Freed, C. R., Breeze, R. E., Rosenberg, N. L., Schneck, S. A., Kriek, E., Qi, J. X., et al. (1992). Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. *N. Engl. J. Med.* 327, 1549–1555. doi: 10.1056/NEJM199211263272202

Freeman, T. B., Cicchetti, F., Hauser, R. A., Deacon, T. W., Li, X. J., Hersch, S. M., et al. (2000). Transplanted fetal striatum in Huntington's disease: phenotypic development and lack of pathology. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13877–13882. doi: 10.1073/pnas.97.25.13877

Goldman, S. A., Nedergaard, M., and Windrem, M. S. (2012). Glial progenitor cell-based treatment and modeling of neurological disease. *Science* 338, 491–495. doi: 10.1126/science.1218071

Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B., Heuer, A., Bramoulle, Y., et al. (2014). Human ESC-derived dopamine neurons show similar preclinical efficacy and potency to fetal neurons when grafted in a rat model of Parkinson's disease. *Cell Stem Cell* 15, 653–665. doi: 10.1016/j.stem.2014.09.017

Hagell, P., Crabb, L., Pogarell, O., Schrag, A., Widner, H., Brooks, D. J., et al. (2000). Health-related quality of life following bilateral intrastriatal transplantation in Parkinson's disease. *Mov. Disord.* 15, 224–229. doi: 10.1002/1531-8257(200003)15:2<224::aid-mds1004>3.0.co;2-w

Hallett, P. J., Cooper, O., Sadi, D., Robertson, H., Mendez, I., and Isacson, O. (2014). Long-term health of dopaminergic neuron transplants in Parkinson's disease patients. *Cell Rep.* 7, 1755–1761. doi: 10.1016/j.celrep.2014.05.027

Hallett, P. J., Deleidi, M., Astradsson, A., Smith, G. A., Cooper, O., Osborn, T. M., et al. (2015). Successful function of autologous iPSC-derived dopamine neurons following transplantation in a non-human primate model of Parkinson's disease. *Cell Stem Cell* 16, 269–274. doi: 10.1016/j.stem.2015.01.018

Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., et al. (2010). Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15921–15926. doi: 10.1073/pnas.1010209107

Hedlund, E., Pruszak, J., Lardaro, T., Ludwig, W., Vinuela, A., Kim, K. S., et al. (2008). Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease. *Stem Cells* 26, 1526–1536. doi: 10.1634/stemcells.2007-0996

Herrington, T. M., Cheng, J. J., and Eskandar, E. N. (2016). Mechanisms of deep brain stimulation. *J. Neurophysiol.* 115, 19–38. doi: 10.1152/jn.00281.2015

Isacson, O., Bjorklund, L. M., and Schumacher, J. M. (2003). Toward full restoration of synaptic and terminal function of the dopaminergic system in Parkinson's disease by stem cells. *Ann. Neurol.* 53(Suppl. 3), S135–S146; discussion S146–138. doi: 10.1002/ana.10482

Isacson, O., and Kordower, J. H. (2008). Future of cell and gene therapies for Parkinson's disease. *Ann. Neurol.* 64(Suppl. 2), S122–S138. doi: 10.1002/ana.21473

Kee, N., Volakakis, N., Kirkeby, A., Dahl, L., Storvall, H., Nolbrant, S., et al. (2017). Single-cell analysis reveals a close relationship between differentiating dopamine and subthalamic nucleus neuronal lineages. *Cell Stem Cell* 20, 29–40. doi: 10.1016/j.stem.2016.10.003

Kefalopoulou, Z., Politis, M., Piccini, P., Mencacci, N., Bhatia, K., Jahanshahi, M., et al. (2014). Long-term clinical outcome of fetal cell transplantation for parkinson disease: two case reports. *JAMA Neurol.* 71, 83–87. doi: 10.1001/jamaneurol.2013.4749

Khakh, B. S., and Deneen, B. (2019). The emerging nature of astrocyte diversity. *Annu. Rev. Neurosci.* 42, 187–207. doi: 10.1146/annurev-neuro-070918-050443

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., et al. (2017). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature* 548, 592–596. doi: 10.1038/nature23664

Kirkeby, A., Nolbrant, S., Tiklova, K., Heuer, A., Kee, N., Cardoso, T., et al. (2017). Predictive markers guide differentiation to improve graft outcome in clinical translation of hESC-based therapy for Parkinson's disease. *Cell Stem Cell* 20, 135–148. doi: 10.1016/j.stem.2016.09.004

Kordower, J., Freeman, T., Chen, E., Mufson, E., Sanberg, P., Hauser, R., et al. (1998). Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Mov. Dis.* 13, 383–393. doi: 10.1002/mds.870130303

Kordower, J., Styren, S., Clarke, M., DeKosky, S., Olanow, C., and Freeman, T. (1997). Fetal grafting for Parkinson's disease: expression of immune markers in two patients with functional fetal nigral implants. *Cell Transpl.* 6, 213–219. doi: 10.1016/s0963-6897(97)00019-5

Kordower, J. H., Chu, Y., Hauser, R. A., Freeman, T. B., and Olanow, C. W. (2008). Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med.* 14, 504–506. doi: 10.1038/nm1747

Kriks, S., Shim, J. W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480, 547–551. doi: 10.1038/nature10648

Kurowska, Z., Englund, E., Widner, H., Lindvall, O., Li, J. Y., and Brundin, P. (2011). Signs of degeneration in 12-22-year old grafts of mesencephalic dopamine neurons in patients with Parkinson's disease. *J. Parkinsons Dis.* 1, 83–92. doi: 10.3233/jpd-2011-11004

Lanza, R., Russell, D. W., and Nagy, A. (2019). Engineering universal cells that evade immune detection. *Nat. Rev. Immunol.* 19, 723–733. doi: 10.1038/s41577-019-0200-1

Li, J. Y., Englund, E., Holton, J. L., Soulet, D., Hagell, P., Lees, A. J., et al. (2008). Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat. Med.* 14, 501–503. doi: 10.1038/nm1746

Li, W., Englund, E., Widner, H., Mattsson, B., van Westen, D., Latt, J., et al. (2016). Extensive graft-derived dopaminergic innervation is maintained 24 years after transplantation in the degenerating parkinsonian brain. *Proc. Natl. Acad. Sci. U.S.A.* 113, 6544–6549. doi: 10.1073/pnas.1605245113

Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., et al. (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247, 574–577. doi: 10.1126/science.2105529

Lindvall, O., Rehncrona, S., Gustavii, B., Brundin, P., Astedt, B., Widner, H., et al. (1988). Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. *Lancet* 2, 1483–1484. doi: 10.1016/s0140-6736(88)90950-6

Majhail, N. S., Mau, L. W., Denzen, E. M., and Arneson, T. J. (2013). Costs of autologous and allogeneic hematopoietic cell transplantation in the United States: a study using a large national private claims database. *Bone Marrow Transpl.* 48, 294–300. doi: 10.1038/bmt.2012.133

Mandel, R. J., Burger, C., and Snyder, R. O. (2008). Viral vectors for in vivo gene transfer in Parkinson's disease: properties and clinical grade production. *Exp. Neurol.* 209, 58–71. doi: 10.1016/j.expneurol.2007.08.008

Mandel, R. J., Rendahl, K. G., Snyder, R. O., and Leff, S. E. (1999). Progress in direct striatal delivery of L-dopa via gene therapy for treatment of Parkinson's disease using recombinant adeno-associated viral vectors. *Exp. Neurol.* 159, 47–64. doi: 10.1006/exnr.1999.7159

Mendez, I., Sanchez-Pernaute, R., Cooper, O., Vinuela, A., Ferrari, D., Bjorklund, L., et al. (2005). Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease. *Brain* 128(Pt 7), 1498–1510. doi: 10.1093/brain/awh510

Mendez, I., Vinuela, A., Astradsson, A., Mukhida, K., Hallett, P., Robertson, H., et al. (2008). Dopamine neurons implanted into people with Parkinson's disease survive without pathology for 14 years. *Nat. Med.* 14, 507–509. doi: 10.1038/nm1752

Montuno, M. A., Kohner, A. B., Foote, K. D., and Okun, M. S. (2013). An algorithm for management of deep brain stimulation battery replacements: devising a web-based battery estimator and clinical symptom approach. *Neuromodulation* 16, 147–153. doi: 10.1111/j.1525-1403.2012.00457.x

Morizane, A., Doi, D., Kikuchi, T., Okita, K., Hotta, A., Kawasaki, T., et al. (2013). Direct comparison of autologous and allogeneic transplantation of iPSC-derived neural cells in the brain of a non-human primate. *Stem Cell Rep.* 1, 283–292. doi: 10.1016/j.stemcr.2013.08.007

Morizane, A., Kikuchi, T., Hayashi, T., Mizuma, H., Takara, S., Doi, H., et al. (2017). MHC matching improves engraftment of iPSC-derived neurons in non-human primates. *Nat. Commun.* 8:385.

Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. *Neuron* 23, 689–702. doi: 10.1016/s0896-6273(01)80028-3

Nolbrant, S., Heuer, A., Parmar, M., and Kirkeby, A. (2017). Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation. *Nat. Protoc.* 12, 1962–1979. doi: 10.1038/nprot.2017.078

Nordstrom, U., Jessell, T. M., and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat. Neurosci.* 5, 525–532. doi: 10.1038/nn0602-854

Nordstrom, U., Maier, E., Jessell, T. M., and Edlund, T. (2006). An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. *PLoS Biol.* 4:e252. doi: 10.1371/journal.pbio.0040252

Olanow, C. W., Goetz, C. G., Kordower, J. H., Stoessl, A. J., Sossi, V., Brin, M. F., et al. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann. Neurol.* 54, 403–414. doi: 10.1002/ana.10720

Osborn, T., Dinesh, D., Moskites, A., MacBain, Z., Moore, M., Brekk, O., et al. (2020). Pre-clinical studies toward autologous midbrain dopamine cell therapy for Parkinson's disease. *StemJournal* (in press).

Osborn, T., and Hallett, P. J. (2017). Seq-ing markers of midbrain dopamine neurons. *Cell Stem Cell* 20, 11–12. doi: 10.1016/j.stem.2016.12.014

Osborn, T. M., Badi, M., Dinesh, D., Thomas, R., Pruszak, J., Astradsson, A., et al. (2017). *Preclinical Characterization of Frozen-Thawed Dopaminergic Cell Preparations for Parkinson's Disease Autologous Cell Replacement Therapy.* Skokie, IL: International Society for Stem Cell Research, F-1125.

Osborn, T. M., Hallett, P. J., Moore, M., Casler, A., Dinesh, D., Skorik, C., et al. (2015). Pre-clinical in vitro and in vivo characterization of xeno-free and foot-print-free iPSC-derived cell preparations for Parkinson's disease cell therapy. *Soc. Neurosci.*

Parmar, M., Grealish, S., and Henchcliffe, C. (2020). The future of stem cell therapies for Parkinson disease. *Nat. Rev. Neurosci.* 21, 103–115. doi: 10.1038/s41583-019-0257-7

Piccini, P., Brooks, D. J., Bjorklund, A., Gunn, R. N., Grasby, P. M., Rimoldi, O., et al. (1999). Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nat. Neurosci.* 2, 1137–1140. doi: 10.1038/16060

Politis, M., Wu, K., Loane, C., Quinn, N. P., Brooks, D. J., Rehncrona, S., et al. (2010). Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. *Sci. Transl. Med.* 2:38ra46. doi: 10.1126/scitranslmed.3000976

Radad, K., Gille, G., and Rausch, W. D. (2005). Short review on dopamine agonists: insight into clinical and research studies relevant to Parkinson's disease. *Pharmacol. Rep.* 57, 701–712.

Redmond, D. E. Jr., Vinuela, A., Kordower, J. H., and Isacson, O. (2008). Influence of cell preparation and target location on the behavioral recovery after striatal transplantation of fetal dopaminergic neurons in a primate model of Parkinson's disease. *Neurobiol. Dis.* 29, 103–116. doi: 10.1016/j.nbd.2007.08.008

Rivetti di Val Cervo, P., Romanov, R. A., Spigolon, G., Masini, D., et al. (2017). Induction of functional dopamine neurons from human astrocytes in vitro and mouse astrocytes in a Parkinson's disease model. *Nat. Biotechnol.* 35, 444–452. doi: 10.1038/nbt.3835

Rowe, J. M., Ciobanu, N., Ascensao, J., Stadtmauer, E. A., Weiner, R. S., Schenkein, D. P., et al. (1994). Recommended guidelines for the management of autologous and allogeneic bone marrow transplantation. A report from the Eastern Cooperative Oncology Group (ECOG). *Ann. Int. Med.* 120, 143–158.

Roy, N. S., Cleren, C., Singh, S. K., Yang, L., Beal, M. F., and Goldman, S. A. (2006). Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat. Med.* 12, 1259–1268. doi: 10.1038/nm1495

Schmidt, R. H., Bjorklund, A., and Stenevi, U. (1981). Intracerebral grafting of dissociated CNS tissue suspensions: a new approach for neuronal transplantation to deep brain sites. *Brain Res.* 218, 347–356. doi: 10.1016/0006-8993(81)91313-5

Schumacher, J., Ellias, S., Palmer, E., Kott, H., Dinsmore, J., Dempsey, P., et al. (2000). Transplantation of embryonic porcine mesencephalic tissue in patients with Parkinson's disease. *Neurology* 54, 1042–1050. doi: 10.1212/wnl.54.5.1042

Soderstrom, K. E., Meredith, G., Freeman, T. B., McGuire, S. O., Collier, T. J., Sortwell, C. E., et al. (2008). The synaptic impact of the host immune response in a parkinsonian allograft rat model: Influence on graft-derived aberrant behaviors. *Neurobiol. Dis.* 32, 229–242. doi: 10.1016/j.nbd.2008.06.018

Sonntag, K. C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R., and Isacson, O. (2007). Enhanced yield of neuroepithelial precursors and midbrain-like dopaminergic neurons from human embryonic stem cells using the bone morphogenic protein antagonist noggin. *Stem Cells* 25, 411–418. doi: 10.1634/stemcells.2006-0380

Strecker, R. E., Sharp, T., Brundin, P., Zetterstrom, T., Ungerstedt, U., and Bjorklund, A. (1987). Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. *Neuroscience* 22, 169–178. doi: 10.1016/0306-4522(87)90207-7

Studer, L. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic-The NYSTEM trial. *Prog. Brain Res.* 230, 191–212. doi: 10.1016/bs.pbr.2017.02.008

Sundberg, M., Bogetofte, H., Lawson, T., Jansson, J., Smith, G., Astradsson, A., et al. (2013). Improved cell therapy protocols for Parkinson's disease based on differentiation efficiency and safety of hESC-, hiPSC-, and non-human primate iPSC-derived dopaminergic neurons. *Stem Cells* 31, 1548–1562. doi: 10.1002/stem.1415

Takahashi, J. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic: the Kyoto trial. *Prog. Brain Res.* 230, 213–226. doi: 10.1016/bs.pbr.2016.11.004

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 10.1016/j.cell.2007.11.019

Tsui, A., and Isacson, O. (2011). Functions of the nigrostriatal dopaminergic synapse and the use of neurotransplantation in Parkinson's disease. *J. Neurol.* 258, 1393–1405. doi: 10.1007/s00415-011-6061-6

Vinuela, A., Hallett, P. J., Reske-Nielsen, C., Patterson, M., Sotnikova, T. D., Caron, M. G., et al. (2008). Implanted reuptake-deficient or wild-type dopaminergic neurons improve ON L-dopa dyskinesias without OFF-dyskinesias in a rat model of Parkinson's disease. *Brain* 131(Pt 12), 3361–3379. doi: 10.1093/brain/awn192

Wakeman, D. R., Hiller, B. M., Marmion, D. J., McMahon, C. W., Corbett, G. T., Mangan, K. P., et al. (2017). Cryopreservation maintains functionality of human iPSC dopamine neurons and rescues parkinsonian phenotypes in vivo. *Stem Cell Rep.* 9, 149–161. doi: 10.1016/j.stemcr.2017.04.033

Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., et al. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5856–5861. doi: 10.1073/pnas.0801677105

Widner, H., Tetrud, J., Rehncrona, S., Snow, B., Brundin, P., Gustavii, B., et al. (1992). Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *N. Engl. J. Med.* 327, 1556–1563. doi: 10.1056/NEJM199211263272203

Yang, D., Zhang, Z. J., Oldenburg, M., Ayala, M., and Zhang, S. C. (2008). Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats. *Stem Cells* 26, 55–63. doi: 10.1634/stemcells.2007-0494

Zetterstrom, T., Brundin, P., Gage, F. H., Sharp, T., Isacson, O., Dunnett, S. B., et al. (1986). In vivo measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. *Brain Res.* 362, 344–349. doi: 10.1016/0006-8993(86)90460-9

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# Transcription Factor-Based Fate Specification and Forward Programming for Neural Regeneration

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Traditionally, *in vitro* generation of donor cells for brain repair has been dominated by the application of extrinsic growth factors and morphogens. Recent advances in cell engineering strategies such as reprogramming of somatic cells into induced pluripotent stem cells and direct cell fate conversion have impressively demonstrated the feasibility to manipulate cell identities by the overexpression of cell fate-determining transcription factors. These strategies are now increasingly implemented for transcription factor-guided differentiation of neural precursors and forward programming of pluripotent stem cells toward specific neural subtypes. This review covers major achievements, pros and cons, as well as future prospects of transcription factor-based cell fate specification and the applicability of these approaches for the generation of donor cells for brain repair.

Keywords: forward programming, transcription factor-driven differentiation, direct cell fate conversion, biomedical application, translation, transplantation, brain repair

## INTRODUCTION

Identifying treatment options for neurological and especially neurodegenerative diseases is one of the most pressing tasks of modern biomedicine. In this context, neural cell replacement has emerged as a particularly promising strategy, which has gained further impetus with the availability of massively scalable human PSCs. A key prerequisite for the use of human PSCs in neural repair is the efficient derivation of disease-specific cell populations. Over the last 20 years, numerous *in vitro* differentiation protocols were established. Classically, they involve extrinsic factors such as morphogens to guide the differentiation process toward a specific cell fate, thereby mimicking regionalization processes during nervous system development. This approach has led to significant advances, for instance, for the generation of midbrain dopamine neurons for the treatment of PD (Kriks et al., 2011; Kirkeby et al., 2012). However, the generation of many neural subtypes is frequently complicated by long differentiation times and complex multi-step growth factor-regimens, which often yield cultures exhibiting a high degree of heterogeneity (see also review by Tao and Zhang, 2016). Thus, many growth factor-based protocols have to be regarded as insufficiently precise when it comes to fine-tuning the specification of distinct neural subtypes, especially considering future biomedical applications.

Since morphogen-based cell specification finally converges on the activation of specific transcriptional programs, TF overexpression by itself represents an alternative method to guide cell

fate acquisition. This idea was further fueled by the ground-breaking discovery by Takahashi and Yamanaka that an ESC-like pluripotent fate can be induced in mouse (Takahashi and Yamanaka, 2006) and human (Takahashi et al., 2007) somatic cells by overexpressing a combination of four different TFs, namely Oct3/4, Sox2, Klf4 and c-Myc. The introduction of the iPSC reprogramming technology had two major implications for the scientific field: First, the feasibility to reprogram terminally differentiated somatic cells into iPSCs hinted at the potential power of exploiting TF overexpression as a tool to manipulate cell fates more globally. Second, it created the general

**Abbreviations:** 6-OHDA, 6-hydroxydopamine; AAVS1, adeno-associated virus integration site 1; Aldh1a1, aldehyde dehydrogenase 1 family member a1; ALDH1L1, aldehyde dehydrogenase 1 family member L1; Aldh2, aldehyde dehydrogenase 2; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ascl1, achaete-scute homolog 1; Atoh1, atonal bHLH TF 1; ATP, adenosine triphosphate; Barhl1, BarH-like homeobox 1; Bcl-XL, B-cell lymphoma-extra large; bHLH, basic helix-loop-helix; Bmp, bone morphogenic protein; Brn2 aka POU3F2, POU domain class 3 transcription factor 2; Brn3a aka POU4F1, POU domain class 4 transcription factor 1; Brn4 aka POU3F4, POU domain class 3 transcription factor 4; ChAT, choline acetyltransferase; c-Myc, avian myelocytomatosis viral oncogene cellular homolog; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CLYBL, citrate lyase beta like; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/Cas9; Ctip2 aka Bcl11b, B-cell lymphoma/leukemia 11b; Cux1, cut like homeobox 1; Dat, dopamine transporter; Darpp32 aka Ppr1b, protein phosphatase 1 regulatory subunit 1b; Dbh, dopamine beta-hydroxylase; Dkk1, Dickkopf-related protein 1; Dlk1, delta like non-canonical Notch ligand 1; Dll, distal-less; Dlx, distal-less homeobox; Dmrt5, doublesex and mab-3-related TF 5; Ebf, early B-cell factor; Egf, epidermal growth factor; Emx2, empty spiracles-homeobox 2; En, engrailed homeobox; ESC, embryonic stem cell; Ezh2, enhancer of zeste homolog 2; Fabp7, fatty acid binding protein 7; Fgf, fibroblast growth factor; Foxa2, forkhead-box-protein 2; Foxg1, forkhead box g1; Foxo1, forkhead box o1; GABA, gamma aminobutyric acid; Gal, galanin; GALC, galactosylceramidase; GDNF, glial cell-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; Girk2, G-protein-related inward-rectifier potassium channel 2; Glast, glutamate aspartate transporter; Gpx1, glutathione peroxidase 1; Gsx2, genetic-screened homeobox 2; HB9, homeobox HB9; HD, Huntington's disease; Hes, hairy and enhancer of split; Il, interleukin; iN, induced neuron; iNPC, induced NPC; iPSC, induced pluripotent stem cell; Isl1, islet-1; Klf4, Krueppel-like factor 4; Krox20 aka Egr2, early growth response protein 2; Lhx, LIM homeobox; Lif, leukemia inhibitory factor; Lmx1, LIM homeobox TF 1; Map2, microtubule-associated protein 2; Mbp, myelin basic protein; MN, motor neuron; MSN, medium spiny neuron; Myod3, myogenic differentiation 3; Myt1l, myelin TF 1-like; Net, norepinephrine transporter; Neun, neuronal nuclei; Neurod, neurogenic differentiation; NfI, nuclear factor I; NG2, neuron-glial antigen 2; Ngn, neurogenin; Nkx2.1, homeobox protein NK-2 homolog A; Nkx2.2, homeobox protein NK-2 homolog B; Nkx6.2, homeobox protein NK-6 homolog B; NPC, neural precursor cell; Nr2f1, nuclear receptor subfamily 2 group f member 1; Ntn1, netrin 1; Nurr1, nuclear receptor related 1; O4 aka CLDN11, claudin 11; Oct3/4, octamer-binding TF 3/4; Olig, oligodendrocyte TF; OPC, oligodendrocyte progenitor cell; Otx, orthodenticle homeobox; Pax, paired box protein; PBX1, pre-B-cell leukemia TF 1; PD, Parkinson's disease; Phox2, paired like homeobox 2; PIEZO2, Piezo-type mechanosensitive ion channel component 2; Pitx3, pituitary homeobox 3; PLP, proteolipid protein; PRC2, polycomb repressive complex 2; Prrx1, paired related homeobox 1; PSC, pluripotent stem cell; RA, retinoic acid; REST, RE1 silencing TF; Rg4 aka UNC119, retinal protein 4; Rnf20, ring finger protein 20; ROSA, reverse oriented splice acceptor; S100β, S100 calcium-binding protein β; Satb2, special AT-rich sequence-binding protein 2; Shh, sonic hedgehog; Smad, mothers against decapentaplegic; Sox, sex determining region Y-box; Stat3, signal transducer and activator of transcription 3; TALENs, transcription activator-like effector nucleases; Tbx, T-box TF; Tcf15, TF 15; TF, transcription factor; Tfap2a, TF AP-2 alpha; Th, tyrosine hydroxylase; Tlx3, T-cell leukemia homeobox 3; TRPM8, transient receptor potential cation channel subfamily melastatin member 8; Tubb3, class III β-tubulin; Tyrp1, tyrosinase-related protein 1; VAChT, vesicular acetylcholine transporter; vGLUT1, vesicular glutamate transporter 1; VIM, vimentin; Vmat, vesicular monoamine transporter; WNT, wingless-int; Zeb, zinc finger E-box-binding homeobox; Zfp, zinc finger protein.

opportunity to derive neural cells from basically any adult human and thus revealed new avenues for disease modeling and personalized biomedicine.
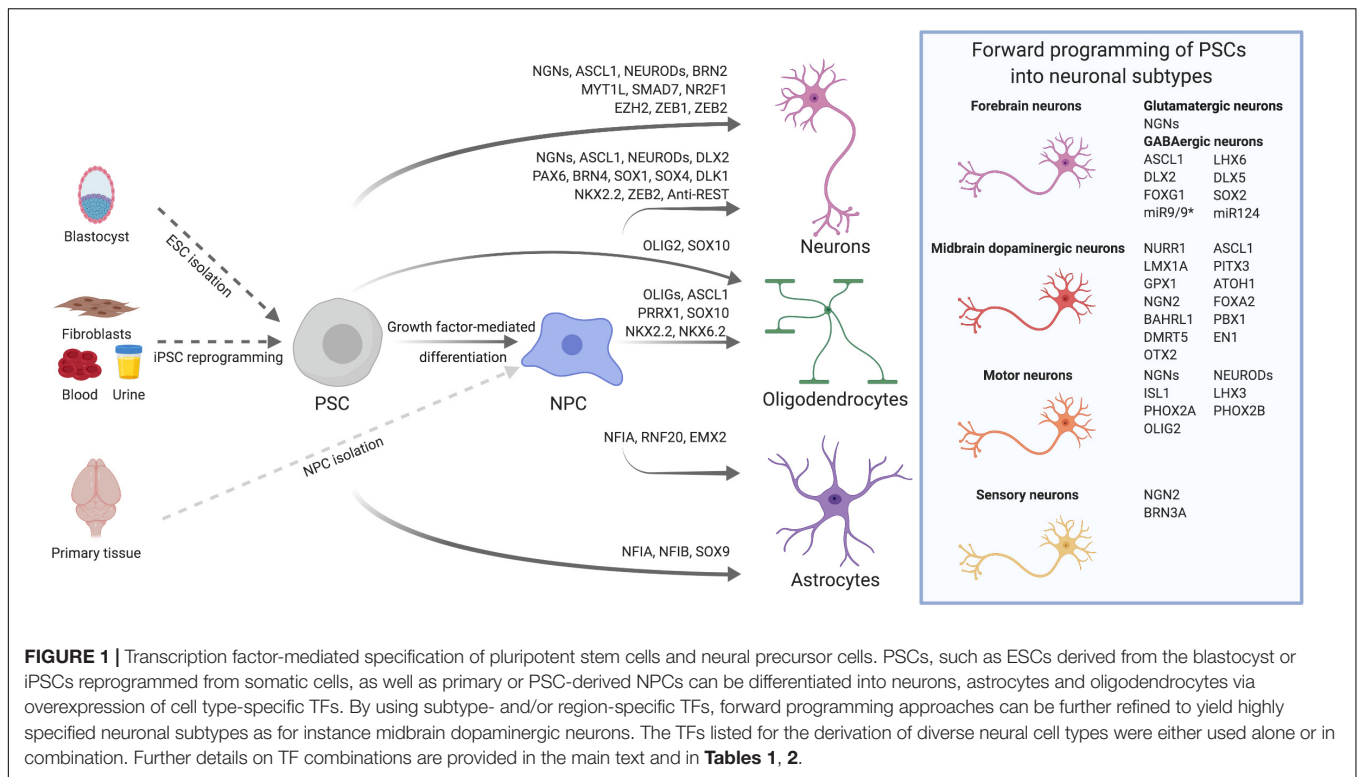
In line with the first idea is the concept of direct cell fate conversion, i.e., the use of TFs to directly convert one somatic cell type into another without transiting a stable, pluripotent state. In fact, direct cell fate conversion has been achieved far before the iPSC technique was even introduced: Davis et al. (1987) successfully converted mouse fibroblasts into myoblasts by overexpressing the TF Myod3. As for neurons, it had already been shown by Magdalena Götz and colleagues in the early 2000s that mouse astrocytes can be directly converted into neurons by overexpressing single neural TFs such as Pax6 (Heins et al., 2002), Olig2 (Buffo et al., 2005), Ngn2 and Ascl1 (Berninger et al., 2007). In 2010, the Wernig lab achieved to derive iNs from mouse fibroblasts via transdifferentiation across germ layers (Vierbuchen et al., 2010). Although in this case Ascl1 overexpression seemed sufficient to drive neuronal conversion, too, the derivation of mature iNs was most efficient when multiple TFs were used simultaneously, such as the combined expression of Ascl1, Brn2 and Myt1l (Vierbuchen et al., 2010). This TF cocktail alone (Pfisterer et al., 2011a,b) or in combination with the bHLH TF NEUROD1 (Pang et al., 2011) was shown to suffice for inducing iNs from human fibroblasts. In combination with SOX2, ASCL1 can also convert human non-neural, brain-resident pericytes into functional iNs (Karow et al., 2012, 2018). How broadly TF overexpression can impact the differentiation of PSCs is illustrated by studies of Minoru Ko and colleagues, who established more than 180 mouse ESC lines, each expressing a distinct TF from the *ROSA* locus after doxycycline induction, which resulted in the specification of a large variety of different somatic cell lineages (in the following also referred to as 'forward programming'; Nishiyama et al., 2009; Correa-Cerro et al., 2011; Yamamizu et al., 2016).

The aim of this review is to give a comprehensive overview on TF-based approaches for the generation of neural cells (**Figure 1**). We will speculate on general mechanisms underlying TF-mediated neuronal differentiation and forward programming, specifically comment on current efforts to derive clinically relevant neuronal subtypes and glial cells, and summarize recent endeavors to apply these cells *in vivo* for brain repair. Finally, we will discuss forward programming as an alternative to direct cell fate conversion, and comment on the achievements as well as remaining hurdles for biomedical translation.

# DERIVATION OF NEURAL CELL TYPES VIA FORWARD PROGRAMMING

Specifying cell fates by TF overexpression is comparably easy to accomplish within one lineage, especially when starting from cell types which are direct progenitors of the target cell type. Almost 20 years ago, Sun et al. (2001) reported about the successful derivation of neurons by retrovirally overexpressing the pro-neural bHLH TF Ngn1 in primary rat cortical NPCs (**Table 1**). Since then, other TFs belonging to the bHLH family have been shown to be capable of forcing neuronal differentiation from

**FIGURE 1 |** Transcription factor-mediated specification of pluripotent stem cells and neural precursor cells. PSCs, such as ESCs derived from the blastocyst or iPSCs reprogrammed from somatic cells, as well as primary or PSC-derived NPCs can be differentiated into neurons, astrocytes and oligodendrocytes via overexpression of cell type-specific TFs. By using subtype- and/or region-specific TFs, forward programming approaches can be further refined to yield highly specified neuronal subtypes as for instance midbrain dopaminergic neurons. The TFs listed for the derivation of diverse neural cell types were either used alone or in combination. Further details on TF combinations are provided in the main text and in **Tables 1**, **2**.

different NPC populations. These TFs include various Ngns such as Ngn1 (Serre et al., 2012; Song et al., 2017), Ngn2 (Geoffroy et al., 2009; Serre et al., 2012; Bolós et al., 2014; Ho et al., 2016; Li X. et al., 2016) and Ngn3 (Serre et al., 2012), Ascl1 (Geoffroy et al., 2009; Kim et al., 2009; Serre et al., 2012; Li X. et al., 2016; Barretto et al., 2020) as well as Neurod TFs (Hsieh et al., 2004).

## bHLH Transcription Factors as Key Instructors of Neuronal Differentiation

Interestingly, also rapid neuronal differentiation of PSCs (this term will be used to describe ESCs and iPSCs together in the following), which are not yet committed to the neural lineage, was shown to be feasible with bHLH TFs. A milestone in the field of neuronal forward programming was reached in 2011, when the groups of Marius Wernig and Thomas Südhof reported that combined overexpression of the TFs Ascl1, Brn2, and Myt1l not only suffices to transdifferentiate mouse fibroblasts into neurons (Vierbuchen et al., 2010) but also efficiently drives neuronal specification from human PSCs (Pang et al., 2011). The authors revealed that ASCL1 is most crucial for neural fate acquisition, whereas the TFs BRN2 and MYT1L rather promote down-stream neuronal maturation. Using the full TF cocktail, electrophysiologically active neurons can be derived from human PSCs after only 6 days of *in vitro* differentiation (Pang et al., 2011). Several other labs subsequently demonstrated that Ascl1 alone can efficiently forward program mouse ESCs (Yamamizu et al., 2013; Teratani-Ota et al., 2016) and human PSCs (Chanda et al., 2014; Robinson et al., 2016) toward a neuronal fate, albeit with

slower differentiation dynamics than the full ASCL1, BRN2, and MYT1L TF combination: In mouse ESCs, overexpression of Ascl1 leads to a sharp increase of neural markers within the first 7 days of induction (Yamamizu et al., 2013; Teratani-Ota et al., 2016), and 11 days after overexpressing Ascl1, about half of the neurons were shown to generate action potentials upon current injection (Yamamizu et al., 2013). In human PSCs, ASCL1-overexpressing cells do not start to express neuronal markers such as TUBB3 and MAP2 before day 9 of differentiation. Still, ASCL1 overexpression alone is sufficient to generate morphologically and functionally mature neurons when ESC-derived immature neuronal cells are cultured in advanced neuronal differentiation medium and are grown on primary glial cells. These neurons not only exhibit mature electrophysiological properties such as spontaneous action potential firing after 4 weeks of differentiation but also respond to exogenous AMPA and GABA application and demonstrate signs of short-term synaptic plasticity, indicating the formation of functional synapses (Chanda et al., 2014).

Overexpression of other bHLH TFs, too, induces rapid neuronal differentiation of PSCs: In one of the first *in vitro* studies employing overexpression of Ngn1 in mouse ESCs, transduced cells underwent morphological rearrangements forming neurite-like structures already within the first 72 h and became electrophysiologically excitable as early as 4 days after transgene induction (Tong et al., 2010). After 5 days of Ngn2 overexpression, mouse ESC-derived cells express the mature neuronal marker Map2, display neuronal electrophysiological properties at day 10, and form synapses in co-culture with primary mouse hippocampal neurons 20 days post induction

| Desired cell type | Starting cell type | Species | Transcription factor used for forward programming | References |
|---|---|---|---|---|
| Neurons (generic) | NPCs | Mouse | Pax6 | Hack et al. (2004) |
| | NPCs | Mouse | Sox1 | Kan et al. (2004) |
| | NPCs | Mouse | Dominant-negative form of REST | Greenway et al. (2007) |
| | NPCs | Mouse | Ascl1 or Ngn2 | Geoffroy et al. (2009) |
| | NPCs | Mouse | Ngn2 | Bolós et al. (2014) |
| | NPCs | Mouse | Sox4 | Braccioli et al. (2018) |
| | NPCs | Mouse | Zeb2 | Yang et al. (2018) |
| | NPCs | Rat | Ngn1 | Sun et al. (2001) |
| | NPCs | Rat | Neurod | Hsieh et al. (2004) |
| | NPCs | Rat | Brn4 | Shi et al. (2010) |
| | NPCs | Rat | Brn4 | Tan et al. (2010) |
| | NPCs (under glia-promoting conditions) | Rat | Dominant-negative form of REST | DeWald et al. (2011) |
| | ESC-derived NPCs | Mouse and human | Dlk1 | Surmacz et al. (2012) |
| | NPCs | Human | ASCL1 | Kim et al. (2009) |
| | NPCs | Human | ASCL1 or NGN1 or NGN2 or NGN3 | Serre et al. (2012) |
| | NPCs | Human | ASCL1 or NGN2 or ASCL1+NGN2 | [1] Li X. et al. (2016) |
| | NPCs (primary and ESC-derived) | Human | shREST or shHAUSP | Huang et al. (2011) |
| | iPSC-derived NPCs | Human | NGN2 | Ho et al. (2016) |
| | iPSC-derived NPCs | Human | ASCL1+DLX2 | Barretto et al. (2020) |
| | ESCs | Mouse | Neurod1 or Neurod2 or Neurod3 | O'Shea (2001) |
| | ESCs | Mouse | Ngn1 | Tong et al. (2010) |
| | ESCs | Mouse | Ngn2 | Thoma et al. (2012) |
| | ESCs | Mouse | Ascl1 or Smad7 or Nr2f1 | Yamamizu et al. (2013) |
| | ESCs | Mouse | Multiple (TF screen) | Teratani-Ota et al. (2016) |
| | ESCs | Mouse | Neurod1 | Pataskar et al. (2016) |
| | ESCs | Mouse | Multiple (TF screen) | Liu et al. (2018) |
| | ESCs | Mouse | sh-lnc-RNA-1604+Zeb1 and/or Zeb2 | Weng et al. (2018) |
| | ESCs | Mouse | Ngn2 or Ascl1 | Aydin et al. (2019) |
| | PSCs | Human | ASCL1+ BRN2+MYT1L | Pang et al. (2011) |
| | PSCs | Human | NGN2 or NEUROD1 | [3] Zhang et al. (2013) |
| | ESCs | Human | ASCL1 | Chanda et al. (2014) |
| | iPSCs | Human | FOXG1+SOX2+ASCL1+DLX5+LHX6 | Colasante et al. (2015) |
| | PSCs | Human | ASCL1+DLX2+LHX6+miR9/9*-124 | [6] Sun et al. (2016) |
| | iPSCs | Human | ASCL1 | Robinson et al. (2016) |
| | iPSCs | Human | NGN2 | Rubio et al. (2016) |
| | PSCs | Human | ASCL1+DLX2 | [4] Yang et al. (2017) |
| | iPSCs | Human | NGN2 | Frega et al. (2017) |
| | PSCs | Human | NGN1 or NGN2 or NGN3 or NEUROD1 or NEUROD2 | Goparaju et al. (2017) |
| | ESCs | Human | NGN1 or NGN2 or NGN3 or NEUROD1 or NEUROD2 | Matsushita et al. (2017) |
| | PSCs | Human | NGN2 | Pawlowski et al. (2017) |
| | iPSCs | Human | NGN2 | Wang et al. (2017) |
| | PSCs | Human | NGN2 | Nehme et al. (2018) |
| | PSCs | Human | LHX6 | [5] Yuan et al. (2018) |
| | ESCs | Human | ZEB1 | [2] Jiang et al. (2018) |
| | iPSCs | Human | NGN2 | Meijer et al. (2019) |
| | iPSCs | Human | NGN2 or ASCL1+DLX2 | Rhee et al. (2019) |
| | iPSCs | Human | NGN2 | Nickolls et al. (2020) |
| Dopaminergic neurons | NPCs | Mouse | Nurr1 | Soldati et al. (2012) |
| | NPCs | Mouse | Foxa2 | Kittappa et al. (2007) |
| | NPCs | Rat | Nurr1 | Sakurada et al. (1999) |

*(Continued)*

**TABLE 1 |** Continued

| Desired cell type | Starting cell type | Species | Transcription factor used for forward programming | References |
|---|---|---|---|---|
| | NPCs | Rat | Nurr1 | [10] Kim et al. (2003) |
| | NPCs | Rat | Nurr1 or Ascl1+Nurr1 or Nurr1+Ngn1 or Nurr1+Ngn2 or Nurr1+Shh or Nurr1+Bcl-XL or Nurr1+Bcl-XL+Shh | [11] Park et al. (2006) |
| | NPCs | Rat | Nurr1 | Bae et al. (2009) |
| | ESC-derived NPCs | Mouse | Dmrt5 | Gennet et al. (2011) |
| | ESC-derived NPCs | Mouse | Lmx1a | Andersson et al. (2006) |
| | ESC-derived NPCs | Mouse | Lmx1a or En1 or Otx2 or Lmx1a+En1 or Lmx1a+Otx2 or Lmx1a+En1+Otx2 or Lmx1a+En1+Otx2+Foxa2 | Panman et al. (2011) |
| | ESC-derived NPCs | Mouse | Lmx1a+Foxa2 or Lmx1a+Foxa2+Barhl1 | Kee et al. (2017) |
| | PSC-derived NPCs | Human | PBX1 | Villaescusa et al. (2016) |
| | ESC-derived NPCs | Human | ASCL1, FOXA2, LMX1A, NGN2, NURR1, OTX2 and PITX3 (alone or in combination) | Azimi et al. (2018) |
| | ESC-derived NPCs | Human | LMX1A | [12] Friling et al. (2009) |
| | ESCs | Mouse | Nurr1 | Chung et al. (2002) |
| | ESCs | Mouse | Nurr1 | [8] Kim et al. (2002) |
| | ESCs | Mouse | Gpx1+Nurr1 | Abasi et al. (2012) |
| | iPSCs | Mouse | Nurr1+Pitx3 | Salemi et al. (2016) |
| | ESCs | Mouse and human | Nurr1+Pitx3 | [9] Martinat et al. (2006) |
| | PSCs | Human | LMX1A | [13] Sánchez-Danés et al. (2012) |
| | iPSCs | Human | mAscl1+mNurr1+mLmx1a | [7] Theka et al. (2013) |
| | PSCs | Human | ATOH1 | Sagal et al. (2014) |
| | iPSCs | Human | NGN2 and/or ATOH1 | Xue et al. (2019) |
| Medium spiny neurons | ESC-derived NPCs | Human | GSX2+EBF1 | [14] Faedo et al. (2017) |
| Motor neurons | ESC-derived NPCs | Mouse | Phox2b or Olig2 | Panman et al. (2011) |
| | ESC-derived NPCs | Mouse and human | Phox2a or Phox2b | Mong et al. (2013) |
| | ESCs-derived NPCs | Human | NGN2+ISL1+LHX3 | Hester et al. (2011) |
| | ESCs | Mouse | Ngn2+Isl1+Lhx3 or Ngn2+Isl1+Phox2a | [15] Mazzoni et al. (2013) |
| | iPSCs | Human | NGN1+NGN2 | Busskamp et al. (2014) |
| | PSCs | Human | NGN1+NGN2+NGN3+NEUROD1+NEUROD2 | Goparaju et al. (2017) |
| | PSCs | Human | NGN2+ISL1+LHX3 | Goto et al. (2017) |
| | iPSCs | Human | NGN2+ISL1+LHX3 or NGN2+ISL1+PHOX2A | De Santis et al. (2018) |
| Sensory neurons | ESC-derived neural crest progenitors | Human | NGN2 | Schrenk-Siemens et al. (2015) |
| | iPSC-derived neural crest progenitors + iPSCs | Human | NGN2+BRN3A | Nickolls et al. (2020) |
| Otic neurons | (Otic) NPCs | Human | NGN1 | Song et al. (2017) |
| Serotonergic neurons | ESC-derived NPCs | Mouse | Nkx2.2 | Panman et al. (2011) |

*Numbers in superscript relate to citations in **Figure 2**.*

(Thoma et al., 2012). The first ground-breaking proof that NGN2 has the same effect in human PSCs was – again – provided by the groups of Marius Wernig and Thomas Südhof in 2013. The authors demonstrated that forward programming human PSCs with NGN2 reproducibly yields neurons with almost 100% purity within 2 weeks, and as was observed in mouse cells, these neurons do not only acquire neuronal-like electrophysiological properties but are also capable of functionally integrating into synaptic networks with cortical mouse neurons. Notably, the authors further reported that overexpressing the bHLH TF NEUROD1 can instruct neuronal differentiation from human

PSCs, too (Zhang et al., 2013). Interestingly, already in 2001, O'Shea (2001) had investigated the neurogenic effect of Neurod TFs by overexpressing Neurod1, Neurod2, and Neurod3 in mouse ESCs and found that all three Neurods suffice to induce immature neuronal-like cells within 72 h.

More recently, other groups have corroborated the finding that NGN2 overexpression suffices to forward program human PSCs to neurons expressing MAP2 and NEUN within 2 to 10 days of differentiation (Rubio et al., 2016; Goparaju et al., 2017; Matsushita et al., 2017; Pawlowski et al., 2017). Neuronal differentiation can be further accelerated by combined

overexpression of NGN1 and NGN2, and in this case, 90% of all cells were found to express MAP2 and the synapse marker synapsin already at day 4 of differentiation. However, additional morphological, transcriptomic, and functional analyses indicate that a majority of the obtained neurons at this early time point are still immature and not yet fully developed (Busskamp et al., 2014). Yet, several labs including our own have revealed that co-culturing NGN2-neurons with glial cells significantly facilitates maturation (Zhang et al., 2013; Busskamp et al., 2014; Meijer et al., 2019; Rhee et al., 2019), and when cultured under appropriate conditions, neurons derived by forward programming can be utilized for sophisticated electrophysical analyses: Cultured on glia microdots, single forward programmed NGN2-neurons form an autaptic system by making synapses onto themselves, which can be used for studying functional features such as synaptic transmission and short-term plasticity (Meijer et al., 2019; Rhee et al., 2019), and networks of forward programmed neurons cultured on multielectrode arrays have also been employed for functional analyses (Frega et al., 2017; Nehme et al., 2018).

## Mechanisms Underlying bHLH Transcription Factor-Mediated Forward Programming

bHLH TFs are named after their common protein structure motif consisting of two α-helices mediating dimerization and a basic domain, which binds to E-box motifs with the consensus sequence CANNTG. The group of bHLH TFs is subdivided according to their ubiquitous versus tissue-specific expression profile, and neural bHLH TFs are further grouped into the *achaete-scute complex* and *atonal* gene families (for further details see review by Dennis et al., 2019). First hints as of why bHLH TFs might be able to orchestrate neuronal fate acquisition were obtained from NPC-to-neuron differentiation paradigms: Ngn1, for instance, specifically binds to E-box motifs at neuronal genes in rat NPCs, acting as a direct transcriptional activator (Sun et al., 2001). In human PSCs, the TFs NGN1, NGN2 and NGN3 seem to even cross-activate each other and induce common pro-neural down-stream targets including other bHLH TFs such as *NEUROD1, NEUROD2* and *NEUROD4* (Busskamp et al., 2014; Goparaju et al., 2017; Xue et al., 2019). Such a synergism might not be restricted to the group of NGNs, since the bHLH TF ATOH1 has been shown to induce both, *NGN2* and *NEUROD1* in human PSCs, and was thus used for forward programming of human PSCs into neurons (Sagal et al., 2014; Xue et al., 2019). These observations might indicate that one common mechanism underlying neuronal forward programming of PSCs with bHLH TFs is the activation of a whole network of cross-regulated bHLH TFs, including the induction of more down-stream Neurod TFs. In line with this hypothesis, no difference was reported comparing the neuronal induction potency of NGN1, NGN2, NGN3, NEUROD1, and NEUROD2 in human ESCs (Matsushita et al., 2017).

In order to elucidate the mechanism by which combined NGN1 and NGN2 overexpression drives neuronal fate acquisition of human iPSCs more in depth, Busskamp et al.

(2014) performed a comprehensive set of experiments dissecting mRNA and miRNA regulation kinetics during the early phase of neural induction. A mRNA network analysis revealed that during the first 4 days of differentiation, destabilization of the pluripotency network is initiated by decreasing *SOX2, NANOG,* and *OCT4* levels, and genes associated with an NPC stage and the gene ontology term 'regulation of neurogenesis' such as *NOTCH1, DLL1, DLL4, HES5, FABP7,* and *NTN1* are temporarily upregulated. This phase of NPC marker induction is very brief, though (as was also observed by Zhang et al., 2013), resulting in a significant downregulation of cell cycle-related genes by day 4, which suggests that PSC-derived cells only traverse a short progenitor-like phase during forward programming. In line with this, neuron-associated genes such as *POU3F2* (also known as *BRN2*), *ZEB1, ISL1, TLX3,* and *POU4F1* (also known as *BRN3a*) are upregulated already in this early phase of reprogramming, whilst inhibitors of neurogenesis as for instance *REST* and *HES1* are repressed. Concomitant with the dynamics of mRNA regulation, the expression of the pluripotency-associated miRNA cluster 302/367 decreases, whereas the abundance of neuronal miRNAs such as miR124, miR96, and miR9 increases upon differentiation (Busskamp et al., 2014).

The molecular consequences of Neurod1 induction were explicitly investigated by Vijay Tiwari's group in mouse ESCs: Neurod1 overexpression influences chromatin accessibility at its target sites by reducing repressive H3K27me3 marks, increasing H3K27ac and recruiting RNA polymerase II. Hence, during the first 24 h of differentiation, the fraction of enhancer and promotor regions directly bound by Neurod1 probably accounts for approximately 25% of all upregulated genes identified by RNA sequencing. Interestingly, upregulated genes are exclusively enriched for neurogenesis-associated gene ontology terms, and consequently, after 48 h, and even in the presence of the pluripotency-promoting factor Lif, Tubb3 expression is induced. Even transient Neurod1 expression for as short as 48 h suffices to stably remodel the epigenetic and transcriptional landscape at Neurod1 targets, which later drive neuronal differentiation of ESCs even in the absence of Neurod1 (Pataskar et al., 2016). Altogether, these data indicate that activation of the bHLH TF Neurod1 might represent one of the crucial entry points for neural fate acquisition in forward programming paradigms.

## Neuronal Forward Programming Factors Beyond the bHLH Family

Neuronal specification from diverse NPC populations can be driven by pro-neural, non-bHLH TFs such as Pax6 (Hack et al., 2004), Brn4 (Shi et al., 2010; Tan et al., 2010) and members of the Sox family of TFs (Kan et al., 2004; Braccioli et al., 2018). Furthermore, overexpression of Dlk1 in mouse and human ESC-derived NPCs facilitates neural specification by promoting cell cycle exit via reduction of Notch, and modulation of BMP signaling (Surmacz et al., 2012). Finally, decreasing REST signaling in human NPC lines induces neurogenesis, too (Huang et al., 2011), and this can most likely be attributed to REST's function as a transcriptional repressor of neuronal

genes such as *Tubb3* (Greenway et al., 2007) and *Neurod2* (DeWald et al., 2011).

Considering these observations, it is not surprising that non-bHLH TFs have also been implemented in forward programming of PSCs. Yamamizu et al. (2013) for instance, identified that doxycycline-mediated induction of Nr2f1 or Smad7 can instruct neuronal differentiation of mouse ESCs. Liu et al. (2018) further performed a comprehensive CRISPR activation screen in mouse ESCs and revealed that besides the bHLH TFs *Ngn1* and *Tcf15*, also non-bHLH TFs such as *Brn2*, *Foxo1*, *Ezh2* and *Zeb1* have neurogenic potential. Zeb1 and Zeb2, for instance, are homologous TFs and downstream effectors of the lncRNA-1604, which regulates neural differentiation by competitive binding with miRNA-200c in mouse ESCs (Weng et al., 2018), and *ZEB1* is also induced after combined overexpression of NGN1 and NGN2 in human iPSCs (Busskamp et al., 2014). Yet, at least for Zeb1 it was shown by other groups that its neuron-promoting effect is comparably weak. When overexpressed in human ESCs, ZEB1 does not immediately decrease pluripotency markers *OCT4, NANOG*, and *SOX2*, and most cells are negative for the early neuronal marker TUBB3 up until day 25 of differentiation (Jiang et al., 2018). Since it was demonstrated that Zeb2 overexpression decreases *Ngn2* expression in embryonic midbrain cells and a dopaminergic cell line (Yang et al., 2018), a negative correlation between these two TFs might account for the observed weak pro-neurogenic effect. Conversely, forward programming by *Ezh2* induction results in the formation of electrophysiological active and synapse-forming neurons, which is comparable to the effect of Ngn1-mediated forward programming. The pro-neurogenic action of Ezh2 might most likely be due to its inhibitory effect on endodermal and mesodermal lineage-associated genes (Liu et al., 2018), which is in line with the fact that the methyltransferase Ezh2 is a core component of the PRC2 complex and involved in transcriptional repression.

In sum, these studies hint at some common mechanisms underlying TF-driven specification of NPCs and PSCs into neurons, which include (i) exit of the original cell fate, (ii) repression of alternative lineage decisions, and (iii) activation of a pro-neuronal transcriptional program.

## Forward Programming Into Clinically Relevant Neuronal Subtypes

When thinking of forward programming as a tool to produce neural cell types for brain repair, it is particularly relevant to thoroughly characterize the exact phenotype of the obtained cells. Already Serre et al. (2012) noted that the four different bHLH TFs NGN1, NGN2, NGN3, and ASCL1 had slightly varying effects on neuronal subtype specification from human primary cortical NPCs, although cultures generally consisted of a mixed population of GABAergic, cholinergic, serotoninergic, adrenergic, and MNs (Serre et al., 2012). This observation is in line with other reports demonstrating divergent effects for different bHLH TFs on neuronal subtype derivation: Overexpression of ASCL1 induces a GABAergic bias in neuronal

cultures differentiating from neurospheres isolated from both human fetal cortex and mesencephalon (Kim et al., 2009), whereas NGN2 overexpression in human iPSC-derived NPCs (Ho et al., 2016) and PSCs (Zhang et al., 2013; Nehme et al., 2018; Meijer et al., 2019; Rhee et al., 2019; Nickolls et al., 2020) leads to the derivation of mostly glutamatergic neurons.

## Cortical Glutamatergic and Forebrain GABAergic Neurons

The results of multiple independent studies indicate that glutamatergic neurons derived by overexpression of NGN2 adopt a telencephalic fate characterized by the expression of cortical layer II/III markers such as FOXG1, BRN2, SATB2, and *CUX1* (Zhang et al., 2013; Frega et al., 2017; Wang et al., 2017; Nehme et al., 2018; Meijer et al., 2019). However, there are reports indicating that forward programming with a combination of NGN1 and NGN2 results in neurons co-expressing vGLUT1 and ChAT (Busskamp et al., 2014). Similarly, mRNA-driven combinatorial overexpression of NGN1, NGN2, NGN3, NEUROD1, and NEUROD2 in human PSCs gives rise to a population of remarkably pure cholinergic MNs (Goparaju et al., 2017), indicating that NGN2 can, in particular in combination with additional TFs and morphogens, instruct other fates than glutamatergic neurons (see also section 'Motor and Sensory Neurons').

Ascl1 – which is expressed in more ventral regions of the telencephalon *in vivo* (Casarosa et al., 1999; Fode et al., 2000) – is alone insufficient to consistently give rise to homogenous cultures of only one specific neuronal subtype and instead results in mixed cultures of MNs, dopaminergic and GABAergic neurons (Yamamizu et al., 2013). However, in a landmark study, Yang et al. (2017) demonstrated that overexpression of ASCL1 in combination with DLX2, a downstream effector of ASCL1, is able to direct human PSCs into remarkably pure cultures of telencephalic forebrain GABAergic neurons. Sun et al. (2016) used a combination of ASCL1 and DLX2 with LHX6 and a synthetic cluster of miRNA-9/9* and miRNA-124 (miR9/9*-124), and found that the induced GABAergic neurons express markers reminiscent of derivatives of the medial ganglionic eminence, but not alternative birthplaces such as the lateral ganglionic eminence, caudal ganglionic eminence or the preoptic area. As somatostatin- and parvalbumin-positive inhibitory interneurons appear to play a particular role in several neurological and neuropsychiatric diseases, the generation of these subtypes would be highly desirable. Colasante et al. (2015) reported that combinatorial overexpression of the five TFs FOXG1, SOX2, ASCL1, DLX5, and LHX6 in human iPSCs gives rise to highly enriched cultures of parvalbumin-expressing inhibitory neurons. Yuan et al. (2018) explored overexpression of LHX6 alone and found that 80% of the human PSC-derived neurons were GABAergic, with a fraction of 21% and 29% of the TUBB3-positive neurons co-expressing parvalbumin and somatostatin, respectively. As with forward programmed excitatory neurons, functional maturation of induced GABAergic neurons can be promoted by co-culture with primary rodent glia (Sun et al., 2016). Interestingly, this process can also be promoted by co-culture with NGN2-forward programmed

excitatory neurons (Yang et al., 2017) – an observation which could suggest that utmost functionality can only be achieved in the context of a heterogenous synaptic network as encountered *in vivo*.

## Midbrain Dopaminergic Neurons

Representing a prime target of PD, midbrain dopamine neurons are a particularly attractive donor cell population for neural repair. However, the efficacy of neuroregeneration seems to heavily depend on the fidelity of neuronal subtype specification. This was recently exemplified by Kirkeby et al. (2017), who revealed that the purity of dopaminergic cell preparations (i.e., the ratio of caudal ventral mesencephalic dopaminergic neurons versus neurons of the diencephalic subthalamic nucleus) is predictive for successful dopaminergic specification and symptom amelioration after transplantation into a mouse model of PD. Although potent extrinsic factor-guided protocols for the derivation of dopaminergic neurons from PSCs exist (Kriks et al., 2011; Kirkeby et al., 2012), there is a necessity to further fine-tune cell fate subspecification. This was nicely illustrated by La Manno et al. (2016), who profiled the developing mouse and human midbrain using single cell RNA sequencing, and delineated multiple molecularly diverse NPC populations and several distinct classes of mature dopaminergic neurons in the midbrain of both species. Notably, the authors compared transcriptomic signatures of human PSC-derived dopaminergic neurons with their *in vivo* counterparts and found that although the morphogen-driven dopaminergic differentiation recapitulated key developmental stages of embryonic dopaminergic lineage specification, the gene expression profile of *in vitro* generated populations still differed from that of native midbrain dopamine neurons. The question remains whether forward programming can further improve the authenticity of specialized dopaminergic neuron subpopulations.

Already beginning in the end of last century, several labs reported that overexpression of the mesencephalic TF Nurr1 in primary adult rat hippocampal NPCs (Sakurada et al., 1999) and primary embryonic rat cortical NPCs (Kim et al., 2003; Park et al., 2006; Bae et al., 2009) promotes the generation of a midbrain dopamine neuron-like phenotype. A similar effect was communicated for primary mouse NPCs isolated from the ganglionic eminence and midbrain as well as mouse ESC-derived NPCs, whereas NPCs from embryonic cortex and spinal cord as well as adult NPCs from the subventricular zone seemed resistant to the pro-dopaminergic patterning effect of Nurr1 (Soldati et al., 2012). Other midbrain-specific TFs that were tested for their potency to instruct dopaminergic fates include (i) Foxa2, which, when overexpressed in mouse primary midbrain-derived and ESC-derived NPCs, was found to boost the derivation of TH-positive neurons (Kittappa et al., 2007), (ii) Lmx1a, which efficiently specifies murine Shh- and Fgf8-treated (Andersson et al., 2006; Panman et al., 2011) as well as human ESC-derived NPCs toward a dopaminergic fate (Friling et al., 2009), and in combination with Foxa2 and Barhl1 drives dopaminergic differentiation from Fgf8- and CHIR99021-exposed murine ESC-derived NPCs (Kee et al., 2017), (iii) En1 and (iv) Otx2, which were reported to drive dopaminergic differentiation of murine

NPCs alone, each in combination with Lmx1a, or as a 3 TF cocktail (Panman et al., 2011), (v) Dmrt5, which does not increase overall neuronal yield after being overexpressed in mouse ESC-derived dopaminergic NPCs but specifically induces an increase of certain midbrain dopaminergic markers on RNA level (Gennet et al., 2011), and (vi) PBX1, which appears to cooperate with NURR1 promoting dopaminergic specification from human PSC-derived NPCs (Villaescusa et al., 2016). Another good example is the recently published study by Azimi et al. (2018), who used magnetically guided mRNA spot delivery to screen single TFs and TF combinations for their capacity to commit human ESC-derived NPCs toward a dopaminergic fate, and revealed that transfection of FOXA2, LMX1A, and PITX3 mRNA results in an increased yield of TH-positive neurons. Combinatorial delivery of these 3 TFs at their respective most effective stage results in almost 68% of TH- and MAP2-double-positive cells (Azimi et al., 2018).

Nurr1 was also the first TF explored in the context of forward programming PSCs toward a dopaminergic fate, and its overexpression in mouse ESCs causes a substantial increase in the number of TH-expressing neurons (Chung et al., 2002; Kim et al., 2002). Exposure to the morphogens Shh and Fgf8 further increases the yield of TH-expressing neurons after Nurr1 overexpression with enrichment in the order of 60% to 80% (Chung et al., 2002; Kim et al., 2002), and enhances the release of dopamine after induced depolarization (Kim et al., 2002). The combination of Nurr1 with other TFs (such as Gpx1), morphogens (as for instance RA) and chemicals (e.g., β-boswellic acid) was reported to boost the yield of dopaminergic neurons, too (Abasi et al., 2012). Especially the combined expression of Nurr1 and Pitx3 was shown to be beneficial for the derivation of dopaminergic neurons from mouse PSCs (Martinat et al., 2006; Salemi et al., 2016). Whereas these TFs alone are sufficient to induce markers expressed early in dopaminergic development such as TH and *Aldh2*, it is only upon co-expression that they synergistically induce more advanced markers such as *Dat* and *Tyrp1* (Martinat et al., 2006). In this co-expression paradigm, too, addition of Shh and Fgf8 increased the induced secretion of dopamine from these cells (Salemi et al., 2016). Notably, and in contrast to overexpression of Nurr1 alone (Kim et al., 2002), co-expression of Nurr1 and Pitx3 (along with exposure to Shh and Fgf8) prevents the derivation of 'contaminating' cell fates such as serotonergic or GABAergic neurons (Martinat et al., 2006).

Sánchez-Danés et al. (2012) analyzed the effect of LMX1A overexpression in human iPSCs and observed a quick down-regulation of *NANOG* with a simultaneous upregulation of *NURR1*, *EN1*, and *TH*, which are all characteristic markers of midbrain dopamine neurons. At day 34 of differentiation, the derived neurons express DAT and TH, show synaptophysin-positive puncta on TH-positive neurites and inducible dopamine release. Although LMX1A overexpression alone enhances the dopaminergic specification of differentiating neurons, it does not result in an increased neuronal yield (Sánchez-Danés et al., 2012).

The group of Vania Broccoli explored forced expression of LMX1A together with NURR1 and ASCL1 in order to boost neuronal induction *per se* as well as dopaminergic induction

in particular. In their study, lentiviral overexpression of this TF cocktail in human iPSCs gave rise to homogenous neuronal cultures expressing a wide range of proteins associated to the dopaminergic lineage such as TH, DAT, ALDH1A1, and GIRK2. Resulting neurons exhibited neuron-like electrophysiological properties and were able to spontaneously release dopamine after 3 weeks of differentiation. However, although the number of TH- and TUBB3-co-expressing cells doubled when applying the TF cocktail (Theka et al., 2013), the overall yield of TH-expressing neurons remained lower as compared to the yield after overexpression of LMX1A alone (Sánchez-Danés et al., 2012). This difference might be attributable to the lack of additional patterning molecules such as SHH and FGF8 in the medium used by Vania Broccoli's group, and/or shorter differentiation times till analysis, and thus warrants further studies on forward programming approaches combining neurogenic and regionalizing TFs.

Finally, it is worth mentioning that the bHLH TF ATOH1 is itself able to induce dopaminergic neurons from human PSCs, especially when combined with exposure to SHH and FGF8b. In combination with these morphogens, ATOH1 overexpression yields up to 82% TH-expressing neurons. In addition to *TH*, *FOXA2*, *NURR1*, *LMX1A*, *DAT*, *VMAT2*, and *OTX2* are significantly upregulated during ATOH1-driven differentiation. Characterization of the growth factor-treated, ATOH1-overexpressing neuronal cultures on a functional level demonstrated that these neurons possess electrophysiological properties similar to primary rat midbrain dopaminergic neurons and exhibit dopamine release after electrical stimulation at day 36 of differentiation, suggesting actual functionality (Sagal et al., 2014). Very recently, this approach was further improved by establishing a protocol based on combined ATOH1 and NGN2 overexpression in human iPSCs via repetitive mRNA transfections (Xue et al., 2019).

## Medium Spiny Neurons

Another neuronal subtype of particular biomedical interest are MSNs, a GABAergic population abundantly found in the striatum and most prominently affected by HD. In human ESC-derived NPCs treated with SHH and the WNT inhibitor DKK1, overexpression of GSX2 and EBF1, two TFs essential for the development of striatal interneurons, actively suppresses the expression of the medial ganglionic eminence progenitor markers PAX6 and NKX2.1 and drives cell cycle exit. After 60 days of long-term differentiation, overexpression of both TFs finally results in MSN progenitor cells expressing ISL1- and CTIP2; by day 80, 38.8% of all cells co-express the MSN markers DARPP32 and CTIP2 (Faedo et al., 2017). However, whether or not this TF combination would be capable of directly specifying undifferentiated PSCs to MSNs merits further investigation.

## Motor and Sensory Neurons

Motor neuron development and specification *in vivo* is relatively well studied (see reviews by Jessell, 2000 and Briscoe and Ericson, 2001), and this knowledge was efficiently exploited for studies focusing on the *in vitro* generation of enriched MN populations, which are affected by degenerative diseases such as amyotrophic lateral sclerosis. Hester et al. (2011) for instance, successfully combined NGN2-driven differentiation of SHH- and RA-treated human PSC-derived NPCs with overexpression of the MN lineage-specific markers ISL1 and LHX3 yielding 60% cells co-expressing the MN markers ISL1 and ChAT at day 13 of differentiation. In 2013, the group of Hynek Wichterle demonstrated that overexpression of this TF combination can also successfully specify the differentiation of mouse ESCs into spinal MNs directly, whereas cranial MNs can be obtained by replacing Lhx3 by Phox2a in this TF cocktail. A systematic comparison of these two different TF combinations by gene array analysis revealed a sharp decrease of pluripotency-associated genes *Oct4* and *Nanog* and an upregulation of pan-MN markers such as *Isl1*, *Ebf1* and *Ebf3* as well as the VAChT in both paradigms, whereas upregulation of *Tbx20*, *Phox2a*, *Phox2b*, *Rg4*, and *Gal* was only detected in neurons subjected to forward programming with Phox2a. The results of this study further indicate that these divergent outcomes are obtained because Isl1 is recruited to different genomic sites when co-expressed with Ngn2 in combination with either Lhx3 or Phox2a. Yet, both MN subpopulations become electrophysiologically functional and capable of forming cholinergic synapses after maturation on cortical mouse astrocytes (Mazzoni et al., 2013). A few years later, Goto et al. (2017) verified that Sendai virus-mediated overexpression of the TF cocktail NGN2, ISL1 and LHX3 in human PSCs, too, promotes the expression of MN markers. More specifically, only the full TF cocktail and the 2-factor combination of NGN2 and LHX3 but neither NGN2 in conjunction with ISL1 nor any of the single TFs resulted in MN derivation. Notably, after 3 weeks of differentiation NGN2/ISL1/LHX3-overexpressing neurons were electrophysiologically active and formed neuromuscular junctions with cultured myocytes (Goto et al., 2017). De Santis et al. (2018) expressed both TF combinations identified by Hynek Wichterle's group (NGN2/ISL1/LHX3 and NGN2/ISL1/PHOX2A) in human iPSCs via Piggy-bac transposable vectors. Concordant with the previous results, iPSCs downregulated the pluripotency marker *NANOG* and upregulated pan-MN genes such as *TUBB3*, *ISL1*, and *ChAT* within the first 3 days of differentiation. By day 5, *HB9* expression was increased when LHX3 was co-expressed, whereas *PHOX2B*, *TBX20*, and *RG4* were detected upon PHOX2A overexpression. Finally, the authors of this study functionally characterized the cranial MNs obtained after 12 to 13 days of NGN2/ISL1/PHOX2A overexpression and observed that these cells were capable of firing action potentials upon current stimulation, and almost half of all analyzed cells even displayed spontaneous glutamatergic postsynaptic currents (De Santis et al., 2018).

Whilst these studies utilized joined overexpression of the neurogenic TF Ngn2 with MN lineage-associated TFs, Goparaju et al. (2017) investigated whether overexpression of generic neurogenic TFs can induce specified neuronal subtypes when combined with fate-modulating extrinsic factors. Indeed, they found that overexpression of NGN1, NGN2, NGN3, NEUROD1, and NEUROD2 in human PSCs combined with RA, forskolin and dual SMAD inhibition via SB431542 and dorsomorphin yields highly pure neuronal cultures expressing the MN markers

HB9, ISL1, and ChAT (Goparaju et al., 2017). Interestingly, the combination of NGN2 overexpression with forskolin and dorsomorphin treatment has been described to even convert human fibroblasts into cholinergic MNs (Liu et al., 2013).

On the other hand, Panman et al. (2011) revealed that overexpression of the MN-associated TFs Phox2b and Olig2 in mouse ESC-derived, posterior-ventral NPCs suffices to specify visceral and somatic MNs, respectively. Further dissecting the role of Phox2 TFs in segregating MNs from other populations of hindbrain neurons, Mong et al. (2013) overexpressed either Phox2a or Phox2b in Nestin-expressing ESC-derived NPCs. Although the expression of both TFs largely overlaps *in vivo*, the sequence of their expression is known to be important for the specification of different neuronal subtypes: Phox2a precedes Phox2b induction during the development of noradrenergic (Pattyn et al., 2000) and midbrain MNs (Pattyn et al., 1997), whereas in hindbrain visceral MNs, Phox2b is induced before Phox2a (see review by Brunet and Pattyn, 2002). Knock-out studies further showed that although Phox2b expression can compensate for effects caused by Phox2a-knock-out in noradrenergic neurons of the locus coeruleus, it does not suffice to rescue the loss of MNs in the midbrain (Coppola et al., 2005). *Vice versa*, Phox2a cannot completely compensate for Phox2b loss during the development of noradrenergic neurons and visceral MNs (Coppola et al., 2005). Concordant with these findings, overexpression of both Phox2 TFs in mouse and human ESC-derived NPCs upregulated the expression of visceral MN markers as for instance *Isl1*, *Nkx6.2*, *Tbx2*, and *Tbx20* when combined with the morphogens Fgf8 and Shh. Conversely, combining Phox2b but not Phox2a overexpression with Bmp7 and Fgf8 treatment increased the expression of genes characteristic for noradrenergic neurons such as *Tfap2a*, *Dbh*, *Tlx3*, and *Net* (Mong et al., 2013).

Very recently, the group of Carsten Bönnemann published a protocol to derive sensory neurons from human iPSCs via forward programming. The authors demonstrated that even in the absence of neuronal lineage-promoting medium conditions, doxycycline-induced expression of NGN2 and BRN3A from the human genomic safe harbor locus *CLYBL* specifies human iPSCs toward a presumable human-specific neuronal subtype of glutamatergic sensory neurons responsive to cold as well as mechanical stimuli. This neuronal phenotype was also acquired when expression of this TF combination was induced in iPSC-derived neural crest progenitors for 14 days. Notably, when iPSC-derived neural crest progenitors were exposed to doxycycline for as short as 24 h, these cells adopted an exclusively PIEZO2-positive but TRPM8-negative touch-sensitive phenotype (Nickolls et al., 2020). This finding is in line with the observation that even a 24-h pulse of NGN2-only overexpression (in combination with a GDNF-based differentiation paradigm) is sufficient to direct human ESC-derived neural crest cells into highly enriched cultures of mechanoreceptive neurons (Schrenk-Siemens et al., 2015).

In sum, whilst these studies impressively illustrate the potential of the forward programming technique to derive distinct neuronal subtypes, they also demonstrate the sensitivity of the approach to subtle alterations in TF combinations and co-administered growth and patterning factors.

## Glial Cells

Astrocytes are crucial for neuronal development, synaptogenesis and synaptic function, brain tissue homeostasis including energy and substrate distribution, and they provide the structural scaffold of the brain parenchyma. Oligodendrocytes are not only crucial for myelination but also axonal maintenance and even immunomodulation (reviewed by Kuhn et al., 2019). Given the plethora of glial functions that are essential for proper brain physiology, the role of these cells in the pathogenesis of neurodegenerative diseases becomes increasingly acknowledged, which contributes to the great interest in producing glial cells for basic and translational research in a fast and efficient manner by TF overexpression (**Table 2**).

### Forward Programming to Astrocytes

As with neurons, astrocytes – and even further specified astrocyte subtypes – can be differentiated from PSCs by multi-step, growth factor-based protocols, which stimulate signaling pathways involved in astrogenesis after initial induction of a neuroectodermal fate (compare, e.g., the elegant protocol by Krencik and Zhang, 2011). Yet, growth factor-based protocols are usually complex and time-consuming, especially if they are aiming at creating non-reactive cells resembling quiescent astrocytes *in vivo*. Hence, there is a need for the derivation of functional astrocytes via forward programming. In primary mouse cortical NPCs, astrocytic commitment can be facilitated via induction of Stat3 by overexpression of Rnf20 (Liang et al., 2018). Overexpression of the TF Emx2 in mouse cortical NPCs regulates Egf and Fgf signaling, which are crucial for maintaining the pool of proliferating astrocyte progenitors (Falcone et al., 2015).

In a landmark study, Canals et al. (2018) lentivirally overexpressed the NFI TF family member NFIB alone or in combination with SOX9 in human PSCs. In this paradigm, PSCs differentiate into mature, post-mitotic astrocytes expressing markers such as GFAP, S100β, VIM, ALDH1L1, and GLAST within 21 days of differentiation. At this differentiation stage, astrocytes further contain glycogen-positive granules comparable to primary astrocytes. Functional assessments of these forward programmed astrocytes between days 14 and 21 of differentiation revealed that the derived cells exhibit typical characteristics of human adult astrocytes such as generation and propagation of spontaneous calcium waves, glutamate uptake, responsiveness to ATP and inflammatory stimuli such as IL-1β, the ability to form functional gap junctions with other astrocytes and the potency to promote synaptogenesis in a co-culture system with iPSC-derived forward programmed neurons (Canals et al., 2018).

Independent of and almost at the same time as the report by Canals et al. (2018), the lab of Su-Chun Zhang published a protocol to derive functional astrocytes from human PSCs by doxycycline-inducible expression of NFIA, another NFI TF family member, via CRISPR/Cas9-mediated targeting of the human *AAVS1* genomic safe harbor locus. By overexpressing SOX9 in addition to NFIA, and combining this forward

**TABLE 2 |** Transcription factors used for promoting glial differentiation of neural precursor cells and pluripotent stem cells *in vitro*.

| Derived cell type | Starting cell type | Species | Transcription factor used for forward programming | References |
|---|---|---|---|---|
| Astrocytes | NPCs | Mouse | Emx2 | Falcone et al. (2015) |
| | NPCs | Mouse | Rnf20 | Liang et al. (2018) |
| | iPSC-derived NPCs | Human | NFIA | [17] Tchieu et al. (2019) |
| | PSCs | Human | NFIB or NFIB+SOX9 | [18] Canals et al. (2018) |
| | PSCs | Human | NFIA or NFIA+SOX9 | [16] Li X. et al. (2018) |
| Oligodendrocytes | NPCs | Mouse | Olig1 | Balasubramaniyan et al. (2004) |
| | NPCs | Mouse | Olig1 or Olig2 or Nkx2.2 | [19] Copray et al. (2006) |
| | NPCs | Mouse | Ascl1 or Olig2 or Nkx2.2 or Ascl1+Olig2 or Ascl1+Nkx2.2 or Olig2+Nkx2.2 | Sugimori et al. (2008) |
| | NPCs | Mouse | Nkx2.2AS | Tochitani and Hayashizaki (2008) |
| | NPCs | Mouse | Olig1 or Olig2 | Maire et al. (2010) |
| | NPCs | Mouse | Ascl1 or Olig2 or Sox10 | Braun et al. (2015) |
| | NPCs | Mouse | Sox10 | [24] Matjusaitis et al. (2019) |
| | NPCs | Mouse | Lnc-158 | Li Y. et al. (2018) |
| | NPCs | Human | OLIG1 or OLIG2 or OLIG1+OLIG2 | [21] Hwang et al. (2009) |
| | NPCs | Human | OLIG2 | [20] Maire et al. (2009) |
| | NPCs | Human | ASCL1 or NKX2.2 or OLIG2 or PRRX1 or SOX10 | [22] Wang et al. (2014) |
| | NPCs | Human | OLIG1 or OLIG2 or OLIG1+OLIG2 | Li et al. (2017) |
| | iPSC-derived NPCs | Human | SOX10 or NKX6.2+OLIG2+SOX10 | [23] Ehrlich et al. (2017) |
| | PSC-derived NPCs | Human | SOX10 | [25] García-León et al. (2018) |
| | iPSCs | Human | OLIG2+SOX10 | Li P. et al. (2016) |
| | PSCs | Human | SOX10 or OLIG2+SOX10 | Pawlowski et al. (2017) |

*Numbers in superscript relate to citations in* **Figure 2**.

programming protocol with a conventional morphogen-driven astrocyte differentiation paradigm, the efficiency of astrocyte generation was significantly increased so that finally around 70% of all cells co-expressed the astrocyte markers GFAP and S100β at day 52 of differentiation. Similar to the astrocytes derived by Canals et al. (2018), their cells could propagate calcium waves, take up free glutamate from the culture medium, and facilitate neurite outgrowth when co-cultured with human iPSC-derived neurons. Interestingly, the authors further reported that transgene induction during the first 10 days of differentiation was dispensable for successful astrocyte induction. Importantly, when they used this transgene induction-free window for morphogen-based patterning, they could generate diverse astrocytic subtypes (i.e., dorsal and ventral forebrain astrocytes as well as spinal astrocytes) within the same time frame (Li X. et al., 2018).

Despite the many similarities between the protocols published by Canals et al. (2018) and Li X. et al. (2018), it is noteworthy that the former protocol leads to the derivation of functional astrocytes much faster than the latter one (2–3 versus >7 weeks). This might have several causes, including the choice of the TFs itself (NFIB versus NFIA), the methods used for TF delivery that could influence total gene dosage (lentiviral expression versus expression from the endogenous *AAVS1* locus) and the efficiency of the concomitant growth factor regimen (e.g., sequential versus combined exposure to FGF and EGF). Thus, the results of these two studies stress the context-dependency of TF-based forward programming. This is further nicely exemplified by the fact that NFIA was recently demonstrated to act as a gliogenic switch in iPSC-derived NPCs, too, facilitating

the fast generation of astrocytes in combination with glia-promoting, LIF-containing medium. Continued overexpression of this TF, however, inhibited astrogenesis from NPCs, probably by inducing premature G1 cell cycle arrest (Tchieu et al., 2019). Surprisingly, overexpression of the long non-coding RNA lnc-158, which is an endogenous antisense RNA of *NFIB* and positively regulates NFIB levels, has been reported to promote the differentiation of primary mouse NPCs into oligodendrocytes instead of astrocytes (Li Y. et al., 2018).

## Promoting Oligodendrogenesis by Transcription Factor Overexpression

Wang et al. (2014) screened 5 TFs (NKX2.2, OLIG2, PRRX1, ASCL1, and SOX10) known to be associated with oligodendrocyte lineage commitment and analyzed their potency to induce OPC markers in primary human NPCs. Whilst all examined TFs repressed astrocytic genes, NKX2.2 and especially ASCL1 induced the expression of neuronal genes in addition to the upregulation of OPC markers. Gene set enrichment analyses of RNA sequencing data further revealed that only SOX10 overexpression induced genes expressed in both primary mouse and human OPCs, whereas ASCL1-induced OPCs expressed markers resembling mouse but not human OPC fate. The authors further demonstrated the superiority of SOX10-induced OPCs by the fact that only this population could be cultured *in vitro* for several passages whilst maintaining its oligodendrocyte differentiation potential (Wang et al., 2014). In line with the results of Wang et al. (2014) are various other reports from different groups demonstrating the oligodendrocyte-promoting

effects of Ascl1 in combination with Olig2 or Nkx2.2 (Sugimori et al., 2008), as well as Ascl1 (Braun et al., 2015), Olig1/2 (Balasubramaniyan et al., 2004; Copray et al., 2006; Sugimori et al., 2008; Hwang et al., 2009; Maire et al., 2009, 2010; Braun et al., 2015; Li et al., 2017), Nkx2.2 (Copray et al., 2006; Sugimori et al., 2008; Tochitani and Hayashizaki, 2008) and Sox10 (Braun et al., 2015; Ehrlich et al., 2017; García-León et al., 2018; Matjusaitis et al., 2019) alone. Interestingly, overexpression of Ascl1 *in vivo* has also been shown to coax endogenous hippocampal NPCs into an oligodendroglial, myelination-competent phenotype (Jessberger et al., 2008; Braun et al., 2015).

Ehrlich et al. (2017) demonstrated that overexpression of SOX10 alone can induce oligodendrocyte differentiation of cultured human iPSC-derived NPCs. Yet, oligodendrocyte derivation is more efficient when SOX10 is overexpressed in combination with OLIG2 and NKX6.2. With this improved protocol, around 60% to 80% of all cells stain positive for GALC and O4 at day 28 of differentiation and O4-enriched oligodendrocytes exhibit the capability to myelinate iPSC-derived neurons after 3 weeks of *in vitro* co-cultivation (Ehrlich et al., 2017). One year after the report of Ehrlich et al. (2017), also the group of Catherine Verfaillie published a protocol to derive oligodendrocytes from human PSC-derived NPCs by lentiviral SOX10 overexpression: García-León et al. (2018) performed RNA sequencing analysis of purified O4-positive cells at day 22 of differentiation, demonstrating that their protocol gives rise to oligodendrocytes that highly resemble intermediate to mature primary human brain-derived oligodendrocytes. Moreover, purified O4-positive oligodendrocytes were able to myelinate human iPSC-derived neurons after 20 days of co-culture. Finally, García-León et al. (2018) created a stable human ESC-line with doxycycline-inducible expression of SOX10 from the endogenous *AAVS1* locus and demonstrated that this approach successfully generates mature oligodendrocytes when transgene expression is induced at the NPC stage. Noteworthy, however, doxycycline-induced expression of SOX10 at the ESC stage was insufficient to give rise to MBP-expressing oligodendrocytes (García-León et al., 2018).

Whereas these studies have identified multiple routes to promote oligodendrocyte differentiation from an NPC stage, direct TF-driven specification of PSCs toward the oligodendrocyte lineage has remained more challenging to achieve. Li P. et al. (2016) reported that combining a multi-step growth factor-based differentiation protocol with SOX10 and OLIG2 overexpression in human iPSCs results in cultures consisting of around 40% O4-positive oligodendrocytes after 4 weeks of differentiation. However, when co-culturing SOX10/OLIG2-induced OPCs with embryonic primary rat cortical neurons, only around 5% of all cells stained positive for O4 at day 14 of co-culture and just 0.5% of all rat axons co-labeled with processes extending from human forward programmed oligodendrocytes (Li P. et al., 2016). One year later, the group of Mark Kotter published a highly controlled SOX10 and OLIG2-driven forward programming protocol for the derivation of oligodendrocytes from human PSCs, which is based on inducible transgene overexpression by dual genomic

safe harbor targeting (i.e., targeting the doxycycline-responsive transcriptional activator to the *ROSA26* and the tetracycline-responsive element-regulated transgenes of interest to the *AAVS1* locus). Interestingly, using this system, the authors could generate proliferative OPCs, which terminally differentiated into almost pure cultures of oligodendroglial cells expressing characteristic markers such as CNP and PLP upon mitogen withdrawal (Pawlowski et al., 2017).

It will be interesting to investigate whether TFs and TF combinations explored in the context of fibroblast-to-glia transdifferentiation can be exploited for forward programming of PSCs. For instance, direct cell fate conversion of rodent fibroblasts into OPCs was achieved by combined overexpression of the TFs Sox10 and Olig2 with either Nkx6.2 (Najm et al., 2013; Matjusaitis et al., 2019) or Zfp536 (Yang et al., 2013). Similarly, myelination-competent Schwann cells can be derived from mouse and human fibroblasts via the combined overexpression of Sox10 and Krox20 (Mazzara et al., 2017; Sowa et al., 2017) and have been shown to accelerate nerve regeneration and motor recovery after transplantation into mice with sciatic nerve transection (Sowa et al., 2017).

Taken together, the results of these studies underpin the potency of some TFs and/or TF combinations for oligodendrocyte specification. Yet, the efficient direct specification of oligodendrocytes form a PSC state deserves further attention. In addition to TFs, miRNAs could be supportive in this process. The miRNAs miR-219 and miR-338 were shown to promote oligodendrocyte maturation by targeting inhibitors of oligodendrogenesis such as Sox6 and Hes5, as well as promoters of neurogenesis as for instance Neurod1, Isl1, Otx2, and Zfp238/RP58 (Zhao et al., 2010).

# *IN VIVO* APPLICATION OF FORWARD PROGRAMMED CELLS: INSIGHTS FROM TRANSPLANTATION STUDIES

As a proof-of-concept for the general applicability of forward programmed cells for neuroregenerative approaches, these cells can be transplanted into healthy animals and monitored for graft survival, maturation and integration (**Figure 2**). Along this line, Zhang et al. (2013) demonstrated that 6 weeks after transplanting human immature neurons (7 days after infecting ESCs with a lentivirus encoding for NGN2) into the mouse striatum, the grafted cells adopted a neuronal phenotype exhibiting dendritic arborizations, axonal outgrowth and electrophysiological functionality, and received inhibitory synaptic input from host striatal interneurons (Zhang et al., 2013). Yuan et al. (2018) transplanted human PSC-derived NPCs, forward programmed by LHX6 overexpression, into the ventral mouse forebrain. Surprisingly, the authors observed that the number of GABAergic interneurons overall was not significantly increased in the doxycycline-induced, forward programmed grafts as compared to uninduced control transplants, and LHX6-overexpressing as well as uninduced control grafts differentiated into all four interneuron subtypes. Still, the forward programmed neurons exhibited spontaneous

**FIGURE 2 |** *In vivo* applications of cell types derived by forward programming. Numbers in superscript relate to references cited in **Tables 1**, **2**.
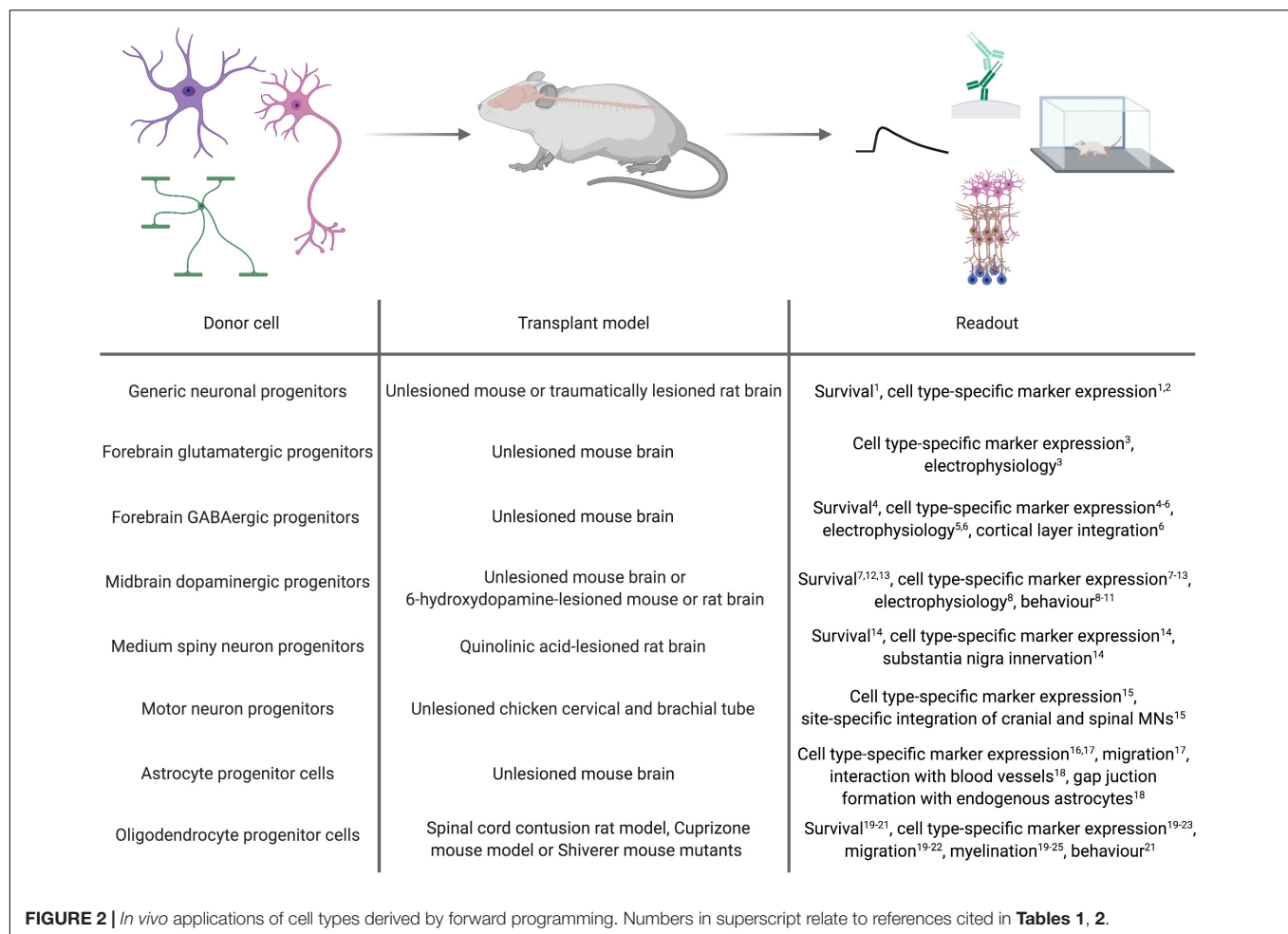
electrophysiological activity and postsynaptic currents reflecting mostly inhibitory GABAergic input (Yuan et al., 2018), demonstrating that forward programmed neurons can – in principal – exhibit proper functionality upon grafting. This notion is substantiated by reports of several other groups: For instance, 14-day-old neurons, derived from human PSCs by ASCL1- and DLX2-overexpression, survive transplantation into the subventricular zone and cerebral cortex of neonatal mice and mature into GABAergic neurons within 3 months post transplantation (Yang et al., 2017), and neurons derived from human PSCs by combined ASCL1, DLX2 and LHX6 overexpression mature into GABAergic neurons *in vivo*, too (Sun et al., 2016). Notably, 2 months after grafting, these GABAergic neurons had functionally integrated into cortical layers V and VI, exhibiting repetitive action potential firing and receiving synaptic input from host neurons (Sun et al., 2016). In an elegant study by the group of Hynek Wichterle, MNs were programmed by overexpressing Ngn2 and Isl1 in combination with either Lhx3 or Phox2a in mouse ESCs, and the resulting cells were grafted into the cervical and brachial tube of chicken embryos 2 days after transgene induction. Already 2 days after transplantation, the grafted cells had spatially segregated and exhibited axonal projections concordant with their MN subclass

identity: Like spinal MNs, Ngn2/Isl1/Lhx3-overexpressing cells accumulated in axial and limb nerve branches and exhibited substantial axonal outgrowth from the ventral root of the spinal cord, whereas Ngn2/Isl1/Phox2a-derived neurons accumulated in the lateral spinal cord and projected axons toward the spinal accessory nerve resembling cranial MNs (Mazzoni et al., 2013).

Whilst transplantation into unlesioned healthy recipients can be a highly useful tool to assess the *in vivo* differentiation and function of forward programmed neurons, studies in the context of a disease model can provide information on their regenerative capacity. First milestones to use forward programmed neurons for experimental neuroregeneration were already achieved as early as 2002, when Kim et al. (2002) transplanted Nurr1-overexpressing mouse ESC-derived neurons in the striatum of 6-OHDA-lesioned rats, an animal model of PD. 4 to 8 weeks post transplantation, the majority of transplanted cells expressed the dopaminergic marker TH, and 5 out of 6 grafts exhibited spontaneous postsynaptic currents. Most importantly, the authors demonstrated that animals transplanted with Nurr1-overexpressing neurons showed improved behavioral recovery compared to animals receiving sham injections or grafts of wild-type cells (Kim et al., 2002). Martinat et al. (2006) reported a

few years later that Nurr1/Pitx3-induced mouse and human ESC-derived NPCs grafted into the striatum of 6-OHDA-lesioned mice resulted in a significant reduction in apomorphine-induced rotation behavior compared to the transplantation of control vector-transduced cells. However, further immunohistochemical analyses of the grafts revealed that in their setting, neurons retained an immature morphology with only a minority of them expressing TH (Martinat et al., 2006). In accordance with this finding, Theka et al. (2013) more recently showed that 12 days after transplanting immature dopaminergic neurons (8 days post inducing ASCL1, NURR1 and LMX1A in human iPSCs) 4 out of 6 grafts survived, and only a fraction of the surviving cells displayed neuronal morphologies and expression of TH (Theka et al., 2013). Similar results were reported by Kim et al. (2003), who demonstrated that although transplantation of rat wild-type midbrain NPCs improves behavior of 6-OHDA-lesioned rats, transplantation of Nurr1-overexpressing midbrain or cortical NPCs does not, presumably because Nurr1-NPC grafts contained fewer TH-positive neurons which additionally exhibited immature morphologies (Kim et al., 2003). Whilst these findings were confirmed by Park et al. (2006), their study further revealed that 8 weeks after transplanting rat NPCs overexpressing a combination of Nurr1, Ascl1 and Shh or Nurr1, Bcl-XL and Shh, dopaminergic specification and dopamine levels are increased and motor deficits decreased compared to transplantation of NPCs overexpressing Nurr1 alone (Park et al., 2006). In a study by Friling et al. (2009) only 50% of all grafts survived after transplanting mouse Lmx1a-overexpressing ESC-derived NPCs into 6-OHDA-lesioned rats. In these grafts, the majority of the transplanted cells co-expressed the dopaminergic markers TH, Pitx3, En1/2, Lmx1a and Vmat, and even non-overlapping positivity for Girk2 and calbindin, indicating generation of both, substantia nigra A9 neurons and ventral tegmental area A10 dopaminergic neurons (Friling et al., 2009). Lastly, NPCs derived from ESCs via forward programming with Lmx1a differentiate into TH, DAT and GIRK2-expressing dopaminergic neurons *in vivo*, too (Sánchez-Danés et al., 2012).

In addition to PD, HD is intensely explored as a candidate disease for neural cell replacement. Since striatal MSNs are the main target of the disease, fast and efficient *in vitro* generation of MSNs is a key prerequisite for a cell therapeutic approach. Faedo et al. (2017) grafted human ESC-derived NPCs carrying inducible GSX2 and EBF1 transgenes into the quinolinic acid-lesioned striatum and observed that 2 months after transplantation, these NPCs had differentiated into GABAergic neurons expressing the striatal MSN markers CTIP2 and DARPP32 and extending projections toward the substantia nigra (Faedo et al., 2017). However, comparable to what has been observed after transplantation of forward programmed dopaminergic neurons (Theka et al., 2013), the number of CTIP2-positive human neurons was not different in GSX2/EBF1-overexpressing transplants versus uninduced control grafts (Faedo et al., 2017). It remains to be investigated whether or not MSNs directly forward programmed from the PSC stage would survive and integrate upon transplantation.

Forward programmed glial cells might be valuable for neuroregenerative interventions, too. For astrocytes, however,

there are only few published reports about the general feasibility of grafting these cells. Yet, these reports demonstrated that forward programmed astrocytes maintain their cellular identity up to 3 months after grafting (Li Y. et al., 2018a; Tchieu et al., 2019) and exhibit astrocyte-specific traits *in vivo* such as their affinity to blood vessels and the formation of functional gap junctions with host astrocytes (Canals et al., 2018). For oligodendrocytes, Copray et al. (2006) reported that Olig2-expressing mouse NPCs grafted into the demyelinated mouse striatum differentiate into mature MBP-positive oligodendrocytes engaging in remyelination (Copray et al., 2006). In line with this, Hwang et al. (2009) demonstrated that OLIG2-expressing NPCs survive transplantation into contused spinal cord better than wild-type NPCs and exhibit increased proliferation and migration into white matter tissue, where they efficiently differentiate into MBP-positive oligodendrocytes promoting myelination. Potentially due to this pro-myelinating effect, transplantation of OLIG2-NPCs improves locomotion after contusive injury compared to sham injection or transplantation of wild-type NPCs (Hwang et al., 2009). Also in Shiverer/Rag mice, which are used as models for myelination disorders, NPCs overexpressing OLIG2 (Maire et al., 2009) or SOX10 (Wang et al., 2014; Matjusaitis et al., 2019) have been shown to differentiate into MBP-positive oligodendrocytes exhibiting ensheathment of host axons. Transplantation of more mature O4-positive oligodendrocytes, which were derived from human NPCs by SOX10 overexpression, promotes myelination in brain slices of Shiverer/Rag mice as well (García-León et al., 2018). In an elegant study, Ehrlich et al. (2017). demonstrated that magnetic activated cell sorting (MACS)-purified O4-positive oligodendrocytes (14 days after induction of the TFs SOX10, OLIG2, and NKX6.2 in human NPCs) not only promote the formation of normally compacted MBP-positive sheaths with nodal structures around host neurons 16 weeks post transplantation but are also capable of remyelination after neurotransplantation in Shiverer/Rag mice treated with the membrane-dissolving chemical lysophosphatidyl-choline, which induces completely demyelinated lesions in white matter tissue (Ehrlich et al., 2017). However, since all of these transplantation studies were conducted with OPCs or oligodendrocytes derived by TF overexpression in NPCs, there is no proof so far that oligodendrocytes forward programmed from PSCs can survive neurotransplantation. This certainly merits further investigations, since OPCs directly converted from somatic fibroblasts were demonstrated to be capable of differentiating into myelinating oligodendroglial cells after transplantation into the brain of Shiverer/Rag mice, too (Najm et al., 2013; Yang et al., 2013).

## FORWARD PROGRAMMING OF PSCs VERSUS PRIMARY CELL FATE CONVERSION

Since forward programming of PSCs can be regarded as a fallout of the technological advances of somatic cell reprogramming into iPSC and direct interconversion of somatic cells within

and across germ layers, it is interesting to reflect on the commonalities and differences of these *in vitro* approaches. From a mechanistic point of view, direct cell fate conversion can be segregated into two different phases, which has been nicely deciphered in several milestone publications in the context of transdifferentiating fibroblasts into neurons via Ascl1, Brn2, and Myt1l overexpression (Vierbuchen et al., 2010; Wapinski et al., 2013; Treutlein et al., 2016; Mall et al., 2017). Here, the fibroblast's chromatin landscape has to be remodeled first in order to become permissive to TF binding at neuron-specific genes. This chromatin opening can be mediated by small molecules acting as epigenetic modifiers, or induced by cell type-specific pioneer TFs, which – by definition – are able to bind to and open up closed chromatin. Second, following the necessary epigenetic rearrangements, the transcriptional landscape has to be modulated in order to activate neuronal genes and inhibit the acquisition of alternative fates, including the repression of fibroblast-specific transcriptional signatures. Notably, many principles regulating the acquisition of a new cell fate during transdifferentiation seem to apply to forward programming, too. Aydin et al. (2019) recently analyzed how Ngn2 and Ascl1 specify mouse ESCs into neurons and demonstrated that both bHLH TFs act as neuronal pioneer TFs binding to genes which are in closed chromatin states in ESCs. By this, Ngn2 and Ascl1 induce and recruit secondary pro-neural TFs such as Brn2, thereby promoting the acquisition and stabilization of a neuronal fate. Interestingly, and in accordance with other publications, Aydin et al. (2019) further report that despite the high similarity of the mechanistic action of these two TFs, Ngn2 and Ascl1 induce quite distinct neuronal programs as the binding patterns of both TFs in ESCs are largely divergent.

Although the general principles underlying forward programming and direct cell fate conversion seem to be quite similar, there are some differences which need to be highlighted (**Table 3**). First, the epigenetic hurdles that have to be overcome for the proper activation of an alternative transcriptional program seem to be lower in PSCs than in terminally differentiated somatic cells (in particular in the case of a trans-germ layer conversion). Thus, although Ascl1, for instance, is sufficient to specify PSCs into neurons, it is comparably inefficient to convert fibroblasts into authentic iNs when overexpressed alone (Liu et al., 2018), and Ngns or Neurods, which are commonly used in forward programming paradigms, seem almost incapable of converting fibroblasts into iNs (Chanda et al., 2014; Liu et al., 2018). Second, from a time perspective, the derivation of neuronal cells from somatic cells is faster via the direct conversion route compared to forward programming via the iPSC stage: In this scenario, transdifferentiation is a one-step-procedure, whereas for forward programming, somatic cells have to be first reprogrammed into iPSCs before they can be differentiated into the desired somatic cell type by TF overexpression. However, due to the intermediate pluripotent stage, the forward programming route is scalable at the iPSC stage and can give rise to highly homogeneous cell batches. Notably, it is still not completely resolved whether forward programming of PSCs involves a stable NPC intermediate, since upregulation of NPC-associated

**TABLE 3 |** Comparative summary of key features important for cell fate engineering.

|  | Forward programming | Direct cell fate conversion |
| --- | --- | --- |
| *Epigenetic barriers for reprogramming* | Low | High |
| *Scalability* | High | Limited depending on proliferative potential of converted product |
| *Degree of standardization that can be reached* | High | Limited depending on proliferative potential of converted product |
| *Preservation of somatic and age memory* | Low | Potentially high |
| *Possible translation into clinical applications* | Indirect (via transplantation) | Direct and indirect (via *in situ* conversion and transplantation of converted cells) |

markers was reported to be very short-lasting (Zhang et al., 2013; Busskamp et al., 2014). Likewise, transient activation of an NPC-like transcriptional program was recently described in the context of direct pericyte-to-neuron conversion (Karow et al., 2018). Interestingly, however, single cell-RNA sequencing of neural cultures derived from human PSCs via NGN2 overexpression recently indicated that even after culturing these cells in neuronal differentiation-promoting medium on mouse glia, a significant fraction of cells can remain in an NPC-like stage resisting neuronal maturation (Nehme et al., 2018). Third, in contrast to forward programming from homogenous iPSC batches, every iN represents a single, post-mitotic direct cell fate conversion event. This means that transdifferentiation-derived cultures represent a mosaic of a vast number of single conversion events, which severely limits the degree of standardization that can be reached with an iN approach. Furthermore, since neurons are post-mitotic, the cell yield of an iN conversion is always limited by (i) the number of starting cells and (ii) the efficiency of the direct conversion approach. However, these drawbacks do not equally apply to all transdifferentiation paradigms. Instead of deriving terminally differentiated iNs, direct cell fate conversion can be used to generate induced neural stem cells (iNSCs; Han et al., 2012; Ring et al., 2012; Shahbazi et al., 2016; Sheng et al., 2018) induced neuronal-like NPCs (iNPCs; Giorgetti et al., 2012), or even iNPCs which are already primed to differentiate toward a specific neuronal subtype such as dopaminergic neurons (Tian et al., 2015). As these cells are stably self-renewing, especially iNSC cultures are almost as scalable and homogeneous as PSCs. Yet, direct conversion into iNSC/iNPCs has the drawback that, as with forward programming, it has to be followed up by a subsequent terminal differentiation step. Here, it is tempting to consider translating previous approaches on facilitating the differentiation of primary or PSC-derived NPCs by TF overexpression to iNSCs/iNPCs. A key issue in all these scenarios remains cell type authenticity, which can be compromised if the converted cells retain a significant degree of epigenetic and transcriptomic memory relating to the fate but also the age of the cell of origin. This memory seems to be largely maintained in fibroblast-derived iNs of different donor ages (Mertens et al., 2015; Yang et al., 2015; Huh et al., 2016; Tang et al., 2017; Kim et al., 2018), whereas it is almost completely reset in iPSCs

(Polo et al., 2010; Lo Sardo et al., 2017; Olova et al., 2019). Recently, directly converted iNSCs were shown to largely reset age-associated cellular signatures, too (Sheng et al., 2018).

## Prospects of Direct Cell Fate Conversion *in vivo*

Direct cell fate conversion cannot only be achieved *in vitro* but also directly *in vivo*. While this concept is distinct from classic forward programming and has received its own coverage in several recent reviews (Heinrich et al., 2015; Grealish et al., 2016; Srivastava and DeWitt, 2016; Barker et al., 2018; Flitsch and Brüstle, 2019; Pesaresi et al., 2019), it might eventually provide a short-cut for brain repair bypassing a transplantation step and is thus worth mentioning here. Several studies have shown that such a transdifferentiation step can be triggered *in vivo* by the direct administration of reprogramming cues like TFs and/or miRNAs to resident rodent brain cells such as astrocytes (Buffo et al., 2005; Kronenberg et al., 2010; Guo et al., 2014; Faiz et al., 2015; Ghasemi-Kasman et al., 2015; Liu et al., 2015; Torper et al., 2015; Gascón et al., 2016) and NG2-positive glial cells (Guo et al., 2014; Heinrich et al., 2014; Torper et al., 2015; Gascón et al., 2016). In this context, the group of Ernest Arenas demonstrated that even the *in vivo* conversion of mouse astrocytes into clinically relevant neuronal subtypes such as midbrain dopaminergic neurons is feasible. The authors overexpressed a TF cocktail comprising Ascl1, Lmx1a, Neurod1, and miRNA-218 in mouse brain astrocytes by stereotactic lentivirus injection and revealed that this does not only successfully elicit transdifferentiation but finally also corrects basal and postsynaptic deficits in dopamine transmission and improves spontaneous motor behavior deficits in 6-OHDA-lesioned PD mice (Rivetti Di Val Cervo et al., 2017). Importantly, the group of Magdalena Götz recently revealed that *in vivo* conversion of mouse cortical astrocytes into neurons can preserve region-and even layer-specific identities (Mattugini et al., 2019). This study also impressively underpins the relevance and potential impact of subspecification within the glial lineage. Lastly, it has recently been shown that *in vivo* cell fate conversion can even be extended beyond germ layer boundaries: Matsuda et al. (2019) reported that brain-resident microglia, which originate from the yolk sac, are amenable to neuronal conversion in the mouse brain (Matsuda et al., 2019). A special variant of the *in vivo* conversion concept is the idea to transplant somatic cells that are already engineered to overexpress specific TFs upon an inducing stimulus and can thus be activated to convert *in situ* (Torper et al., 2013). Taken together, these reports underline the enormous biomedical potential of both, direct *in vitro* and *in vivo* conversion of somatic cells.

## CHALLENGES FOR FUTURE CLINICAL APPLICATION

### Cell Type Subspecification, Authenticity, and Maturity

Forward programming comes with the significant benefit of being fast enough to be able to provide autologous cells from human patients for cell replacement therapies. However, on the benchwork side, one significant limitation of some currently available forward programming protocols is the reduced purity of the obtained cultures, especially when it comes to deriving highly specified neuronal subtypes. In some cases, subspecification might be augmented by the use of morphogens and small molecules. On the one hand, these molecules can help to properly regionalize (intermediate) NPC stages, as they might provide additional phenotype-instructing differentiation cues, which could otherwise only be delivered by combining several TFs upstream of the lineage-relevant signaling pathways. This was nicely exemplified by the group of Johan Ericson and Thomas Perlmann, who showed that overexpression of Lmx1a in ESC-derived mouse NPCs suffices to instruct dopaminergic neuron differentiation when combined with Shh and Fgf8 treatment, whereas caudalized NPCs only adopt a dopaminergic phenotype if the three midbrain-associated TFs Lmx1a, En1, and Otx2 are overexpressed in combination (Panman et al., 2011). On the other hand, small molecules might act as epigenetic modifiers and facilitate forward programming by either re-activating lineage-instructive genes, which are in an unfavorable epigenetic state or even completely silenced in PSCs, or by repressing alternative lineages (similar to the mode of action that has been described for the TF EZH2; Liu et al., 2018). In direct cell fate conversion, for instance, iN derivation from human fibroblasts via NGN2 overexpression is only successful if combined with the treatment with two small molecules, namely forskolin and dorsomorphin, which modulate chromatin accessibility at NGN2 target sites (Liu et al., 2013; Smith et al., 2016).

Another challenge for the clinical application of forward programmed cells might be the degree of cellular authenticity and maturity that can be achieved. One way to improve neuronal maturation *in vitro* is to co-culture forward programmed neurons with glial cells. Notably, the species from which the glial cells are retrieved might influence their maturation-promoting effect: In a recent study, culturing human glutamatergic neurons (differentiated via a classical morphogen-based approach) on mouse astrocytes was found to be superior to co-culture with rat astrocytes, whereas co-culture with human astrocytes did not support neuronal survival beyond 4 weeks. Interestingly, GABAergic neurons did not show this selective response to glial co-culture (Rhee et al., 2019), and whether or not these effects also apply to forward programmed neurons is still to be determined.

Further studies are also required in order to clarify to what extent forward programmed neurons resemble their physiological *in vivo* counterparts. For example, in a recent study 4-week-old NGN2-forward programmed human neurons grown in an autapse setting displayed surprising morphological and functional properties, including systematic multi-peak excitatory postsynaptic currents originating from neurons possessing multiple axons, which were evident in about 25% of all cells analyzed (Meijer et al., 2019; Rhee et al., 2019). Increased axonal lengths and small diameters contributed to the observed phenomenon, and further resulted in extended synaptic delays as compared to mouse forebrain cortical neurons. Moreover, detection of a slow component contributing to these unusual

electrophysiological kinetics indicated that the NGN2-neurons possess the ability to co-release GABA and glutamate from the same synapse (Rhee et al., 2019). Although there are neurons *in vivo*, which grow multiple axons and/or co-release different neurotransmitters from the same synapse, these findings deserve further attention.

## Transgene Delivery, Stability of Programmed Phenotypes and Safety

From a translational perspective, extrinsic factor-driven protocols, such as the delivery of differentiation cues by growth factors, morphogens and/or small molecules but also TF-based approaches employing mRNAs or proteins rather than integrating constructs might be easier to pass regulatory hurdles associated with the implementation of clinical trials. So far, however, most TF-based approaches have been relying on genetic modification of the target cell either by the use of integrating viruses or gene editing techniques such as CRISPR/Cas9. A main advantage of integrating a fate-instructing TF by techniques such as TALENs or CRISPR/Cas9 is better control on the integration site. Accordingly, some studies successfully integrated the forward programming-conveying TFs in the human genomic safe harbor *AAVS1* locus with high precision (Wang et al., 2017; García-León et al., 2018; Li Y. et al., 2018a; Meijer et al., 2019; Rhee et al., 2019). While integration-free methods such as mRNA (Goparaju et al., 2017; Matsushita et al., 2017; Xue et al., 2019) or protein delivery (Robinson et al., 2016) are appealing, they come with their own pros and cons. For instance, mRNAs are rapidly translated but subsequently also timely degraded after entering the target cells, and significant protocol adaptations might be necessary to enable efficient transfection at all (Xue et al., 2019). Protein delivery, on the other hand, is technically challenging but also the only technique that circumvents potential post-translational regulation (Robinson et al., 2016).

Another challenge is the maintenance of inducible transgene activation in the grafted cells until the point where the cellular phenotype of the transplanted cells becomes stable and transgene-independent. In principle, this could be tackled by grafting cells at later stages of *in vitro* specification. However, advanced pre-differentiation is typically associated with decreased survival and integration of the grafted cells. Thus, it might be beneficial to implement modalities for continuous delivery of TFs, e.g., by repetitive virus injection or slow-release depots in form of scaffolds binding or encapsulating fate-specifying proteins such as TFs and/or morphogens (see review by Bruggeman et al., 2019). Prolonged provision of fate-specifying factors beyond the timepoint of transplantation might also enhance the *in vivo* stability of neuronal subtype identities: Although there are TF-based protocols available to produce quite specific neuronal subtypes *in vitro*, the results of several studies suggest that TF-mediated acquisition and maintenance of subtype specification might be less efficient in transplanted neurons compared to a pure *in vitro* scenario

(Martinat et al., 2006; Theka et al., 2013; Faedo et al., 2017; Yuan et al., 2018).

Another issue to be tackled when it comes to clinical transplantation is the fact that although grafting NPCs is more efficient than transplantation of terminally differentiated mature cells – a notion particularly relevant for neurons (for a more comprehensive commentary, see Björklund and Lindvall, 2000) – transplantation of still immature cells such as differentiating PSCs and NPCs can increase the risk of uncontrolled overgrowth (Friling et al., 2009). While growth factor-based protocols might be more vulnerable to this complication, teratoma formation after transplantation has also been observed in the context of TF overexpression paradigms (Martinat et al., 2006; Friling et al., 2009).

## CONCLUDING REMARKS

TF overexpression in NPCs and TF-based forward programming of PSCs are valuable techniques to derive specialized and comparably mature neural cells within short time frames and thus provide powerful alternatives to classic growth factor-mediated PSC differentiation and direct (somatic) cell fate conversion. Besides generating precious insights into how cell fates are established and controlled by transcriptional and epigenetic regulation, one major asset of these approaches is that they can provide new donor sources for brain repair. Yet, a number of issues need to be addressed more deeply before forward programming can be implemented in a clinical setting.

## AUTHOR CONTRIBUTIONS

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# REFERENCES

Abasi, M., Massumi, M., Riazi, G., and Amini, H. (2012). The synergistic effect of beta-boswellic acid and Nurr1 overexpression on dopaminergic programming of antioxidant glutathione peroxidase-1-expressing murine embryonic stem cells. *Neuroscience* 222, 404–416. doi: 10.1016/j.neuroscience.2012.07.009

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., et al. (2006). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* 124, 393–405. doi: 10.1016/j.cell.2005.10.037

Aydin, B., Kakumanu, A., Rossillo, M., Moreno-Estellés, M., Garipler, G., Ringstad, N., et al. (2019). Proneural factors Ascl1 and Neurog2 contribute to neuronal subtype identities by establishing distinct chromatin landscapes. *Nat. Neurosci.* 22, 897–908. doi: 10.1038/s41593-019-0399-y

Azimi, S. M., Sheridan, S. D., Ghannad-Rezaie, M., Eimon, P. M., and Yanik, M. F. (2018). Combinatorial programming of human neuronal progenitors using magnetically-guided stoichiometric mRNA delivery. *eLife* 7:e31922. doi: 10.7554/eLife.31922

Bae, E. J., Lee, H. S., Park, C. H., and Lee, S. H. (2009). Orphan nuclear receptor Nurr1 induces neuron differentiation from embryonic cortical precursor cells via an extrinsic paracrine mechanism. *FEBS Lett.* 583, 1505–1510. doi: 10.1016/j.febslet.2009.04.004

Balasubramaniyan, V., Timmer, N., Kust, B., Boddeke, E., and Copray, S. (2004). Transient expression of Olig1 initiates the differentiation of neural stem cells into oligodendrocyte progenitor cells. *Stem Cells* 2, 812–822. doi: 10.1634/stemcells.22-6-878

Barker, R. A., Götz, M., and Parmar, M. (2018). New approaches for brain repair - from rescue to reprogramming. *Nature* 557, 329–334. doi: 10.1038/s41586-018-0087-1

Barretto, N., Zhang, H., Powell, S. K., Fernando, M. B., Zhang, S., Flaherty, E. K., et al. (2020). ASCL1- and DLX2-induced GABAergic neurons from hiPSC-derived NPCs. *J. Neurosci. Methods* 334:108548. doi: 10.1016/j.jneumeth.2019.108548

Berninger, B., Costa, M. R., Koch, U., Schroeder, T., Sutor, B., Grothe, B., et al. (2007). Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. *J. Neurosci.* 27, 8654–8664. doi: 10.1523/JNEUROSCI.1615-07.2007

Björklund, A., and Lindvall, O. (2000). Cell replacement therapies for central nervous system disorders. *Nat. Neurosci.* 3, 537–544. doi: 10.1038/75705

Bolós, M., Hu, Y., Young, K. M., Foa, L., and Small, D. H. (2014). Neurogenin 2 mediates amyloid-β precursor protein-stimulated neurogenesis. *J. Biol. Chem.* 289, 31253–31261. doi: 10.1074/jbc.M114.581918

Braccioli, L., Vervoort, S. J., Puma, G., Nijboer, C. H., and Coffer, P. J. (2018). SOX4 inhibits oligodendrocyte differentiation of embryonic neural stem cells in vitro by inducing Hes5 expression. *Stem Cell Res.* 33, 110–119. doi: 10.1016/j.scr.2018.10.005

Braun, S. M. G., Pilz, G. A., Machado, R. A. C., Moss, J., Becher, B., Toni, N., et al. (2015). Programming hippocampal neural stem/progenitor cells into oligodendrocytes enhances remyelination in the adult brain after injury. *Cell Rep.* 11, 1679–1685. doi: 10.1016/j.celrep.2015.05.024

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* 11, 43–49. doi: 10.1016/s0959-4388(00)00172-0

Bruggeman, K. F., Moriarty, N., Dowd, E., Nisbet, D. R., and Parish, C. L. (2019). Harnessing stem cells and biomaterials to promote neural repair. *Br. J. Pharmacol.* 176, 355–368. doi: 10.1111/bph.14545

Brunet, J. F., and Pattyn, A. (2002). Phox2 genes - from patterning to connectivity. *Curr. Opin. Genet. Dev.* 12, 435–440. doi: 10.1016/s0959-437x(02)00322-2

Buffo, A., Vosko, M. R., Ertürk, D., Hamann, G. F., Jucker, M., Rowitch, D., et al. (2005). Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18183–18188. doi: 10.1073/pnas.0506535102

Busskamp, V., Lewis, N. E., Guye, P., Ng, A. H., Shipman, S. L., Byrne, S. M., et al. (2014). Rapid neurogenesis through transcriptional activation in human stem cells. *Mol. Syst. Biol.* 10:760. doi: 10.15252/msb.20145508

Canals, I., Ginisty, A., Quist, E., Timmerman, R., Fritze, J., Miskinyte, G., et al. (2018). Rapid and efficient induction of functional astrocytes from human pluripotent stem cells. *Nat. Methods* 15, 693–696. doi: 10.1038/s41592-018-0103-2

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* 126, 525–534. doi: 10.1371/journal.pcbi.0020117

Chanda, S., Ang, C. E., Davila, J., Pak, C., Mall, M., Lee, Q. Y., et al. (2014). Generation of induced neuronal cells by the single reprogramming factor ASCL1. *Stem Cell Rep.* 3, 282–296. doi: 10.1016/j.stemcr.2014.05.020

Chung, S., Sonntag, K. C., Andersson, T., Bjorklund, L. M., Park, J. J., Kim, D. W., et al. (2002). Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. *Eur. J. Neurosci.* 16, 1829–1838. doi: 10.1046/j.1460-9568.2002.02255.x

Colasante, G., Lignani, G., Rubio, A., Medrihan, L., Yekhlef, L., Sessa, A., et al. (2015). Rapid conversion of fibroblasts into functional forebrain GABAergic interneurons by direct genetic reprogramming. *Cell Stem Cell* 17, 719–734. doi: 10.1016/j.stem.2015.09.002

Coppola, E., Pattyn, A., Guthrie, S. C., Goridis, C., and Studer, M. (2005). Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. *EMBO J.* 24, 4392–4403. doi: 10.1038/sj.emboj.7600897

Copray, S., Balasubramaniyan, V., Levenga, J., de Bruijn, J., Liem, R., and Boddeke, E. (2006). Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. *Stem Cells* 24, 1001–1010. doi: 10.1634/stemcells.2005-0239

Correa-Cerro, L. S., Piao, Y., Sharov, A. A., Nishiyama, A., Cadet, J. S., Yu, H., et al. (2011). Generation of mouse ES cell lines engineered for the forced induction of transcription factors. *Sci. Rep.* 1:167. doi: 10.1038/srep00167

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51, 987–1000. doi: 10.1016/0092-8674(87)90585-X

De Santis, R., Garone, M. G., Pagani, F., de Turris, V., Di Angelantonio, S., and Rosa, A. (2018). Direct conversion of human pluripotent stem cells into cranial motor neurons using a piggyBac vector. *Stem Cell Res.* 29, 189–196. doi: 10.1016/j.scr.2018.04.012

Dennis, D. J., Han, S., and Schuurmans, C. (2019). bHLH transcription factors in neural development, disease, and reprogramming. *Brain Res.* 1705, 48–65. doi: 10.1016/j.brainres.2018.03.013

DeWald, L. E., Rodriguez, J. P., and Levine, J. M. (2011). The RE1 binding protein REST regulates oligodendrocyte differentiation. *J. Neurosci.* 31, 3470–3483. doi: 10.1523/JNEUROSCI.2768-10.2011

Ehrlich, M., Mozafari, S., Glatza, M., Starost, L., Velychko, S., Hallmann, A. L., et al. (2017). Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 114, E2243–E2252. doi: 10.1073/pnas.1614412114

Faedo, A., Laporta, A., Segnali, A., Galimberti, M., Besusso, D., Cesana, E., et al. (2017). Differentiation of human telencephalic progenitor cells into MSNs by inducible expression of Gsx2 and Ebf1. *Proc. Natl. Acad. Sci. U.S.A.* 114, E1234–E1242. doi: 10.1073/pnas.1611473114

Faiz, M., Sachewsky, N., Gascón, S., Bang, K. W. A., Morshead, C. M., and Nagy, A. (2015). Adult neural stem cells from the subventricular zone give rise to reactive astrocytes in the cortex after stroke. *Cell Stem Cell* 17, 624–634. doi: 10.1016/j.stem.2015.08.002

Falcone, C., Filippis, C., Granzotto, M., and Mallamaci, A. (2015). Emx2 expression levels in NSCs modulate astrogenesis rates by regulating EgfR and Fgf9. *Glia* 63, 412–422. doi: 10.1002/glia.22761

Flitsch, L. J., and Brüstle, O. (2019). Evolving principles underlying neural lineage conversion and their relevance for biomedical translation. *F1000Res.* 8:1548. doi: 10.12688/f1000research.18926.1

Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. J., and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* 14, 67–80. doi: 10.1101/gad.14.1.67

Frega, M., van Gestel, S. H. C., Linda, K., van der Raadt, J., Keller, J., Van Rhijn, J. R., et al. (2017). Rapid neuronal differentiation of induced pluripotent stem cells for measuring network activity on micro-electrode arrays. *J. Vis. Exp.* 119:54900. doi: 10.3791/54900

Friling, S., Andersson, E., Thompson, L. H., Jönsson, M. E., Hebsgaard, J. B., Nanou, E., et al. (2009). Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7613–7618. doi: 10.1073/pnas.0902396106

García-León, J. A., Kumar, M., Boon, R., Chau, D., One, J., Wolfs, E., et al. (2018). SOX10 single transcription factor-based fast and efficient generation

of oligodendrocytes from human pluripotent stem cells. *Stem Cell Rep.* 10, 655–672. doi: 10.1016/j.stemcr.2017.12.014

Gascón, S., Murenu, E., Masserdotti, G., Ortega, F., Russo, G. L., Petrik, D., et al. (2016). Identification and successful negotiation of a metabolic checkpoint in direct neuronal reprogramming. *Cell Stem Cell* 18, 396–409. doi: 10.1016/j.stem. 2015.12.003

Gennet, N., Gale, E., Nan, X., Farley, E., Takacs, K., Oberwallner, B., et al. (2011). Doublesex and mab-3-related transcription factor 5 promotes midbrain dopaminergic identity in pluripotent stem cells by enforcing a ventral-medial progenitor fate. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9131–9136. doi: 10.1073/pnas. 1016679108

Geoffroy, C. G., Critchley, J. A., Castro, D. S., Ramelli, S., Barraclough, C., Descombes, P., et al. (2009). Engineering of dominant active basic helix-loop-helix proteins that are resistant to negative regulation by postnatal central nervous system antineurogenic cues. *Stem Cells* 27, 847–856. doi: 10.1002/ stem.17

Ghasemi-Kasman, M., Hajikaram, M., Baharvand, H., and Javan, M. (2015). MicroRNA-mediated in vitro and in vivo direct conversion of astrocytes to neuroblasts. *PLoS One* 10:e0127878. doi: 10.1371/journal.pone.0127878

Giorgetti, A., Marchetto, M. C. N., Li, M., Yu, D., Fazzina, R., Mu, Y., et al. (2012). Cord blood-derived neuronal cells by ectopic expression of Sox2 and c-Myc. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12556–12561. doi: 10.1073/pnas.1209523109

Goparaju, S. K., Kohda, K., Ibata, K., Soma, A., Nakatake, Y., Akiyama, T., et al. (2017). Rapid differentiation of human pluripotent stem cells into functional neurons by mRNAs encoding transcription factors. *Sci. Rep.* 7:42367. doi: 10. 1038/srep42367

Goto, K., Imamura, K., Komatsu, K., Mitani, K., Aiba, K., Nakatsuji, N., et al. (2017). Simple derivation of spinal motor neurons from ESCs/iPSCs using Sendai virus vectors. *Mol. Ther. Methods Clin. Dev.* 4, 115–125. doi: 10.1016/ j.omtm.2016.12.007

Grealish, S., Drouin-Ouellet, J., and Parmar, M. (2016). Brain repair and reprogramming: the route to clinical translation. *J. Intern. Med.* 280, 265–275. doi: 10.1111/joim.12475

Greenway, D. J., Street, M., Jeffries, A., and Buckley, N. J. (2007). RE1 silencing transcription factor maintains a repressive chromatin environment in embryonic hippocampal neural stem cells. *Stem Cells* 25, 354–363. doi: 10.1634/stemcells.2006-0207

Guo, Z., Zhang, L., Wu, Z., Chen, Y., Wang, F., and Chen, G. (2014). In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. *Cell Stem Cell* 14, 188–202. doi: 10.1016/j.stem.2013.12.001

Hack, M. A., Sugimori, M., Lundberg, C., Nakafuku, M., and Götz, M. (2004). Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol. Cell. Neurosci.* 25, 664–678. doi: 10.1016/j.mcn.2003.12.012

Han, D. W., Tapia, N., Hermann, A., Hemmer, K., Höing, S., Araúzo-Bravo, M. J., et al. (2012). Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* 10, 465–472. doi: 10.1016/j.stem.2012.02.021

Heinrich, C., Bergami, M., Gascón, S., Lepier, A., Viganò, F., Dimou, L., et al. (2014). Sox2-mediated conversion of NG2 glia into induced neurons in the injured adult cerebral cortex. *Stem Cell Rep.* 3, 1000–1014. doi: 10.1016/j.stemcr. 2014.10.007

Heinrich, C., Spagnoli, F. M., and Berninger, B. (2015). In vivo reprogramming for tissue repair. *Nat. Cell Biol.* 17, 204–211. doi: 10.1038/ncb3108

Heins, N., Malatesta, P., Cecconi, F., Nakafuku, M., Tucker, K. L., Hack, M. A., et al. (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat. Neurosci.* 5, 308–315. doi: 10.1038/nn828

Hester, M. E., Murtha, M. J., Song, S., Rao, M., Miranda, C. J., Meyer, K., et al. (2011). Rapid and efficient generation of functional motor neurons from human pluripotent stem cells using gene delivered transcription factor codes. *Mol. Ther.* 19, 1905–1912. doi: 10.1038/mt.2011.135

Ho, S. M., Hartley, B. J., Tcw, J., Beaumont, M., Stafford, K., Slesinger, P. A., et al. (2016). Rapid Ngn2-induction of excitatory neurons from hiPSC-derived neural progenitor cells. *Methods* 101, 113–124. doi: 10.1016/j.ymeth.2015. 11.019

Hsieh, J., Nakashima, K., Kuwabara, T., Mejia, E., and Gage, F. H. (2004). Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16659–16664. doi: 10.1073/pnas.0407643101

Huang, Z., Wu, Q., Guryanova, O. A., Cheng, L., Shou, W., Rich, J. N., et al. (2011). Deubiquitinase HAUSP stabilizes REST and promotes maintenance of neural progenitor cells. *Nat. Cell Biol.* 13, 142–152. doi: 10.1038/ncb2153

Huh, C. J., Zhang, B., Victor, M. B., Dahiya, S., Batista, L. F. Z., Horvath, S., et al. (2016). Maintenance of age in human neurons generated by microRNA-based neuronal conversion of fibroblasts. *eLife* 5:e18648. doi: 10.7554/eLife.18648

Hwang, D. H., Kim, B. G., Kim, E. J., Lee, S. I., Joo, I. S., Suh-Kim, H., et al. (2009). Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. *BMC Neurosci.* 10:117. doi: 10.1186/1471-2202-10-117

Jessberger, S., Toni, N., Clemenson, G. D., Ray, J., and Gage, F. H. (2008). Directed differentiation of hippocampal stem/progenitor cells in the adult brain. *Nat. Neurosci.* 11, 888–893. doi: 10.1038/nn.2148

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* 1, 20–29. doi: 10.1038/35049541

Jiang, Y., Yan, L., Xia, L., Lu, X., Zhu, W., Ding, D., et al. (2018). Zinc finger E-box–binding homeobox 1 (ZEB1) is required for neural differentiation of human embryonic stem cells. *J. Biol. Chem.* 293, 19317–19329. doi: 10.1074/jbc.RA118. 005498

Kan, L., Israsena, N., Zhang, Z., Hu, M., Zhao, L. R., Jalali, A., et al. (2004). Sox1 acts through multiple independent pathways to promote neurogenesis. *Dev. Biol.* 269, 580–594. doi: 10.1016/j.ydbio.2004.02.005

Karow, M., Camp, J. G., Falk, S., Gerber, T., Pataskar, A., Gac-Santel, M., et al. (2018). Direct pericyte-to-neuron reprogramming via unfolding of a neural stem cell-like program. *Nat. Neurosci.* 21, 932–940. doi: 10.1038/s41593-018-0168-3

Karow, M., Sánchez, R., Schichor, C., Masserdotti, G., Ortega, F., Heinrich, C., et al. (2012). Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells. *Cell Stem Cell* 11, 471–476. doi: 10.1016/j.stem.2012. 07.007

Kee, N., Volakakis, N., Kirkeby, A., Dahl, L., Storvall, H., Nolbrant, S., et al. (2017). Single-cell analysis reveals a close relationship between differentiating dopamine and subthalamic nucleus neuronal lineages. *Cell Stem Cell* 20, 29–40. doi: 10.1016/j.stem.2016.10.003

Kim, H.-J., McMillan, E., Han, F., and Svendsen, C. N. (2009). Regionally specified human neural progenitor cells derived from the mesencephalon and forebrain undergo increased neurogenesis following overexpression of ASCL1. *Stem Cells* 27, 390–398. doi: 10.1634/stemcells.2007-1047

Kim, J. H., Auerbach, J. M., Rodríguez-Gómez, J. A., Velasco, I., Gavin, D., Lumelsky, N., et al. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418, 50–56. doi: 10.1038/nature00900

Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., et al. (2003). Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *J. Neurochem.* 85, 1443–1454. doi: 10. 1046/j.1471-4159.2003.01780.x

Kim, Y., Zheng, X., Ansari, Z., Bunnell, M. C., Herdy, J. R., Traxler, L., et al. (2018). Mitochondrial aging defects emerge in directly reprogrammed human neurons due to their metabolic profile. *Cell Rep.* 23, 2550–2558. doi: 10.1016/j.celrep. 2018.04.105

Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., et al. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. *Cell Rep.* 1, 703–714. doi: 10.1016/j.celrep.2012.04.009

Kirkeby, A., Nolbrant, S., Tiklova, K., Heuer, A., Kee, N., Cardoso, T., et al. (2017). Predictive markers guide differentiation to improve graft outcome in clinical translation of hESC-based therapy for Parkinson's disease. *Cell Stem Cell* 20, 135–148. doi: 10.1016/j.stem.2016.09.004

Kittappa, R., Chang, W. W., Awatramani, R. B., and McKay, R. D. G. (2007). The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. *PLoS Biol.* 5:e325. doi: 10.1371/journal.pbio.0050325

Krencik, R., and Zhang, S.-C. (2011). Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. *Nat. Protoc.* 6, 1710–1717. doi: 10.1038/jid.2014.371

Kriks, S., Shim, J. W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480, 547–551. doi: 10.1038/nature10648

Kronenberg, G., Gertz, K., Cheung, G., Buffo, A., Kettenmann, H., Götz, M., et al. (2010). Modulation of fate determinants Olig2 and Pax6 in resident glia evokes spiking neuroblasts in a model of mild brain ischemia. *Stroke* 41, 2944–2949. doi: 10.1161/STROKEAHA.110.583039

Kuhn, S., Gritti, L., Crooks, D., and Dombrowski, Y. (2019). Oligodendrocytes in development, myelin generation and beyond. *Cells* 8:E1424. doi: 10.3390/cells8111424

La Manno, G., Gyllborg, D., Codeluppi, S., Nishimura, K., Salto, C., Zeisel, A., et al. (2016). Molecular diversity of midbrain development in mouse, human, and stem cells. *Cell* 167, 566–580.e19. doi: 10.1016/j.cell.2016.09.027

Li, P., Li, M., Tang, X., Wang, S., Zhang, Y. A., and Chen, Z. (2016). Accelerated generation of oligodendrocyte progenitor cells from human induced pluripotent stem cells by forced expression of Sox10 and Olig2. *Sci. China Life Sci.* 59, 1131–1138. doi: 10.1007/s11427-016-0165-3

Li, X., Tao, Y., Bradley, R., Du, Z., Tao, Y., Kong, L., et al. (2018). Fast generation of functional subtype astrocytes from human pluripotent stem cells. *Stem Cell Rep.* 11, 998–1008. doi: 10.1016/j.stemcr.2018.08.019

Li, X., Tzeng, S. Y., Liu, X., Tammia, M., Cheng, Y.-H., Rolfe, A., et al. (2016). Nanoparticle-mediated transcriptional modifications enhances neuronal differentiation of human neural stem cells following transplantation in rat brain. *Biomaterials* 84, 157–166. doi: 10.1016/j.physbeh.2017.03.040

Li, Y., Guo, B., Yang, R., Xiao, Z., Gao, X., Yu, J., et al. (2018). A novel long noncoding RNA lnc158 promotes the differentiation of mouse neural precursor cells into oligodendrocytes by targeting nuclear factor-IB. *Neuroreport* 29, 1121–1128. doi: 10.1097/WNR.0000000000001083

Li, X., Tzeng, S. Y., Zamboni, C. G., Koliatsos, V. E., Ming, G., Green, J. J., et al. (2017). Enhancing oligodendrocyte differentiation by transient transcription activation via DNA nanoparticle-mediated transfection. *Acta Biomater.* 54, 249–258. doi: 10.1016/j.actbio.2017.03.032

Liang, Q., Xia, W., Li, W., and Jiao, J. (2018). RNF20 controls astrocytic differentiation through epigenetic regulation of STAT3 in the developing brain. *Cell Death Differ.* 25, 294–306. doi: 10.1038/cdd.2017.157

Liu, M. L., Zang, T., Zou, Y., Chang, J. C., Gibson, J. R., Huber, K. M., et al. (2013). Small molecules enable neurogenin 2 to efficiently convert human fibroblasts into cholinergic neurons. *Nat. Commun.* 4:2183. doi: 10.1038/ncomms3183

Liu, Y., Miao, Q., Yuan, J., Han, S., Zhang, P., Li, S., et al. (2015). Ascl1 converts dorsal midbrain astrocytes into functional neurons in vivo. *J. Neurosci.* 35, 9336–9355. doi: 10.1523/JNEUROSCI.3975-14.2015

Liu, Y., Yu, C., Daley, T. P., Wang, F., Cao, W. S., Bhate, S., et al. (2018). CRISPR activation screens systematically identify factors that drive neuronal fate and reprogramming. *Cell Stem Cell* 23, 758–771. doi: 10.1016/j.stem.2018.09.003

Lo Sardo, V., Ferguson, W., Erikson, G. A., Topol, E. J., Baldwin, K. K., and Torkamani, A. (2017). Influence of donor age on induced pluripotent stem cells. *Nat. Biotechnol.* 35, 69–74. doi: 10.1038/nbt.3749

Maire, C. L., Buchet, D., Kerninon, C., Deboux, C., Evercooren, A. B. V., and Nait-Oumesmar, B. (2009). Directing human neural stem/precursor cells into oligodendrocytes by overexpression of Olig2 transcription factor. *J. Neurosci. Res.* 87, 3438–3446. doi: 10.1002/jnr.22194

Maire, C. L., Wegener, A., Kerninon, C., and Oumesmar, B. N. (2010). Gain-of-function of Olig transcription factors enhances oligodendrogenesis and myelination. *Stem Cells* 28, 1611–1622. doi: 10.1002/stem.480

Mall, M., Kareta, M. S., Chanda, S., Ahlenius, H., Perotti, N., Zhou, B., et al. (2017). Myt1l safeguards neuronal identity by actively repressing many non-neuronal fates. *Nature* 544, 245–249. doi: 10.1038/nature21722

Martinat, C., Bacci, J. J., Leete, T., Kim, J., Vanti, W. B., Newman, A. H., et al. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2874–2879. doi: 10.1073/pnas.0511153103

Matjusaitis, M., Wagstaff, L. J., Martella, A., Baranowski, B., Blin, C., Gogolok, S., et al. (2019). Reprogramming of fibroblasts to oligodendrocyte progenitor-like cells using CRISPR/Cas9-based synthetic transcription factors. *Stem Cell Rep.* 10, 390–405. doi: 10.1016/j.stemcr.2019.10.010

Matsuda, T., Irie, T., Katsurabayashi, S., Hayashi, Y., Nagai, T., Hamazaki, N., et al. (2019). Pioneer factor NeuroD1 rearranges transcriptional and epigenetic profiles to execute microglia-neuron conversion. *Neuron* 101, 472–485. doi: 10.1016/j.neuron.2018.12.010

Matsushita, M., Nakatake, Y., Arai, I., Ibata, K., Kohda, K., Goparaju, S. K., et al. (2017). Neural differentiation of human embryonic stem cells induced by the transgene-mediated overexpression of single transcription factors. *Biochem. Biophys. Res. Commun.* 490, 296–301. doi: 10.1016/j.bbrc.2017.06.039

Mattugini, N., Bocchi, R., Scheuss, V., Russo, G. L., Torper, O., Lao, C. L., et al. (2019). Inducing different neuronal subtypes from astrocytes in the injured mouse cerebral cortex. *Neuron* 103, 1086–1095.e5. doi: 10.1016/j.neuron.2019.08.009

Mazzara, P. G., Massimino, L., Pellegatta, M., Ronchi, G., Ricca, A., Iannielli, A., et al. (2017). Two factor-based reprogramming of rodent and human fibroblasts into Schwann cells. *Nat. Commun.* 8:14088. doi: 10.1038/ncomms14088

Mazzoni, E. O., Mahony, S., Closser, M., Morrison, C. A., Nedelec, S., Williams, D. J., et al. (2013). Synergistic binding of transcription factors to cell-specific enhancers programs motor neuron identity. *Nat. Neurosci.* 16, 1219–1227. doi: 10.1038/nn.3467

Meijer, M., Rehbach, K., Brunner, J. W., Classen, J. A., Lammertse, H. C. A., van Linge, L. A., et al. (2019). A single-cell model for synaptic transmission and plasticity in human iPSC-derived neurons. *Cell Rep.* 27, 2199–2211. doi: 10.1016/j.celrep.2019.04.058

Mertens, J., Paquola, A. C. M., Ku, M., Hatch, E., Böhnke, L., Ladjevardi, S., et al. (2015). Directly reprogrammed human neurons retain aging-associated transcriptomic signatures and reveal age-related nucleocytoplasmic defects. *Cell Stem Cell* 17, 705–718. doi: 10.1016/j.stem.2015.09.001

Mong, J., Panman, L., Alekseenko, Z., Kee, N., Stanton, L. W., Ericson, J., et al. (2013). Transcription factor-induced lineage programming of noradrenaline and motor neurons from embryonic stem cells. *Stem Cells* 32, 604–622. doi: 10.1002/stem.1585

Najm, F. J., Lager, A. M., Zaremba, A., Wyatt, K., Caprariello, A. V., Factor, D. C., et al. (2013). Transcription factor-mediated reprogramming of fibroblasts to expandable, myelinogenic oligodendrocyte progenitor cells. *Nat. Biotechnol.* 31, 426–433. doi: 10.1038/nbt.2561

Nehme, R., Zuccaro, E., Ghosh, S. D., Li, C., Sherwood, J. L., Pietilainen, O., et al. (2018). Combining NGN2 programming with developmental patterning generates human excitatory neurons with NMDAR-mediated synaptic transmission. *Cell Rep.* 23, 2509–2523. doi: 10.1016/j.celrep.2018.04.066

Nickolls, A. R., Lee, M. M., Espinoza, D. F., Szczot, M., Lam, R. M., Wang, Q., et al. (2020). Transcriptional programming of human mechanosensory neuron subtypes from pluripotent stem cells. *Cell Rep.* 30, 932–946.e7. doi: 10.1016/j.celrep.2019.12.062

Nishiyama, A., Xin, L., Sharov, A. A., Thomas, M., Mowrer, G., Meyers, E., et al. (2009). Uncovering early response of gene regulatory networks in ESCs by systematic induction of transcription factors. *Cell Stem Cell* 5, 420–433. doi: 10.1016/j.stem.2009.07.012

Olova, N., Simpson, D. J., Marioni, R. E., and Chandra, T. (2019). Partial reprogramming induces a steady decline in epigenetic age before loss of somatic identity. *Aging Cell* 18:e12877. doi: 10.1111/acel.12877

O'Shea, K. S. (2001). Neuronal differentiation of mouse embryonic stem cells: lineage selection and forced differentiation paradigms. *Blood Cells Mol. Dis.* 27, 705–712. doi: 10.1006/bcmd.2001.0435

Pang, Z. P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D. R., Yang, T. Q., et al. (2011). Induction of human neuronal cells by defined transcription factors. *Nature* 476, 220–223. doi: 10.1038/nature10202.Induction

Panman, L., Andersson, E., Alekseenko, Z., Hedlund, E., Kee, N., Mong, J., et al. (2011). Transcription factor-induced lineage selection of stem-cell-derived neural progenitor cells. *Cell Stem Cell* 8, 663–675. doi: 10.1016/j.stem.2011.04.001

Park, C.-H., Kang, J. S., Shin, Y. H., Chang, M. Y., Chung, S., Koh, H.-C., et al. (2006). Acquisition of in vitro and in vivo functionality of Nurr1-induced dopamine neurons. *FASEB J.* 20, 2553–2555. doi: 10.1096/fj.06-6159fje

Pataskar, A., Jung, J., Smialowski, P., Noack, F., Calegari, F., Straub, T., et al. (2016). NeuroD1 reprograms chromatin and transcription factor landscapes to induce the neuronal program. *EMBO J.* 35, 24–45. doi: 10.15252/embj.201591206

Pattyn, A., Goridis, C., and Brunet, J. F. (2000). Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. *Mol. Cell. Neurosci.* 15, 235–243. doi: 10.1006/mcne.1999.0826

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. *Development* 124, 4065–4075.

Pawlowski, M., Ortmann, D., Bertero, A., Tavares, J. M., Pedersen, R. A., Vallier, L., et al. (2017). Inducible and deterministic forward programming of human pluripotent stem cells into neurons, skeletal myocytes, and oligodendrocytes. *Stem Cell Rep.* 8, 803–812. doi: 10.1016/j.stemcr.2017.02.016

Pesaresi, M., Sebastian-Perez, R., and Cosma, M. P. (2019). Dedifferentiation, transdifferentiation and cell fusion: in vivo reprogramming strategies for regenerative medicine. *FEBS J.* 286, 1074–1093. doi: 10.1111/febs.14633

Pfisterer, U., Kirkeby, A., Torper, O., Wood, J., Nelander, J., Dufour, A., et al. (2011a). Direct conversion of human fibroblasts to dopaminergic neurons. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10343–10348. doi: 10.1073/pnas.1105135108

Pfisterer, U., Wood, J., Nihlberg, K., Hallgren, O., Bjermer, L., Westergren-Thorsson, G., et al. (2011b). Efficient induction of functional neurons from adult human fibroblasts. *Cell Cycle* 10, 3311–3316. doi: 10.4161/cc.10.19.17584

Polo, J. M., Liu, S., Figueroa, M. E., Kulalert, W., Eminli, S., Tan, K. Y., et al. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat. Biotechnol.* 28, 848–855. doi: 10.1038/nbt.1667

Rhee, H. J., Shaib, A. H., Rehbach, K., Lee, C., Seif, P., Thomas, C., et al. (2019). An autaptic culture system for standardized analyses of iPSC-derived human neurons. *Cell Rep.* 27, 2212–2228.e7. doi: 10.1016/j.celrep.2019.04.059

Ring, K. L., Tong, L. M., Balestra, M. E., Javier, R., Andrews-Zwilling, Y., Li, G., et al. (2012). Direct reprogramming of mouse and human fibroblasts into multipotent neural cells with a single factor. *Cell Stem Cell* 11, 100–109. doi: 10.1016/j.stem.2012.05.018

Rivetti Di Val Cervo, P., Romanov, R. A., Spigolon, G., Masini, D., Martín-Montañez, E., Toledo, E. M., et al. (2017). Induction of functional dopamine neurons from human astrocytes in vitro and mouse astrocytes in a Parkinson's disease model. *Nat. Biotechnol.* 35, 444–452. doi: 10.1038/nbt.3835

Robinson, M., Chapani, P., Styan, T., Vaidyanathan, R., and Willerth, S. M. (2016). Functionalizing Ascl1 with novel intracellular protein delivery technology for promoting neuronal differentiation of human induced pluripotent stem cells. *Stem Cell Rev. Rep.* 12, 476–483. doi: 10.1007/s12015-016-9655-7

Rubio, A., Luoni, M., Giannelli, S. G., Radice, I., Iannielli, A., Cancellieri, C., et al. (2016). Rapid and efficient CRISPR/Cas9 gene inactivation in human neurons during human pluripotent stem cell differentiation and direct reprogramming. *Sci. Rep.* 6:37540. doi: 10.1038/srep37540

Sagal, J., Zhan, X., Xu, J., Tilghman, J., Karuppagounder, S. S., Chen, L., et al. (2014). Proneural transcription factor Atoh1 drives highly efficient differentiation of human pluripotent stem cells into dopaminergic neurons. *Stem Cells Transl. Med.* 3, 888–898. doi: 10.5966/sctm.2013-0213

Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D., and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* 126, 4017–4026.

Salemi, S., Baktash, P., Rajaei, B., Noori, M., Amini, H., Shamsara, M., et al. (2016). Efficient generation of dopaminergic-like neurons by overexpression of Nurr1 and Pitx3 in mouse induced pluripotent stem cells. *Neurosci. Lett.* 626, 126–134. doi: 10.1016/j.neulet.2016.05.032

Sánchez-Danés, A., Consiglio, A., Richaud, Y., Rodríguez-Pizà, I., Dehay, B., Edel, M., et al. (2012). Efficient generation of A9 midbrain dopaminergic neurons by lentiviral delivery of LMX1A in human embryonic stem cells and induced pluripotent stem cells. *Hum. Gene Ther.* 23, 56–69. doi: 10.1089/hum.2011.054

Schrenk-Siemens, K., Wende, H., Prato, V., Song, K., Rostock, C., Loewer, A., et al. (2015). PIEZO2 is required for mechanotransduction in human stem cell-derived touch receptors. *Nat. Neurosci.* 18, 10–16. doi: 10.1038/nn.3894

Serre, A., Snyder, E. Y., Mallet, J., and Buchet, D. (2012). Overexpression of basic helix-loop-helix transcription factors enhances neuronal differentiation of fetal human neural progenitor cells in various ways. *Stem Cells Dev.* 21, 539–553. doi: 10.1089/scd.2011.0079

Shahbazi, E., Moradi, S., Nemati, S., Satarian, L., Basiri, M., Gourabi, H., et al. (2016). Conversion of human fibroblasts to stably self-renewing neural stem cells with a single zinc-finger transcription factor. *Stem Cell Rep.* 6, 539–551. doi: 10.1016/j.stemcr.2016.02.013

Sheng, C., Jungverdorben, J., Wiethoff, H., Lin, Q., Flitsch, L. J., Eckert, D., et al. (2018). A stably self-renewing adult blood-derived induced neural stem cell exhibiting patternability and epigenetic rejuvenation. *Nat. Commun.* 9:4047. doi: 10.1038/s41467-018-06398-5

Shi, J., Jin, G., Zhu, H., Tian, M., Zhang, X., Qin, J., et al. (2010). The role of Brn-4 in the regulation of neural stem cell differentiation into neurons. *Neurosci. Res.* 67, 8–17. doi: 10.1016/j.neures.2010.01.007

Smith, D. K., Yang, J., Liu, M. L., and Zhang, C. L. (2016). Small molecules modulate chromatin accessibility to promote NEUROG2-mediated fibroblast-to-neuron reprogramming. *Stem Cell Rep.* 7, 955–969. doi: 10.1016/j.stemcr.2016.09.013

Soldati, C., Cacci, E., Biagioni, S., Carucci, N., Lupo, G., Perrone-Capano, C., et al. (2012). Restriction of neural precursor ability to respond to Nurr1 by early regional specification. *PLoS One* 7:e51798. doi: 10.1371/journal.pone.0051798

Song, Z., Jadali, A., Fritzsch, B., and Kwan, K. Y. (2017). NEUROG1 regulates CDK2 to promote proliferation in otic progenitors. *Stem Cell Rep.* 9, 1516–1529. doi: 10.1016/j.stemcr.2017.09.011

Sowa, Y., Kishida, T., Tomita, K., Yamamoto, K., Numajiri, T., and Mazda, O. (2017). Direct conversion of human fibroblasts into Schwann cells that facilitate regeneration of injured peripheral nerve in vivo. *Stem Cells Transl. Med.* 6, 1207–1216. doi: 10.5966/sctm.2012-0084

Srivastava, D., and DeWitt, N. (2016). In vivo cellular reprogramming: the next generation. *Cell* 166, 1386–1396. doi: 10.1016/j.cell.2016.08.055

Sugimori, M., Nagao, M., Parras, C. M., Nakatani, H., Lebel, M., Guillemot, F., et al. (2008). Ascl1 is required for oligodendrocyte development in the spinal cord. *Development* 135, 1271–1281. doi: 10.1242/dev.015370

Sun, A. X., Yuan, Q., Tan, S., Xiao, Y., Wang, D., Khoo, A. T. T., et al. (2016). Direct induction and functional maturation of forebrain GABAergic neurons from human pluripotent stem cells. *Cell Rep.* 16, 1942–1953. doi: 10.1016/j.celrep.2016.07.035

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., et al. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 104, 365–376. doi: 10.1016/s0092-8674(01)00224-0

Surmacz, B., Noisa, P., Risner-Janiczek, J. R., Hui, K., Ungless, M., Cui, W., et al. (2012). DLK1 promotes neurogenesis of human and mouse pluripotent stem cell-derived neural progenitors via modulating Notch and BMP signalling. *Stem Cell Rev. Rep.* 8, 459–471. doi: 10.1007/s12015-011-9298-7

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 10.1016/j.cell.2007.11.019

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Tan, X. F., Qin, J. B., Jin, G. H., Tian, M. L., Li, H. M., Zhu, H. X., et al. (2010). Effects of Brn-4 on the neuronal differentiation of neural stem cells derived from rat midbrain. *Cell Biol. Int.* 34, 877–882. doi: 10.1042/cbi20100214

Tang, Y., Liu, M. L., Zang, T., and Zhang, C. L. (2017). Direct reprogramming rather than iPSC-based reprogramming maintains aging hallmarks in human motor neurons. *Front. Mol. Neurosci.* 10:359. doi: 10.3389/fnmol.2017.00359

Tao, Y., and Zhang, S. C. (2016). Neural subtype specification from human pluripotent stem cells. *Cell Stem Cell* 19, 573–586. doi: 10.1016/j.stem.2016.10.015

Tchieu, J., Calder, E. L., Guttikonda, S. R., Gutzwiller, E. M., Aromolaran, K. A., Steinbeck, J. A., et al. (2019). NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells. *Nat. Biotechnol.* 37, 267–275. doi: 10.1038/s41587-019-0035-0

Teratani-Ota, Y., Yamamizu, K., Piao, Y., Sharova, L., Amano, M., Yu, H., et al. (2016). Induction of specific neuron types by overexpression of single transcription factors. *In Vitro Cell. Dev. Biol. Anim.* 52, 961–973. doi: 10.1007/s11626-016-0056-7

Theka, I., Caiazzo, M., Dvoretskova, E., Leo, D., Ungaro, F., Curreli, S., et al. (2013). Rapid generation of functional dopaminergic neurons from human induced pluripotent stem cells through a single-step procedure using cell lineage transcription factors. *Stem Cells Transl. Med.* 2, 473–479. doi: 10.5966/sctm.2012-0133

Thoma, E. C., Wischmeyer, E., Offen, N., Maurus, K., Sirén, A. L., Schartl, M., et al. (2012). Ectopic expression of neurogenin 2 alone is sufficient to induce differentiation of embryonic stem cells into mature neurons. *PLoS One* 7:e38651. doi: 10.1371/journal.pone.0038651

Tian, C., Li, Y., Huang, Y., Wang, Y., Chen, D., Liu, J., et al. (2015). Selective generation of dopaminergic precursors from mouse fibroblasts by direct lineage conversion. *Sci. Rep.* 5:12622. doi: 10.1038/srep12622

Tochitani, S., and Hayashizaki, Y. (2008). Nkx2.2 antisense RNA overexpression enhanced oligodendrocytic differentiation. *Biochem. Biophys. Res. Commun.* 372, 691–696. doi: 10.1016/j.bbrc.2008.05.127

Tong, M., Hernandez, J. L., Purcell, E. K., Altschuler, R. A., and Duncan, R. K. (2010). The intrinsic electrophysiological properties of neurons derived from mouse embryonic stem cells overexpressing neurogenin-1. *Am. J. Physiol. Physiol.* 299, C1335–C1344. doi: 10.1152/ajpcell.00207.2010

Torper, O., Ottosson, D. R., Pereira, M., Lau, S., Cardoso, T., Grealish, S., et al. (2015). In vivo reprogramming of striatal NG2 glia into functional neurons that integrate into local host circuitry. *Cell Rep.* 12, 474–481. doi: 10.1016/j.celrep.2015.06.040

Torper, O., Pfisterer, U., Wolf, D. A., Pereira, M., Lau, S., Jakobsson, J., et al. (2013). Generation of induced neurons via direct conversion in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7038–7043. doi: 10.1073/pnas.1303829110

Treutlein, B., Lee, Q. Y., Camp, J. G., Mall, M., Koh, W., Shariati, S. A. M., et al. (2016). Dissecting direct reprogramming from fibroblast to neuron using single-cell RNA-seq. *Nature* 534, 391–395. doi: 10.1038/nature18323

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035–1041. doi: 10.1038/nature08797

Villaescusa, J. C., Li, B., Toledo, E. M., Rivetti di Val Cervo, P., Yang, S., Stott, S. R., et al. (2016). A PBX1 transcriptional network controls dopaminergic neuron development and is impaired in Parkinson's disease. *EMBO J.* 35, 1963–1978. doi: 10.15252/embj.201593725

Wang, C., Ward, M. E., Chen, R., Liu, K., Tracy, T. E., Chen, X., et al. (2017). Scalable production of iPSC-derived human neurons to identify tau-lowering compounds by high-content screening. *Stem Cell Rep.* 9, 1221–1233. doi: 10.1016/j.stemcr.2017.08.019

Wang, J., Pol, S. U., Haberman, A. K., Wang, C., O'Bara, M. A., and Sim, F. J. (2014). Transcription factor induction of human oligodendrocyte progenitor fate and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2885–E2894. doi: 10.1073/pnas.1408295111

Wapinski, O. L., Vierbuchen, T., Qu, K., Lee, Q. Y., Chanda, S., Fuentes, D. R., et al. (2013). Hierarchical mechanisms for direct reprogramming of fibroblasts to neurons. *Cell* 155, 621–635. doi: 10.1016/j.cell.2013.09.028

Weng, R., Lu, C., Liu, X., Li, G., Lan, Y., Qiao, J., et al. (2018). Long noncoding RNA-1604 orchestrates neural differentiation through the miR-200c/ZEB axis. *Stem Cells* 36, 325–336. doi: 10.1002/stem.2749

Xue, Y., Zhan, X., Sun, S., Karuppagounder, S. S., Xia, S., Dawson, V. L., et al. (2019). Synthetic mRNAs drive highly efficient iPS cell differentiation to dopaminergic neurons. *Stem Cells Transl. Med.* 8, 112–123. doi: 10.1002/sctm.18-0036

Yamamizu, K., Piao, Y., Sharov, A. A., Zsiros, V., Yu, H., Nakazawa, K., et al. (2013). Identification of transcription factors for lineage-specific ESC differentiation. *Stem Cell Rep.* 1, 545–559. doi: 10.1016/j.stemcr.2013.10.006

Yamamizu, K., Sharov, A. A., Piao, Y., Amano, M., Yu, H., Nishiyama, A., et al. (2016). Generation and gene expression profiling of 48 transcription-factor-inducible mouse embryonic stem cell lines. *Sci. Rep.* 6:25667. doi: 10.1038/srep25667

Yang, N., Chanda, S., Marro, S., Ng, Y. H., Janas, J. A., Haag, D., et al. (2017). Generation of pure GABAergic neurons by transcription factor programming. *Nat. Methods* 14, 621–628. doi: 10.1038/nmeth.4291

Yang, N., Zuchero, J. B., Ahlenius, H., Marro, S., Ng, Y. H., Vierbuchen, T., et al. (2013). Generation of oligodendroglial cells by direct lineage conversion. *Nat. Biotechnol.* 31, 434–439. doi: 10.1038/nbt.2564

Yang, S., Toledo, E. M., Rosmaninho, P., Peng, C., Uhlén, P., Castro, D. S., et al. (2018). A Zeb2-miR-200c loop controls midbrain dopaminergic neuron neurogenesis and migration. *Commun. Biol.* 1:75. doi: 10.1038/s42003-018-0080-0

Yang, Y., Jiao, J., Gao, R., Le, R., Kou, X., Zhao, Y., et al. (2015). Enhanced rejuvenation in induced pluripotent stem cell-derived neurons compared with directly converted neurons from an aged mouse. *Stem Cells Dev.* 24, 2767–2777. doi: 10.1089/scd.2015.0137

Yuan, F., Chen, X., Fang, K. H., Wang, Y., Lin, M., Xu, S. B., et al. (2018). Induction of human somatostatin and parvalbumin neurons by expressing a single transcription factor LIM homeobox 6. *eLife* 7:e37382. doi: 10.7554/eLife.37382

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., et al. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron* 78, 785–798. doi: 10.1016/j.neuron.2013.05.029

Zhao, X., He, X., Han, X., Yu, Y., Ye, F., Chen, Y., et al. (2010). MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron* 65, 612–626. doi: 10.1016/j.neuron.2010.02.018

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# Neuronal Replacement as a Tool for Basal Ganglia Circuitry Repair: 40 Years in Perspective

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The ability of new neurons to promote repair of brain circuitry depends on their capacity to re-establish afferent and efferent connections with the host. In this review article, we give an overview of past and current efforts to restore damaged connectivity in the adult mammalian brain using implants of fetal neuroblasts or stem cell-derived neuronal precursors, with a focus on strategies aimed to repair damaged basal ganglia circuitry induced by lesions that mimic the pathology seen in humans affected by Parkinson's or Huntington's disease. Early work performed in rodents showed that neuroblasts obtained from striatal primordia or fetal ventral mesencephalon can become anatomically and functionally integrated into lesioned striatal and nigral circuitry, establish afferent and efferent connections with the lesioned host, and reverse the lesion-induced behavioral impairments. Recent progress in the generation of striatal and nigral progenitors from pluripotent stem cells have provided compelling evidence that they can survive and mature in the lesioned brain and re-establish afferent and efferent axonal connectivity with a remarkable degree of specificity. The studies of cell-based circuitry repair are now entering a new phase. The introduction of genetic and virus-based techniques for brain connectomics has opened entirely new possibilities for studies of graft-host integration and connectivity, and the access to more refined experimental techniques, such as chemo- and optogenetics, has provided new powerful tools to study the capacity of grafted neurons to impact the function of the host brain. Progress in this field will help to guide the efforts to develop therapeutic strategies for cell-based repair in Huntington's and Parkinson's disease and other neurodegenerative conditions involving damage to basal ganglia circuitry.

Keywords: embryonic stem cells, induced pluripotent stem cells, regenerative medicine, striatum, substantia nigra, nigrostriatal pathway, dopamine

## INTRODUCTION

The idea that new cells—neurons, neuroblasts, or immature neural precursors—can be used to repair damaged neural circuitry in the brain goes back to the late 1970s. These early studies were performed in rodents and made use of neuroblasts obtained from the developing CNS. The experiments pursued in our lab here in Lund were focused on the use of tissue dissected from the developing ventral mesencephalon or fetal striatal primordia (i.e., the ganglionic eminences)

to replace the lost nigral or striatal neurons and restore axonal connectivity in rats with damage to the basal ganglia circuitry, similar to that seen in patients with Parkinson's or Huntington's disease.

Neural transplantation is a classic approach in cold-blooded vertebrates, salamanders, fish, and frogs, that goes back to the early decades of the last century. Similar studies in mammals were initially unsuccessful due to shortcomings of the methods used at the time, and it was not until the 1970s, with the introduction of histochemical methods and modern tract-tracing techniques, that the effective tools for the study of survival and growth of neural tissue became available (for reviews of these early developments see Thompson and Björklund, 2015; Dunnett and Björklund, 2017). Subsequent progress has been critically dependent on the development of increasingly more refined and powerful techniques for studies of neuronal connectivity that has taken place over the last decades. The early studies used methods that allowed selective visualization of specific neuronal systems, defined by their transmitter content, or on the use of species-specific antibodies that allow immunohistochemical staining of, e.g., mouse, pig or human neurons and their axonal projections in the rodent brain or spinal cord. These methods were combined with classic anterograde or retrograde tracers injected into the grafted tissue or selected anatomical targets in the host brain. Decades later, the possibility to create transgenic animals and cell lines expressing fluorescent reporters, such as green fluorescent protein (GFP), provided a new set of powerful and versatile tools to trace axonal projections, making it possible to study the connectivity of neural grafts with a sensitivity and specificity that went beyond what had been possible with classic tract-tracing techniques.

Contrary to the prevailing notion at the time, the results obtained in these pioneering studies provided compelling evidence that new neurons can be anatomically integrated into damaged brain circuitry, and that neurons developing from implanted neuroblasts exhibit a remarkable capacity to recreate functional efferent and afferent connections with the damaged host brain. From the very beginning of these studies, the striatum, and its cortical, thalamic and brain stem connections, proved to be a very useful testbed for the development of this cell-based repair approach. There were tools available that allowed selective damage to components of this circuitry, and a battery of tests for striatum-related motor and cognitive behaviors, suitable for monitoring of behavioral impairment and recovery in lesioned rats and mice, had been developed. The two most commonly used brain lesion models—ablation of striatal projection neurons using excitotoxic lesions, induced by ibotenic acid (IBO) or quinolinic acid (QUIN), and damage to the nigrostriatal dopamine (DA) system using the 6-hydroxydopamine (6-OHDA) neurotoxin—were also attractive in that they replicated some of the key pathology seen in patients with Huntington's and Parkinson's disease.

During the last decade, the development of new genetic and viral techniques for the study of brain connectomics has opened new possibilities, and as a result, we are now entering a new phase in the study of cell-based brain repair, and at the same time, the approach using transplants of DA neurons derived from pluripotent stem cells (PSCs) is now entering the clinic in patients with Parkinson's disease. The purpose of the present review is to summarize the progress made in this field, from the early days to the present, with a focus on the studies performed over the last four decades in our lab here in Lund, and discuss the possibilities and challenges for the development of cell-based therapies for brain circuitry repair.

## BASAL GANGLIA CIRCUITRY AND MOTOR CONTROL

In rodents, striatal connectivity is organized in a way that is very well suited for the exploration of repair strategies. The striatum contains a well-defined mixture of short-axoned interneurons and long-axoned spiny projection neurons that link the striatum with three major downstream targets, the external and internal globus pallidus and the substantia nigra. As illustrated schematically in **Figure 1A**, the projection neurons (which constitute more than 90% of all neurons in the striatum) are of two subtypes: the D1 receptor-bearing striatonigral neurons that innervate the internal globus and the pars reticulata of the substantia nigra, and the D2 receptor-bearing striatopallidal neurons which innervate the external globus pallidus. These projections are strictly unilateral, which means that a lesion on one side will leave the contralateral side intact to serve as an internal control. The projection neurons receive inputs from local interneurons, as well as from four major extrastriatal sources: glutamatergic excitatory afferents from neocortex and thalamus, and modulatory afferents from dopaminergic neurons in the ventral midbrain and serotonergic neurons in the pontine raphe nuclei (for review see Silberberg and Bolam, 2015).

The striatum plays a central role in the planning and execution of movement, as well as in the acquisition of motor skills and habits (for recent reviews see Redgrave et al., 2010; Graybiel and Grafton, 2015). As illustrated in **Figure 1**, cortical control is channeled through the striatum and its principal downstream targets, external and internal pallidum, and substantia nigra. The cortico-striatal inputs are topographically organized, such that areas associated with the sensorimotor function project to the dorsolateral part of the striatum, associative inputs to the dorsomedial part, and limbic inputs to the ventromedial part (including nc. accumbens). Consistent with this anatomical arrangement the striatum can be subdivided into three parts that subserve different aspects of motor behavior: *a dorsolateral part* regulating habitual, automatic movements (corresponding to the post-commissural putamen in humans); *a dorsomedial part* mediating associative and goal-directed behaviors (corresponding the rostral putamen and caudate nucleus in humans); and *a ventromedial/nc accumbens part* involved in motivational and emotional behavior (corresponding to the ventral striatum in humans; Redgrave et al., 2010; Graybiel and Grafton, 2015). These subsectors of the cortico-striatal machinery are functionally interconnected: their outputs converge in the downstream targets, globus pallidus, and substantia nigra, and they interact in the execution of coordinated motor behavior.
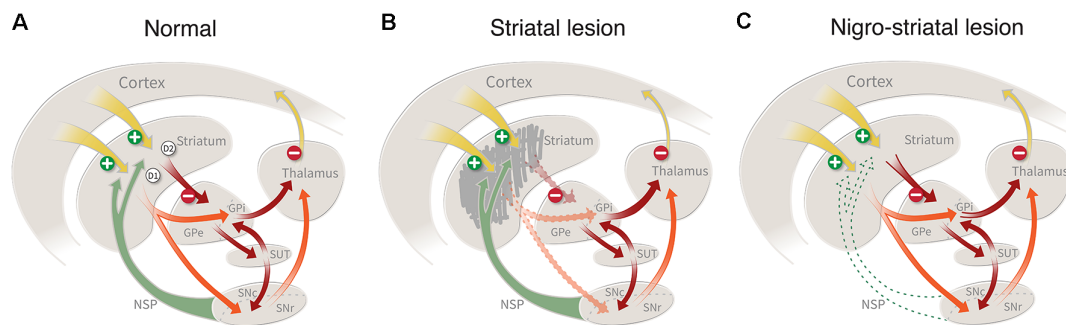
**FIGURE 1 | (A)** Striatal connectivity comprises two major neuronal circuits: the cortico-striato-pallido-thalamic circuit and the cortico-striato-nigro-thalamic circuit, which in turn are interlinked by an important regulatory hub, the subthalamic nucleus. The striatal projection neurons (which constitute more than 90% of all neurons in the striatum) are of two types: the D1 receptor-bearing striatonigral neurons that innervate the internal globus and the pars reticulata of the substantia nigra, and the D2 receptor-bearing striatopallidal neurons which innervate the external globus pallidus. **(B)** Damage to the striatal projection neurons, caused by an intrastriatal injection of ibotenic acid (IBO) or quinolinic acid (QUIN), will disrupt these two circuits and result in a disinhibition of the downstream targets, the pallidum and the substantia nigra, pars reticulata. This lesion mimics the striatal damage seen in patients with Huntington's disease. **(C)** Lesion of the nigrostriatal dopamine (DA) pathway, induced by the injection of 6-hydroxydopamine (6-OHDA) or MPTP, removes an important regulatory control, resulting in motor impairments similar to those seen on patients with Parkinson's disease.

According to the classic model shown in **Figure 1A**, the two subtypes of striatal projection neurons are proposed to exert opposing influences on motor function, such that the neurons projecting to the internal pallidum and substantia nigra, called the *direct pathway*, act to facilitate movement, and the neurons innervation the external pallidum, the *indirect pathway*, serve to inhibit movement (Albin et al., 1989; DeLong and Wichmann, 2015). Although the functional interactions between these two output pathways are more complex than suggested by this simplistic model (see Redgrave et al., 2010), it seems clear that the overall inhibitory control exerted by the GABAergic projection neurons over their downstream targets is a key element in the initiation and execution of movement. As a consequence, damage to the cortico-striato-pallidal circuit induced by ablation of the striatal projection neurons will result in a disinhibition of the affected pallidal and nigral target cells and impaired motor function (**Figure 1B**; Chevalier and Deniau, 1990; Redgrave et al., 2010).

The DA neurons of the substantia nigra and ventral tegmental area (VTA) provide important modulatory control of striatal function. Importantly, the DA neurons are not themselves part of the motor execution pathway—i.e., the striatopallidal and striatonigral loops—meaning that removal of the DA input (as seen in nigra-lesioned animals or PD patients) will leave this circuitry intact, but in a dysfunctional state, resulting in a hypokinetic syndrome characterized by a difficulty to initiate and perform movements (**Figure 1C**). The nigrostriatal pathway exhibits a general medial-to-lateral topography, and the part that is most severely affected in PD is the lateral portion, i.e., the part that projects to the dorsolateral (sensorimotor) part of the striatum (Kish et al., 1988). This pattern of DA neuron loss is consistent with the fact that it is the execution of automatic movements and motor habits that are most severely, and also early, affected in PD patients (Marsden, 1982; Rodriguez-Oroz et al., 2009). Consequently, restoration of striatal DA neurotransmission, by drugs or DA neuron transplants, is the main therapeutic strategy for the recovery of motor function in patients with PD.

# RECONSTRUCTION OF BASAL GANGLIA CIRCUITRY IN ANIMALS WITH STRIATAL LESIONS

Ablation of the striatal projection neurons disrupts major pathways for the execution of functions initiated at the level of the cerebral cortex and causes impairment of sensorimotor, associative, or limbic behaviors. These impairments are caused by the removal of inhibitory control of functionally related downstream targets, the substantia nigra, and the external and internal pallidum (see above). Conceived in this way, the impairments seen in striatum-lesioned animals can be viewed as a "disconnection syndrome" caused by a disruption of the cortico-striato-pallido/nigro-thalamic circuits that subserve these functions. The cell-based repair strategies pursued in this model seek to restore connectivity and function in these execution pathways.

The excitotoxic lesion used in these studies causes a rapid neuron death followed by a gradual shrinkage of the striatal volume within the following weeks (Isacson et al., 1985). When applied unilaterally, as shown in **Figure 2A**, the lesion will cause motor asymmetry and a distinct impairment in contralateral paw use that can be monitored in the so-called staircase test, and when applied bilaterally the rats will develop a hyperactivity syndrome accompanied by pronounced cognitive and motivational impairments (for review see Dunnett et al., 2000). The cells used for grafting in this model are obtained from the fetal striatal primordia residing in the ganglionic eminence (GE). GE, however, is a heterogeneous structure that gives rise not only to neurons in the striatum but also to other areas. Thus, the lateral part of this structure, lateral ganglionic eminence (LGE), is the source of the striatal projection neurons (as well as interneurons destined for the olfactory bulb), and the medial
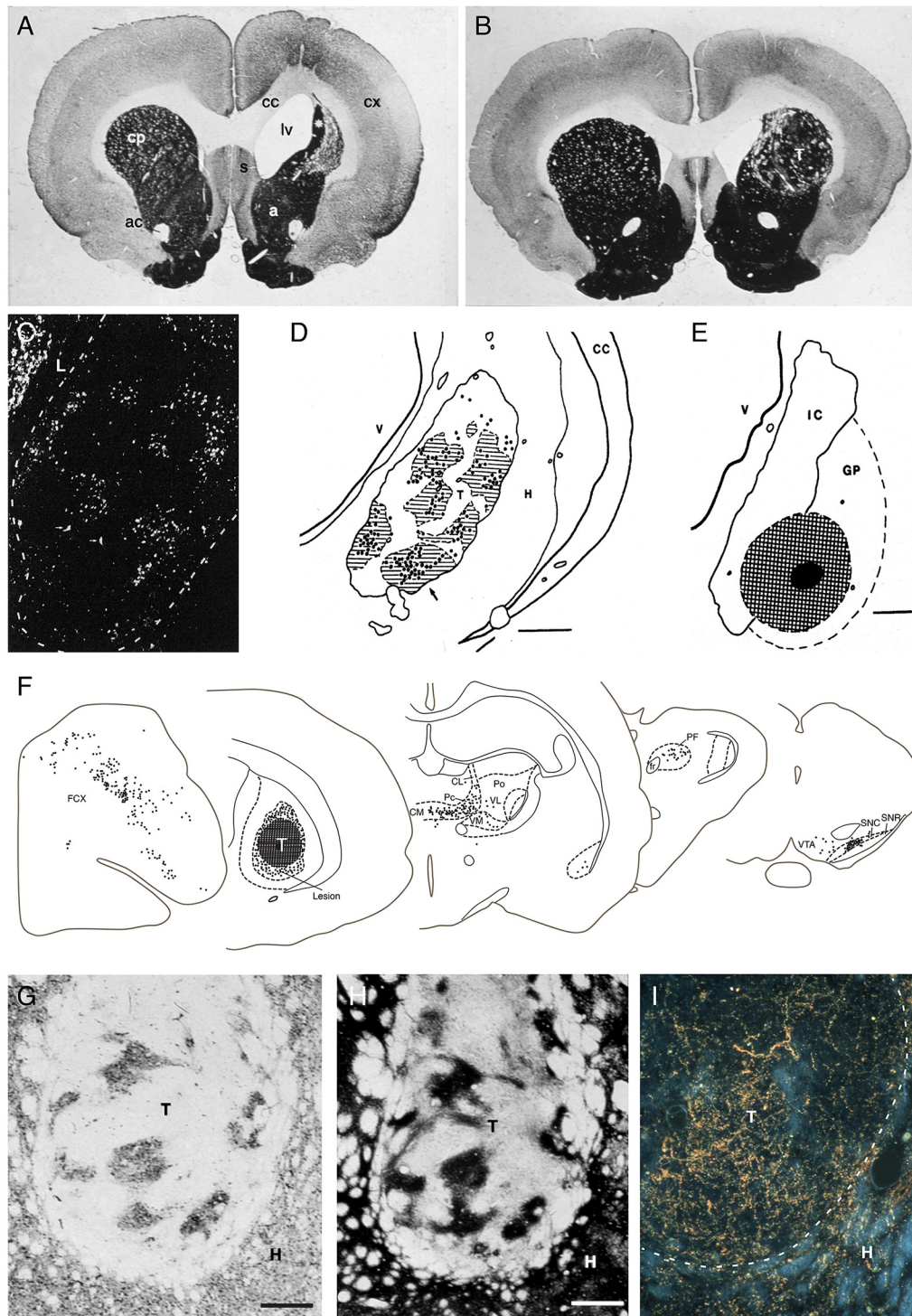
**FIGURE 2 | (A,B)** The loss of striatal neurons, and the reduction in overall striatal volume, seen in the excitotoxin lesioned striatum (as shown in **A**), are largely restored by the transplanted fetal striatal primordium (**B**; AchE stain, 6 months survival. T = transplant). **(C–E)** Injection of the retrograde axonal tracer FluoroGold into the Globus pallidus (hatched area in **E**) results in the labeling of large numbers of DARPP-32+ striatal projection neurons in the graft (bright spots in **C**). The vast majority of these are located within the DARPP-32+ patches in the grafts (striped area in **D**). **(F)** Injection of a retrograde axonal tracer into the striatal graft (hatched area, T) reveal extensive afferent inputs from the same regions of the host brain that innervate the striatum in the intact brain, including the frontoparietal cortex (FCX), the intralaminar thalamic nuclei (CL, CM, PF, Pc, Po, VL, VM), and the substantia nigra (SNC), with a distribution that is closely similar to that seen in the intact animal. **(G–I)** The DARPP-32+ areas of the grafts **(G)** are densely innervated by TH+ axons from the host nigrostriatal pathway **(H)**, as well as from the host frontal cortex (labeled with the anterograde tracer PHA-L in **I**) adapted from Wictorin et al. (1989b) and Wictorin and Björklund (1989).

part, the MGE, is the source of the striatal interneurons, and it contributes also to the formation of interneurons in the cortex, pallidum and other ventral forebrain regions.

## Development and Composition of the Striatal Grafts

In our studies, we have used GE cells obtained from 14 to 15 day old rat fetuses and injected them into the lesioned site as a crude cell suspension. These graft deposits grow and mature with a time-course that is fairly close to normal striatum: they expand 5- to 8-fold in size over the first 3 weeks and reach their final size and maturation after 2–3 months (Isacson et al., 1984, 1986; Labandeira-Garcia et al., 1991). At this time they have expanded to occupy a major part of the lesioned and neuron-depleted part of the host striatum (**Figure 2B**). Interestingly, the growth and development of the grafts depend on the conditions at the transplantation site: grafts placed in the non-lesioned striatum are initially (at 4 days) of the same size, but they fail to grow in size at longer survival times (Labandeira-Garcia et al., 1991).

Neurons expressing characteristic markers of striatal projection neurons and interneurons are distributed in patches throughout the graft tissue, suggesting that the grafted striatal primordia can continue their fetal development in their new location. Further studies have shown that the striatal component is derived from the LGE, while the non-striatal areas, which express neuronal markers characteristic of cerebral cortex and globus pallidus, is largely derived from the MGE (Graybiel et al., 1989; Wictorin et al., 1989b; Campbell et al., 1995; Olsson et al., 1995). Thus, the proportion of the grafts expressing striatal markers can be greatly enhanced, from 30% to 40% in whole GE grafts up to 80–90%, if the dissection of the GE is restricted to the dorsal part of the LGE (Pakzaban et al., 1993; Olsson et al., 1995).

The neuronal composition of the striatum-like areas is notably similar to that seen in the intact striatum. As in the intact striatum, the vast majority of the neurons (over 90%) are of the DARPP-32 positive medium spiny type, and the two major subtypes, the pre-proenkephalin (PPE) and pre-protachykinin (PPT) expressing neurons, which are the characteristic markers of the striatopallidal and striatonigral projection neurons, respectively, are present in similar proportions, around 50%, as in the host (Campbell et al., 1992, 1995; Liu et al., 1992). The interneuron population has not been fully characterized, but it has been shown to include the characteristic type of large-sized cholinergic (ChAT positive) interneurons, as well as various types of GABAergic (GAD positive) interneurons that are seen to make connections within the grafts (Roberts and Difiglia, 1988; Graybiel et al., 1989; Helm et al., 1992; Clarke and Dunnett, 1993).

## Afferent Host-to-Graft Connectivity

The anatomical integration of the GE grafts has been extensively investigated using a combination of classic anterograde and retrograde tracers. These studies show extensive afferent inputs from the same regions of the host brain that innervate the striatum in the intact brain, including the frontoparietal cortex, the intralaminar thalamic nuclei, the basolateral amygdala,

substantia nigra and the dorsal raphe nucleus, with a distribution that is closely similar to that seen in the intact animal (**Figures 2F–I**; Wictorin et al., 1988, 1989a; Wictorin and Björklund, 1989; Labandeira-Garcia et al., 1991). Simultaneous injection of two retrograde tracers, rhodamine-labeled beads into the grafts and True Blue into the adjacent, spared striatum, showed that the vast majority, 60–100%, of the cells in thalamus, substantia nigra and dorsal raphe were double-labeled, indicating that the host inputs to the striatal grafts are derived from axons that remain in the neuron-depleted area, i.e., from the very same neurons that project to the area of the striatum was the graft is placed.

Ultrastructural studies have shown that the cortical and thalamic afferents from asymmetric synaptic contacts with the grafted striatal neurons. As in the intact striatum, the contacts are made on both dendritic spines and shafts with a predominance of spine synapses, although the relative proportion of spine synapses tends to be lower than in the normal striatum (Wictorin et al., 1989a; Xu et al., 1991; Clarke and Dunnett, 1993; Dunnett and Björklund, 2017). The nigral TH-positive afferents provide a dense innervation of the DARPP32-positive striatum-like patches (**Figures 2G,H**) and have been shown to make synaptic contacts onto dendrites and spines of medium spiny grafted neurons (Wictorin et al., 1988, 1989b). Interestingly the cortical and dopaminergic inputs were seen to converge onto the same spines and shafts of neurons projecting to the host globus pallidus, similar to the connectivity seen in the intact striatum (Clarke et al., 1988b; Clarke and Dunnett, 1993). The functionality of the cortical input is supported by electrophysiological recordings (Rutherford et al., 1987), and also by the use of the c-Fos expression as a cellular marker of stimulation-induced functional activity (Labandeira-Garcia and Guerra, 1994) showing prominent activation of c-Fos in the DARPP-32 positive neurons induced by stimulation of the frontal cortex. The density of c-Fos positive nuclei within the DARPP-32 positive striatum-like patches was around 60% of that seen in the intact striatum, suggesting that the host cortical inputs can exert a wide-spread control of the grafted striatal projection neurons.

## Efferent Graft-to-Host Connectivity

The efferent projections of the grafted neurons are most efficiently visualized in xenograft experiments where the mouse, pig, or human GE are transplanted to the lesioned rat striatum and visualized by immunostaining using species-specific antibodies. This allows the grafted cells and the graft-derived axonal projections to be traced with high sensitivity and in their entirety. The results obtained using this approach (Wictorin et al., 1991) reveal a remarkable specificity in the axonal outgrowth pattern. The axons grow exclusively in one direction, caudally, and are confined to a single bundle that extends along with the myelinated fiber bundles of the internal capsule, ramifying into branches of terminals within the globus pallidus. Studies using a combination of anterograde and retrograde axonal tracers have shown that the outgrowing axons are derived almost exclusively from the DARPP-32 positive GABAergic medium spiny neurons (**Figures 2C–E**;

Wictorin et al., 1989c; Campbell et al., 1995) and that they make synaptic contacts with dendritic shafts and spines of the host pallidal neurons, similar to the ones made by the striatopallidal connection in the intact animal (Wictorin et al., 1989a). The axonal outgrowth from grafts of rat or mouse GE tissue does not reach beyond the globus pallidus, which is in contrast to the more extensive outgrowth obtained from grafts of fetal human or pig GE tissue which extends along the nigrostriatal pathway to the substantia nigra, indicating that the growth capacity of the striatal neurons reflects their size in the donor species, small in rodents but much larger in pigs and human (Wictorin et al., 1990; Isacson and Deacon, 1996).

## Dopaminergic Regulation of Graft Function

These findings show that the key components of the cortico-striato-pallidal circuitry are re-established in the fetal GE grafts (**Figures 3A,B**). An additional essential component is the host dopaminergic input. Immunohistochemistry shows that the striatum-like regions of the GE grafts receive a dense synaptic innervation from the host substantia nigra that converges with the cortical input onto the dendrites and spines of the medium spiny neurons in the graft (**Figures 2G,H**; Wictorin et al., 1989b; Clarke and Dunnett, 1993).

Several lines of evidence suggest that the host DA innervation is functional and that it is likely to play the same regulatory role as in the intact striatum. One approach has been to use cellular markers of neuronal function, such as neuropeptide mRNA and c-Fos expression, to monitor the level of afferent dopaminergic control. The DA afferents are known to exert a differential regulation over the two major output pathways: inhibitory for the D2 receptor and PPEmRNA expressing striatopallidal neurons, and excitatory for the D1 receptor and PPTmRNA expressing striatonigral neurons (**Figure 3B**). The dopaminergic control of these two transcripts—down-regulation of PPEmRNA in the D2 neurons and up-regulation of PPTmRNA in the D1 neurons—is as efficient in the striatal grafts as in the normal striatum (Campbell et al., 1992; Liu et al., 1992). In further support, it has been shown that DA releasing drugs (amphetamine and cocaine), which are known to induce c-Fos expression selectively in the D1 bearing striatonigral neurons, are as effective in the grafted animals as in the intact striatum. This effect was abolished by the 6-OHDA lesion of the host nigrostriatal input (Liu et al., 1991; Mandel et al., 1992).

Together, these data show that the striatal efferent projections are re-established by the GE grafts and that they are under the control of the host DA system (**Figures 3A,B**). The sparse graft projection to the host substantia nigra, however, suggests that the functional effects obtained with rat fetal GE grafts are mediated primarily by their pallidal connection.

## Behavioral Evidence for Circuitry Repair

Graft-induced functional recovery has been observed in behavioral tasks at different levels of complexity: locomotor activity, skilled paw use, habit learning, and conditioned motivational behaviors (for review see Dunnett et al., 2000; Reddington et al., 2014). The ability of the GE grafts to restore function across this range of unconditioned and conditioned

motor behaviors constitute the very best example, so far, of functional circuitry repair. As discussed above, the automatic and habitual motor behaviors (monitored in locomotor and paw use tests), habit learning, and conditioned motivational behaviors, are in the intact striatum mediated by different subsectors of the cortico-striatal machinery, converging onto their principal downstream targets, globus pallidus and substantia nigra. The integrated system converging onto the globus pallidus seems to be efficiently restored in the transplanted animals.

On the simplest level, the *locomotor hyperactivity* induced by bilateral striatal lesions can be described as the removal of a tonic inhibitory control of the striatal downstream targets, exerted by the GABAergic striatal projection neurons. This disinhibitory effect is supported by the observation that the activity of the external globus pallidus is markedly increased in striatum-lesioned rats (Isacson et al., 1984; Nakao et al., 1999). Viewed in this way, the normalization of locomotor activity seen in the GE transplanted rats is readily explained by the restitution of inhibitory control of the previously denervated pallidal neurons, mediated by the GABAergic striatopallidal connection formed by the DARPP-32 positive neurons in the grafts (see e.g., Isacson et al., 1986; Reading and Dunnett, 1995; Nakao et al., 1999).

The results obtained in the *skilled paw use test* (**Figure 3C**) are particularly interesting since the performance in this more complex test depends on the integrity of all major components of the striatal machinery, not only the striatum but also the cortico-striatal afferents and the nigral dopaminergic innervation (Whishaw et al., 1986). The recovery seen in the GE grafted rats in this test, therefore, is likely to reflect the functionality of the entire cortico-striato-pallidal circuit, as well as a functional dopaminergic control of this pathway (Montoya et al., 1990; Döbrössy and Dunnett, 2005; Klein et al., 2013). As illustrated in **Figure 3D**, the recovery is well correlated with the volume of DARRPP-32 positive striatal areas and the total number of DARPP-32 positive neurons in the grafts (Nakao et al., 1996; Fricker et al., 1997), and is not observed in rats with grafts of non-striatal tissue (Montoya et al., 1990). The quality of paw reaching, including all components of the movement, grasping and retrieval, was fully restored and equal to the performance of the intact controls (Klein et al., 2013).

The ability of the GE grafts to promote recovery in the paw reaching task is also interesting because it combines elements of motivation, learning, and acquisition of motor habits, as well as the execution of skilled motor behavior, i.e., the separate and interacting elements of skilled, complex movement that are subserved by different sectors of the striatum. It seems possible that a graft placed in the lateral sector of the striatum can interact with the ventromedial striatum/nucleus accumbens sector of the host (which is largely spared in these experiments), and that this interaction can take place at the level of the globus pallidus where the outputs of these parallel circuits converge (see above).

These findings indicate that the host cortical inputs play an important role in the graft-induced recovery of more complex motor behavior. This is further supported by studies using the *delayed alternation task* (**Figure 3E**), a classical learning task that is critically dependent on the prefrontal cortex and its
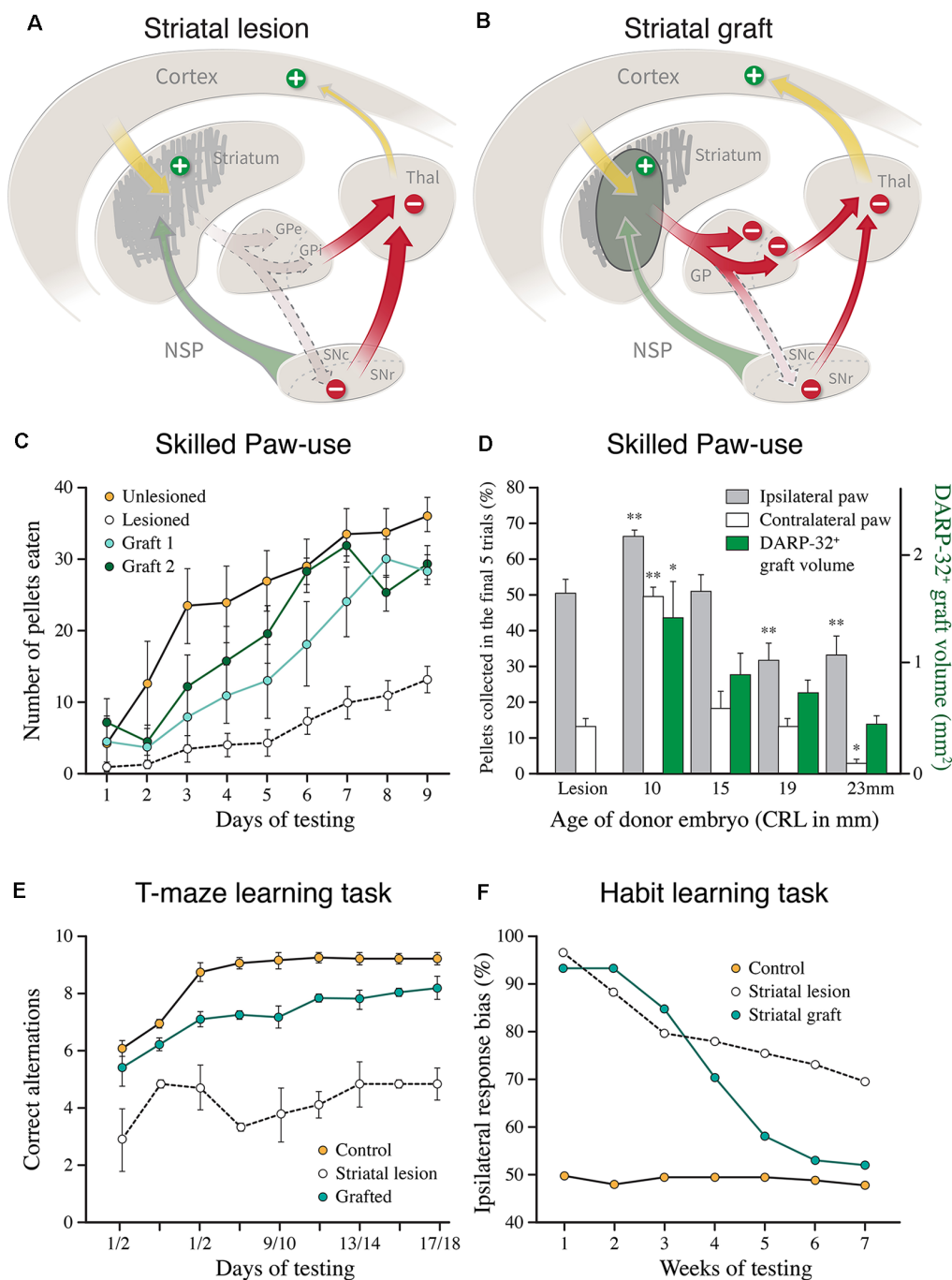
**FIGURE 3 | (A,B)** Cartoons illustrating the disinhibitory effect of the excitotoxic striatal lesion on the downstream targets, globus pallidus (GPe and GPi), and substantia nigra pars reticulata (SNc), and the reversal of this effect induced by the striatal graft. **(C)** Recovery of skilled motor performance is the paw reaching test, as seen in two groups of lesioned and grafted rats, using grafts derived from the lateral ganglionic eminence (LGE) only (modified from Nakao et al., 1996). **(D)** Recovery of the use of the paw contralateral to the lesion and grafted side (open bars) is well correlated to the volume of the DARPP-32+ portion of the fetal GE grafts, obtained from the whole GE at different donor ages (green bars; modified from Fricker et al., 1997). **(E)** Graft-induced recovery in the performance of delayed alternation in the classic T-maze task in rats with bilateral striatal lesions and transplants (modified from Isacson et al., 1986). **(F)** Graft-induced recovery of habit learning in rats with unilateral striatal lesions and transplants. In this test, the grafted animals had to relearn the task over a similar period, 6–8 weeks, as seen in intact rats learning the same task for the first time (modified from Brasted et al., 1999). *$p < 0.05$, **$p < 0.01$.

connections with the medial striatum. Bilateral striatal lesions induce a marked and permanent impairment in this test,

performed either in a T-maze (as shown in **Figure 3E**), or in an operant Skinner box. GE grafts have been shown to restore

the ability of the grafted animals to perform this task (Isacson et al., 1986; Dunnett and White, 2006), and that this recovery is matched by extensive afferent input from the host prefrontal cortex (Dunnett and White, 2006).

Further evidence for circuit reconstruction, and support for the concept of "learning to use the transplant," comes from studies on *habit learning*. The formation and maintenance of motor habits is a characteristic feature of striatal function (Knowlton et al., 1996; Packard and Knowlton, 2002). Studies in Steve Dunnetts lab have used a well-designed stimulus-response association task to explore the loss and recovery of this aspect of motor behavior in rats with unilateral striatal lesions and transplants (for a recent review see Dunnett and Björklund, 2017). These studies show that well-learned motor habits are lost in rats with striatal lesions, but that they can be relearned in the presence of a striatal graft (**Figure 3F**; Brasted et al., 1999, 2000). When tested several months after lesion and transplant the previously trained animals were as impaired as the lesion-only controls. The transplanted rats, however, were able to relearn the task over a similar period, 6–8 weeks, as seen in intact rats learning the same task for the first time, whereas the lesioned rats were unable to relearn even after extensive additional training. These data suggest that the GE grafts re-constitute a new habit-learning system that becomes functionally integrated into the lesioned host circuitry.

Taken together, these studies show that the GE grafts are remarkably effective in restoring both simple automatic and more complex motor behaviors of the type that normally depends on a well-functioning cortico-striato-pallidal circuitry, as well as a functional DA input. It is important, however, to keep in mind that the recovery in most cases is only partial and that the relearning seen in the delayed alternation and habit learning tasks is variable and level off, at a level that is below the optimal performance in the non-lesioned controls. This is perhaps not so surprising given that the fetal rodent GE grafts used in these studies restore only a relatively small fraction of the lost striatal neurons, and that their efferent connectivity is limited to the globus pallidus. i.e., the target of the indirect pathway (**Figure 3B**) while the prime target of the direct pathway, the pars reticulata of the substantia nigra, remains poorly innervated, although both major types of striatal projection neurons, the D1 and D2 expressing ones, are present in fairly equal numbers in these grafts (Campbell et al., 1992, 1995; Liu et al., 1992). Nakao et al. (1996) have estimated that the number of DARPP-32 positive projection neurons in well-functioning grafts amounts to around 30–40% of the lost neurons. This figure was obtained from grafts derived from the lateral part of the GE which yields a higher proportion of DARPP-32 positive tissue in the grafts, in this case about 60% of total graft volume. In most studies using grafts derived from the whole GE the DARPP-32 positive part is less than that, indicating that significant recovery also in complex motor behavior is obtained with grafts that replace as little as 20–30% of the lost striatal projection neurons. Despite these anatomical shortcomings, it is notable that the graft-induced recovery obtained in the best cases matches well that seen in non-lesioned controls.

Although the DARPP-32 positive striatal projection neurons constitute an essential component of a functional striatal graft, it remains unclear to what extent other complementary striatal neuron types, the GABAergicg, and cholinergic interneurons, in particular, may play a role. This remains to be explored, but it is interesting to note that the extent of recovery in the skilled paw use test (as reviewed above) appears to be similar in animals receiving transplants of the whole GE (**Figure 3D**; Fricker et al., 1997) or the lateral GE only (**Figure 3C**; Nakao et al., 1996). As mentioned earlier, these two graft types differ in their interneuron content: the medial part of the GE is the source of the striatal GABAergic and cholinergic interneurons, and as a result, these types of neurons are more or less lacking in the LGE grafts.

## RECONSTRUCTION OF THE NIGROSTRIATAL DOPAMINE PATHWAY IN ANIMAL MODELS OF PD

The nigrostriatal system has been in the focus of brain repair studies since the 1980s when the first exploratory intracerebral DA neuron grafting experiments were performed. It is experimentally attractive for several reasons: there are effective tools, the 6-OHDA and MPTP neurotoxins, that allow complete and selective lesions of the nigral DA projection, and there are accurate and relatively simple behavioral tests to monitor the effects of lesions and transplants. Moreover, the nigrostriatal pathway is strictly unilateral making it possible to use the non-lesioned side as an internal control. The motor impairments induced by lesions of the nigral DA system, which resemble the core motor symptoms in PD, are particularly interesting in the perspective of cell-based repair since they are due to loss of a component of the striatal circuitry that is regulatory, and thus not by itself part of the motor execution machinery. It is common to compare the role of the striatal DA input to the clutch in a car: it is needed to put the engine in gear, while the driving part of the motor is still intact. Thus, in DA lesioned animals as well as in PD patients, the cortico-striato-pallido/nigral circuitry that initiates and executes movements is intact, but its use is impaired or blocked due to the lack of the DA-mediated activating input, which regulates the threshold for the initiation of movement.

This modulatory role of the nigral DA neurons suggests the possibility to reverse the DA-lesion induced motor impairments, at least to some extent, using relatively simple approaches that restore DA neurotransmission. This is supported by the fact that L-DOPA therapy works well in PD patients, showing that tonic activation of striatal DA receptors is sufficient to provide significant symptomatic improvement, at least in the early stages of the disease.

The idea to use transplants of DA neurons to restore striatal DA neurotransmission goes back to the late 1970s. In these early studies, performed in 6-OHDA lesioned rats, fetal ventral mesencephalic (VM) tissue was transplanted either as a solid piece in direct contact with the striatum or as a cell suspension injected into the striatum. The grafted DA neuroblasts continued

to develop in their new location and were seen to form an extensive functional axonal network in the surrounding host striatum. In their ectopic location, however, they were not in the correct position to reconstruct the entire nigrostriatal pathway and its full repertoire of afferent inputs (for review of these early studies see Winkler et al., 2000; Thompson and Björklund, 2012). More complete circuitry repair, as achieved in the striatal transplantation model (see above), is more of a challenge in the nigrostriatal system, since the axons of the nigral DA neurons extend over a relatively long distance along the nigrostriatal pathway to reach the striatum.

Full circuitry reconstruction requires that the grafted neurons are anatomically and functionally integrated into host neural circuitry. The presence of host afferent inputs to intra-striatal DA neuron transplants was initially studied using classical tract-tracing techniques. Although these studies have generated quite a lot of interesting information, it is only now, with the introduction of the monosynaptic rabies tracing technique (Wickersham et al., 2007), that we have access to a tool that can give us a more complete and also more detailed picture of the extent and identity of the afferent inputs to the grafts.

## Graft Composition

The fetal VM tissue commonly used in these studies contains the early progenitors of two major DA neuron types, the A9 neurons of the substantia nigra, and the A10 neurons that reside in the VTA and the medial part of the substantia nigra. In adult rodents, the two populations are present in roughly equal proportions (Björklund and Dunnett, 2007). In mature VM grafts the A9 neurons (Girk2+) are more numerous than the A10 (calbindin+) neurons, usually in the proportion of 2:1 (Grealish et al., 2010; Bye et al., 2012). The two types typically cluster together, with the A9 neurons located in the periphery and the A10 neurons residing in the graft core.

The DA neuron population, however, represents only a minority of the cells contained in mature VM grafts. The non-DA neuron population has not been fully characterized, but it is known that it contains both serotonin-, GABA-, enkephalin- and substance-P-containing neurons, as well as other types that cannot be readily identified based on neurochemical phenotype (for review see Thompson and Björklund, 2012). The relative proportion of serotonin neurons varies depending on the dissection of the VM tissue piece, from about 15–20% of the number of DA neurons as seen in standard VM preparations to about 50% of the DA component when dissection limit of the VM piece extends further caudally. The GABAergic component is likely to contain, in addition to local interneurons, two populations of projection neurons: the GABAergic neurons of the pars reticulata (normally projecting to thalamus and tectum) and the GABAergic neurons present in the VTA that constitute about 1/3 of the neurons in the VTA and are known to project widely to forebrain and brainstem targets (Taylor et al., 2014).

The standard VM grafts are also rich in cells with a glia-like morphology, some of which express the astrocyte marker GFAP. Although it is known from cell culture studies that astrocytes can provide essential trophic support for midbrain DA neurons (see e.g., Takeshima et al., 1994), experiments where the glial

component is eliminated by cell sorting before grafting, or when fetal tissue form very early embryos before the onset of gliogenesis are used, suggest that the DA neurons survive and differentiate also in the virtual absence of glial cells (Thompson et al., 2006; Grealish et al., 2014).

## Target-Directed Axonal Outgrowth Patterns

Regardless of their placement—in striatum or nigra—the graft-derived axonal projections are remarkably specific for their growth trajectory, as well as their innervated targets. The most striking examples come from observations made in rats with transplants of human VM tissue implanted at different sites along the nigrostriatal pathway (Wictorin et al., 1992; Grealish et al., 2014). Grafts placed either in the ventral midbrain or into the bundle project almost exclusively in the rostral direction and extend their axons along the course of the nigrostriatal pathway, toward the striatum, and along with the medial forebrain bundle (MFB) toward the normally DA-innervated areas of the limbic forebrain, matching well the distribution of DA projections in the intact brain (**Figure 4**).

The areas innervated by the VM grafts include territories that normally receive afferents from either the A9 neurons in the substantia nigra (i.e., dorsolateral caudate-putamen; CPu in **Figure 4**) or the A10 neurons in the VTA (i.e., nc. accumbens and prefrontal cortex; NAc and PFC in **Figure 4**). Both A9 and A10 neurons are present in the graft (see above) and there is evidence from tracing studies that the innervations derived from the two subtypes match the normal projection patterns, such that the A9 neurons innervate caudate-putamen and the A10 neurons cortical and limbic areas (Thompson et al., 2005; Grealish et al., 2010, 2014). These observations indicate the presence of axon guidance and target recognition mechanisms in the DA-denervated forebrain that can guide the growing axons to their appropriate targets and that these mechanisms are sufficiently specific and refined to distinguish between the A9 and A10 subtypes.

The demarcation of the innervated territories is remarkably precise: axons extending along the bundles of the internal capsule are seen to branch into fine-caliber beaded terminals as they pass the border between the globus pallidus and the caudate-putamen (**Figures 4B–D**), and in animals, with grafts placed in the caudate-putamen the graft-derived TH-positive innervation stops precisely at the border to the globus pallidus (Gage et al., 1983; Wictorin et al., 1992). Notably, studies comparing the performance of VM grafts in the intact and the DA-denervated striatum have shown that the density and extent of the graft-derived innervation are markedly increased in the absence of the intrinsic nigral input (Doucet et al., 1990; Thompson et al., 2005).

Although not studied in detail, it is clear that also the non-DA neurons in the VM grafts contribute to graft-host connectivity (Thompson et al., 2008; Grealish et al., 2014). Retrograde tracing studies indicate that these non-dopaminergic projections originate from GABAergic neurons contained in the grafts, and their wide-spread projection patterns, as revealed by species-specific antibodies in mouse-to-rat and human-to-rat grafts, suggest that they, at least in part, are derived

from GABAergic neurons identical to the ones normally residing in the VTA and the pars reticulata of the substantia nigra (Thompson et al., 2008).

## Afferent Host-to-Graft Connectivity

From the outset, it has been assumed that DA neurons transplanted ectopically into the striatum are largely devoid of regulatory afferent inputs. In support of this idea, the early microdialysis studies showed that DA is released from intrastriatal VM grafts in an autoregulated fashion, suggesting that the grafted DA neurons maintain the capacity for regulated transmitter release even in the absence of afferent inputs (Zetterström et al., 1986; Strecker et al., 1987). A subsequent study in awake, behaving animals, however, showed that their activity as monitored by changes in DA release can be modulated during different types of behavior (Cenci et al., 1994). Although the behavior-related responses were lower in magnitude, and also less consistent than in intact rats, they suggest that the grafted neurons are reached by physiologically relevant inputs from the host brain. This finding is in line with electrophysiological studies showing that the majority of intrastriatal grafted DA neurons maintain their firing properties, including pacemaker activity, and that they show electrophysiological responses following stimulation of either host striatum or cortex (Fisher et al., 1991; Sorensen et al., 2005).

Anatomical data on the extent of host afferent inputs to fetal VM grafts are very sparse and limited to a single tract-tracing study (Doucet et al., 1989). As discussed further below, more recent studies performed using the more powerful monosynaptic rabies tracing method have shown abundant host inputs to human embryonic stem cell (hESC)-derived DA neuron grafts placed either in the striatum or the ventral midbrain (Grealish et al., 2015; Cardoso et al., 2018; Adler et al., 2019). For technical reasons, it has so far not been possible to perform similar rabies-based tracing studies on fetal VM grafts. The Doucet et al.'s (1989) study, using Phaseolus Vulgaris Leucoagglutinin (PHA-L) as an anterograde tracer, provides evidence for a fairly rich synaptic innervation from the host frontal cortex, as well as a limited serotonergic input to intrastriatal VM grafts, but other potential sources of afferents were not explored in this study. Nevertheless, the data we have from rabies tracing studies of hESC-derived DA neuron grafts, as discussed below, point to the capacity of grafted DA neurons to become extensively integrated into host circuitry.

## Restoration of DA Neurotransmission

Studies performed during the 1980s and 1990s had shown that the intrastriatal implanted nigral DA neurons are actively secreting their transmitter, and that part of this release may take place at morphologically normal synaptic sites. More direct evidence that graft-derived DA release is functional was obtained in studies using quantitative receptor-ligand binding techniques and *in situ* hybridization histochemistry. Lesions of the nigrostriatal DA pathway or blockade of striatal DA neurotransmission are known to lead to long-lasting postsynaptic modifications in the striatal target neurons. One such modification is the denervation-induced upregulation of

postsynaptic DA receptors located on the striatal target neurons. This receptor supersensitivity is normalized by DA neuron grafts, most pronounced for the D2 receptors (Dawson et al., 1991; Rioux et al., 1991; Chritin et al., 1992; Savasta et al., 1992). This is consistent with the ability of DA neuron grafts to reduce turning behavior in unilateral 6-OHDA lesioned rats in response to either D1 or D2 (or mixed) receptor agonists. Rioux et al. (1991) reported parallel reductions in D1 and D2 agonist-induced turning and striatal receptor ligand-binding in rats with long-term surviving fetal VM grafts, suggesting that these parameters are closely linked.

The long-lasting functional changes seen in response to dopaminergic denervation include an increase in GABA turnover, accompanied by an increase in glutamic acid decarboxylase (GAD) enzyme activity and GAD mRNA expression in the DA-denervated striatum. Similarly, the enkephalin neurons, which constitute a subset of the D2 expressing GABAergic projection neurons projecting to the globus pallidus, express increased peptide levels as well as increased proenkephalin mRNA. This is maintained over a long time indicating that enkephalin synthesis is permanently increased in the striatopallidal projection neurons after removal of the striatal DA afferents. These changes are completely reversed by intrastriatal nigral transplants, thus providing further support that the grafted nigral neurons can restore normal inhibitory control over the host striatopallidal projection neurons (Cenci et al., 1993, 1997; Winkler et al., 2003).

The substance P containing striatonigral neurons respond to DA denervation in the opposite direction, i.e., by a reduction in peptide levels and RNA message. These effects are consistent with the current view that the two major subsets of striatal projection neurons are differentially regulated by the host nigral DA afferents, such that the striatopallidal enkephalin neurons are tonically inhibited and the striatonigral substance P neurons tonically activated by the DA input. In behaviorally functional grafts (as assessed by the rotation test) the effect on substance P synthesis is partial and restricted to the area receiving a dense DA input, while the increased enkephalin synthesis is fully normalized also outside the graft-reinnervated area, indicating that DA released from the graft-derived terminal network can reach functional levels over areas that extend well beyond that covered by the outgrowing fibers (Cenci et al., 1993; Strömberg et al., 2000).

Taken together, these findings point to two complementary modes of action: the diffuse release of DA by so-called volume transmission, acting primarily on the high-affinity D2 receptors, and regulated DA release at specialized synaptic sites involving both D1 and D2 receptors. As discussed further below, both mechanisms are likely needed to obtain the full extent of graft-induced behavioral recovery.

## Behavioral Correlates of Circuitry Repair

Drug-induced rotation is the most commonly used test to assess DA neuron function. In this test, an active turning behavior is induced in animals with a unilateral lesion of the nigrostriatal pathway using either the DA releasing drug, amphetamine, or the D1/D2 receptor agonist, apomorphine. In both cases, the
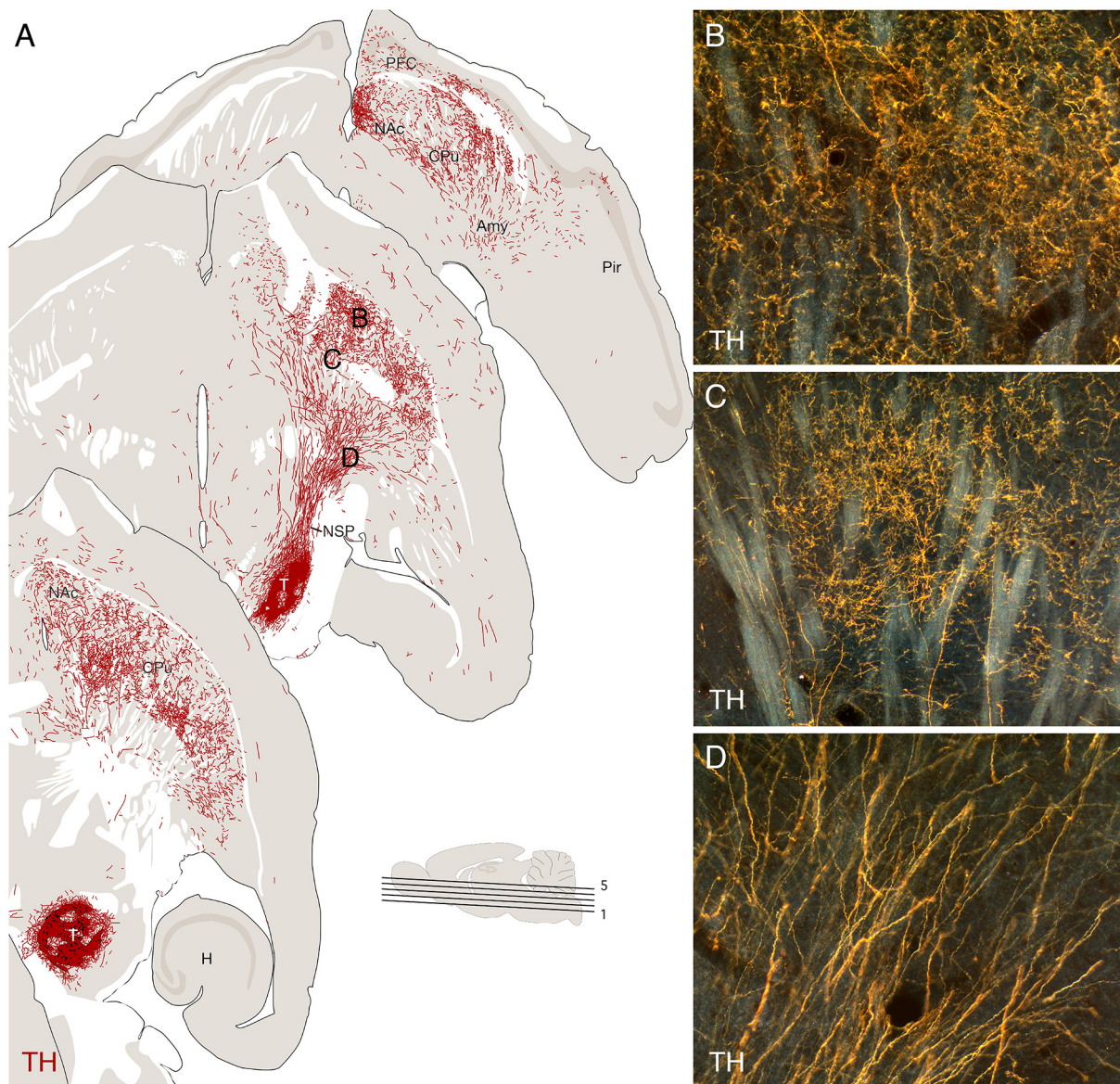
**FIGURE 4 |** Re-establishment of the nigrostriatal pathway from a transplant of mouse fetal ventral mesencephalic (VM), implanted as a cell suspension in substantia nigra of a 6-OHDA lesioned mouse, 16 weeks post-grafting. The VM tissue was obtained from a transgenic Pitx3-green fluorescent protein (GFP) mouse allowing the outgrowing axons to be visualized using GFP immunostaining, as illustrated in the computer-assisted drawings derived from three horizontal sections in panel **(A)**. The micrographs in panels **(B–D)** are taken from the areas marked in panel **(A)**. Amy, amygdala; CPu, caudate-putamen; H, hippocampus; NAc, nc. Accumbens; NSP, nigrostriatal pathway; Pir, piriform cortex. Modified and redrawn from Thompson et al. (2009).

functional recovery obtained with intrastriatal or intranigral VM grafts is readily explained by tonic activation of DA receptors mediated by volume transmission. Indeed, Savasta et al. (1992) have shown that the recovery in the agonist-induced rotation is well correlated with the normalization of D2 receptor binding in the grafted striatum and that this normalization involves also the non-reinnervated areas. As reviewed elsewhere (Björklund and Dunnett, 2019), full recovery in the drug-induced rotation is obtained with transplants that contain less than 500 DA neurons (i.e., about 4% of

the normal number of nigral DA neurons in the rat) and restore as little as 5% of the normal striatal DA content. These transplants are too small to induce recovery in the standard tests of spontaneous motor or sensorimotor behavior. Significant recovery in these tests is seen only in animals with transplants rich in DA neurons that provide more extensive striatal reinnervation, suggesting that the extent of reinnervation, i.e., functional synaptic inputs, play an important role. This is further supported by extracellular recordings showing that the increased striatal neuron activity seen in the lesioned rats

have normalized in the graft reinnervated area, but not in the non-reinnervated part (Di Loreto et al., 1996; Strömberg et al., 2000). In line with these observations, it has been shown that the functional impact depends on the area innervated by the grafted DA neurons. As illustrated in **Figure 5**, it has been shown that the recovery of individual components of the DA lesion syndrome is determined by the subregion reinnervated by the transplant; dorsomedial vs. ventrolateral striatum in the rat (Mandel et al., 1990), and caudate vs. putamen in the marmoset (Annett et al., 1995).
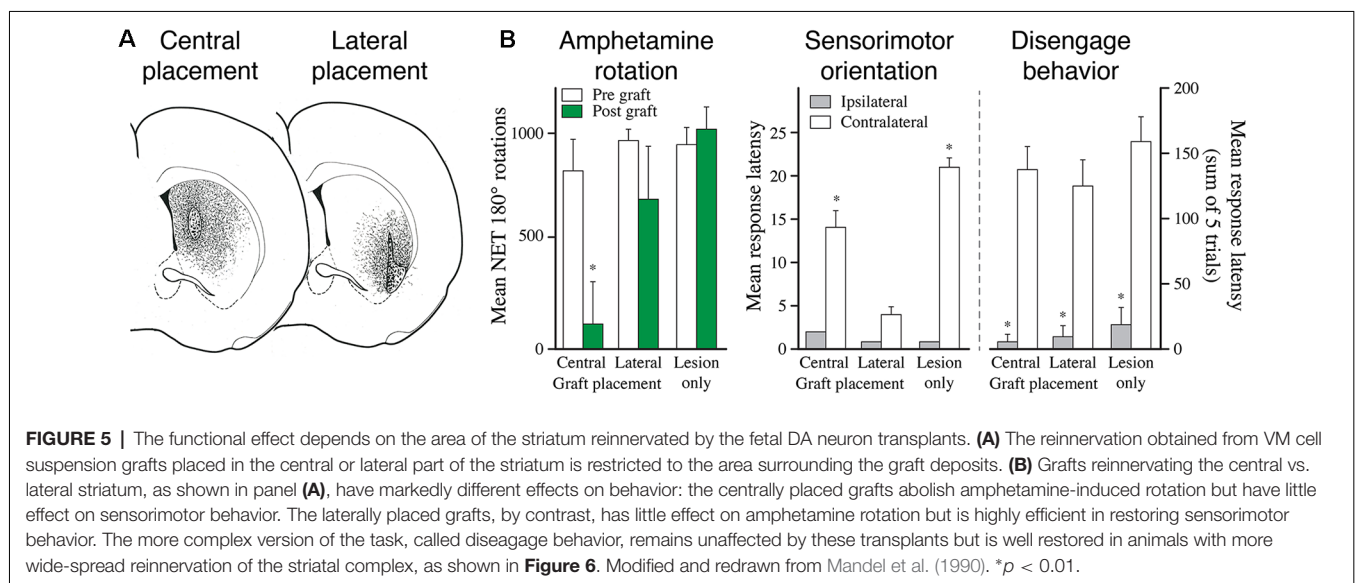
The synaptic contacts formed by the grafted DA neurons on the host striatal projection neurons resemble, in part, those present in the intact striatum, although their relative distribution is different—less frequent on dendrites and more abundant on the neuronal perikarya (Freund et al., 1985; Mahalik et al., 1985; Clarke et al., 1988a). The synapses made on dendritic spines, which constitutes about 40% of all TH-positive synapses formed, resemble those seen in normal animals, both in that they make contacts with spine necks and in that they are associated with an asymmetric TH-negative synapse contacting the spine head. This resembles the arrangement seen in the intact striatum where dopaminergic and cortical glutamatergic inputs converge onto the same spines (Smith and Bolam, 1990; Xu et al., 2012), suggesting the creation of a local microcircuitry allowing the graft-derived DA innervation to interact with the host corticostriatal input.

In a more recent study, Rylander et al. (2013) have added an interesting dimension to these early findings, showing that a region-specific and DA-dependent long-term potentiation (LTP), which is abolished in 6-OHDA lesioned rats, is fully restored in the grafted animals. This form of synaptic plasticity is NMDA receptor (i.e., glutamate) dependent and mediated by D1 receptors. Interestingly, this effect was limited to the most densely reinnervated (ventrolateral) part of the striatum where the TH-positive innervation density was restored to over 60%. This is consistent with observations in animals with partial 6-OHDA lesions showing that induction of LTP in the striatal projection neurons is critically dependent on a rich DA innervation (Paillé et al., 2010). It seems possible that this mechanism is involved in the establishment and maintenance of stimulus-response habits, which is a characteristic feature of the dorsolateral sector of the striatum (Redgrave et al., 2010; see above). In line with this idea, Dowd and Dunnett (2004) have shown that DA neuron transplants targeting this striatal subregion are efficient in restoring learned, goal-directed behavior, assessed in a task that involves the selection, initiation, and execution of conditioned responses on either side of rat's head, a habit learning task that is likely to depend on the local interaction between dopaminergic and glutaminergic (i.e., cortical) afferents.

## Limitations to the Functionality of the Intrastriatal DA Neuron Grafts

Available data indicate that a striatal DA reinnervation in the order of 20–30% of normal is necessary to obtain measurable improvements in tests of spontaneous motor behavior, such a forelimb stepping, and paw-use. As illustrated in **Figure 6**, such a more wide-spread reinnervation can be obtained with VM tissue implanted at multiple sites in the striatum. Using multiple graft placements it is possible to restore striatal DA innervation density up to 60–70% of normal throughout the striatum, but even in such cases the recovery of more complex motor behavior is incomplete (Winkler et al., 1999). One factor that may play a role is the extent of the graft-derived reinnervation outside the caudate-putamen. The innervation generated by intrastriatal DA neuron grafts in the MFB lesioned rats (the lesion most commonly used in these studies) is confined to the dorsal striatum, while other DA-innervated forebrain areas, including nc. accumbens, olfactory tubercle, and the frontal and cingulate cortex, remain denervated. Indeed, in rats with partial 6-OHDA lesions where the projections to limbic and cortical areas are left intact, it has been shown that the magnitude of graft-induced



**FIGURE 5 |** The functional effect depends on the area of the striatum reinnervated by the fetal DA neuron transplants. **(A)** The reinnervation obtained from VM cell suspension grafts placed in the central or lateral part of the striatum is restricted to the area surrounding the graft deposits. **(B)** Grafts reinnervating the central vs. lateral striatum, as shown in panel **(A)**, have markedly different effects on behavior: the centrally placed grafts abolish amphetamine-induced rotation but have little effect on sensorimotor behavior. The laterally placed grafts, by contrast, has little effect on amphetamine rotation but is highly efficient in restoring sensorimotor behavior. The more complex version of the task, called diseagage behavior, remains unaffected by these transplants but is well restored in animals with more wide-spread reinnervation of the striatal complex, as shown in **Figure 6**. Modified and redrawn from Mandel et al. (1990). *$p < 0.01$.
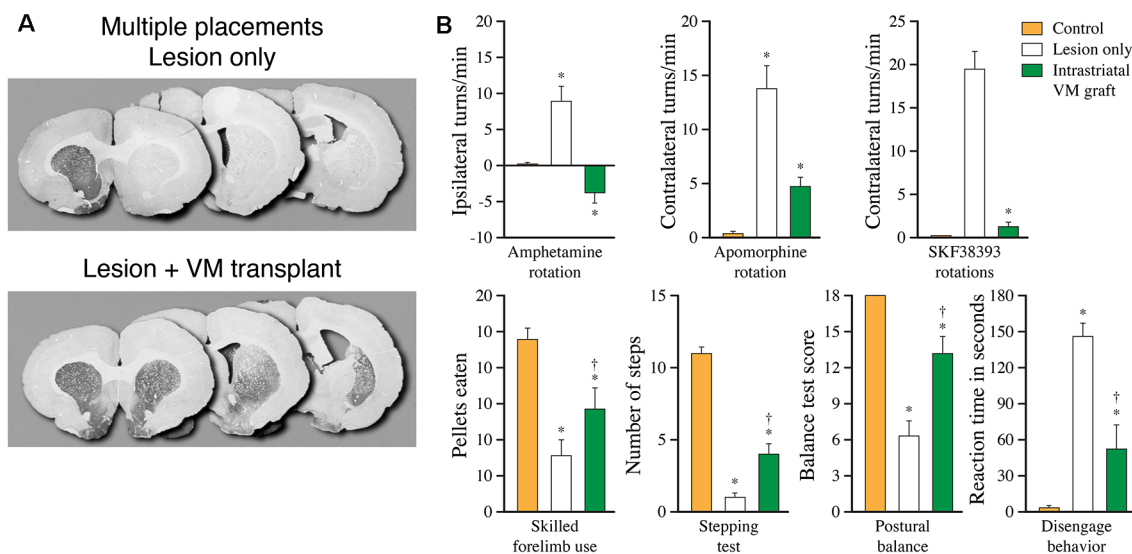
**FIGURE 6 |** More complete recovery can be obtained by spreading the graft tissue over multiple implantation sites. **(A)** Extent of graft induced reinnervation obtained with fetal VM grafts spread over seven injection sites distributed over the entire striatum, including the nc. accumbens, 10 months post-grafting. **(B)** In these animals, significant functional recovery is seen in a broad range of drug-induced and spontaneous motor tests, but remains incomplete in most of the tests Data compiled from Winkler et al. (1999). *Different from Control at $p < 0.05$; †different from Lesion only at $p < 0.05$.

recovery is more pronounced as assessed in the stepping and paw-use tests. Importantly, when the lesion, in a second step, was extended to remove the spared limbic and cortical projection is fully recovered animals, the graft-induced improvement in the two tests was partially lost (Kirik et al., 2001; Breysse et al., 2007). These data suggest that the extent of denervation outside the dorsal striatum will have an impact on the functional outcome, not only in the experimental setting but also in grafted PD patients. In support of this idea, Piccini et al. (2005) have reported that the best functional outcome in grafted PD patients is seen in subjects where the DA innervation in areas outside the grafted region is well preserved, as determined by FluoroDopa PET.

The most obvious limitation to the functionality of the intrastriatal grafts, however, is their ectopic location. As discussed further below, it has been shown that hESC-derived DA neuron grafts receive abundant host afferent inputs even in this ectopic location (Grealish et al., 2014; Adler et al., 2019). Full circuitry reconstruction, however, will require that the cells are placed in their normal location, i.e., the ventral midbrain. Progress in this field has been hampered by technical problems. In the early studies using rat fetal DA neurons grafted to the substantia nigra (Nikkhah et al., 1994; Winkler et al., 1999) no or very limited axonal growth was observed along the nigrostriatal pathway. From these studies, it became clear that fetal *rat* DA neurons do not have the growth capacity, or do not grow for a sufficiently long time, to reach the striatum in adult rats (although they manage to do so in neonatal rats; Bentlage et al., 1999). Fetal *mouse* DA neurons, by contrast, grow axons efficiently along the nigrostriatal pathway in adult mice (see **Figure 4**), but fail to do so in adult rats (Gaillard et al., 2009; Thompson et al., 2009). Nevertheless, it is possible to achieve the full reconstruction of the nigrostriatal pathway in rats using

DA neurons of either human or porcine origin, i.e., neurons that intrinsically possess greater growth capacity and extend their growing axons over a longer period (Wictorin et al., 1989b; Isacson et al., 1995; Grealish et al., 2014).

The extent and origins of afferent inputs to intranigral grafts of fetal VM tissue have so far not been explored, and the functional impact of intranigral VM grafts has been limited to a single study in the mouse showing recovery in the amphetamine rotation test (Thompson et al., 2009). With the current emphasis on cells derived from pluripotent human stem cells (hPSCs), work along this line is likely to be pursued in studies using hPSCs rather than fetal VM tissue grafts. With the introduction of the monosynaptic rabies tracing technique, we have now access to a tool that can give us a more complete and also more detailed picture of the regulatory afferent inputs to intranigral DA neuron grafts.

# STUDIES USING CELLS DERIVED FROM HUMAN PSCs

The studies reviewed in the previous sections were mostly performed during the last decades of the past century. They were all based on the use of cells from the fetal brain, and the clinical trials in PD and HD patients have also been based on the use of fetal tissue. The experience gained from these trials has been highly valuable and provided important proof-of-principle that the neuronal replacement approach can work, at least in the case of PD. However, the use of fetal tissues for transplantation in patients is both ethically and practically problematic—they are difficult to obtain in sufficient numbers, impossible to standardize and quality control, and cannot be scaled up for routine clinical use. Further progress in this field, therefore,

is critically dependent on the development of a scalable cell source specifically produced for use in patients. The discovery and derivation of hESCs in 1998 (Thomson et al., 1998) and the subsequent generation of induced pluripotent stem cells (iPSCs) in 2006 (Takahashi and Yamanaka, 2006) have revolutionized the field and provided new powerful tools for the derivation of virtually any cell type in the body, including the ones relevant for basal ganglia repair.

## Generation of Midbrain DA and Striatal Projection Neurons From Human PSCs

The development of protocols for the generation of midbrain DA (mDA) neurons and striatal projection neurons from hPSCs have progressed in parallel. The first attempts to generate mDA neurons from hESCs were based on protocols developed for mouse ESCs (see e.g., Kawasaki et al., 2000; Kim et al., 2002). Although they generated relatively large numbers of TH-expressing neurons *in vitro*, their midbrain properties were not clear, and they performed poorly after grafting. It is known from early experimental work that fully functional DA neuron transplants need to be of the midbrain A9 and A10 type. Thus, DA neurons of other phenotypes (hypothalamic or forebrain) do not show the ability to reinnervate the striatum in a target-specific manner, a property that is necessary for behavioral recovery.

An important breakthrough came in 2007–2008 with the discovery of the midbrain floor plate cell as the unique cellular progenitor of mDA neurons (Ono et al., 2007; Bonilla et al., 2008), which led to the development of protocols for the generation of floorplate cells (Fasano et al., 2010) and subsequently to DA neurons with an authentic midbrain phenotype (Cooper et al., 2010; Kriks et al., 2011; Kirkeby et al., 2012). These floorplate-derived cells express specific markers of mDA progenitors *in vitro* and their performance—survival, growth, and function—after transplantation in rodent and primate PD models matches very well that seen with authentic fetal mDA neurons (see e.g., Sundberg et al., 2013; Grealish et al., 2014; Chen et al., 2016; Kikuchi et al., 2017). With further refinement and transfer to production under GMP-compliant conditions completed, these cell products are now ready for use in patient trials (Kirkeby et al., 2017b; Studer, 2017; Takahashi, 2017).

The development of protocols for the generation of hPSC-derived striatal projection neurons is based on the same principle, i.e., to mimic the endogenous developmental process that takes place in the fetal ganglionic eminence as closely as possible (for review see Li and Rosser, 2017). In the most efficient protocols published so far striatal projection neurons, characterized by their DARPP-32 expression in combination with CTIP2 and/or GABA, are generated by modulation of the Sonic Hedgehog (SHH) and/or WNT signaling pathways (Ma et al., 2012; Delli Carri et al., 2013; Nicoleau et al., 2013; Adil et al., 2018; Wu et al., 2018). In an interesting alternative approach, Arber et al. (2015) have replaced the ventralizing factor SSH with a lateralizing factor, Activin A, to obtain cells with a striatal projection neuron fate.

The hESC-derived striatal progenitors survive and mature after transplantation to the lesioned rat or mouse striatum. The cells proliferate over the first weeks and as a result, the grafts expand in size to compensate for the striatal projection neuron cell loss caused by the excitotoxic lesion. Continued cell proliferation and signs of graft overgrowth have been an issue in some cases (Delli Carri et al., 2013; Nicoleau et al., 2013) suggesting that these graft preparations contain immature cells that fail to terminally differentiate. The percentage of DARPP-32+/CTIP2+ neurons generated in these protocols vary between 20 and 60%. Also, the grafts have been shown to contain other neuronal types, including GABAergic interneurons (Besusso et al., 2020), and in one case also a significant component (27%) of GFAP-positive astrocytes (Adil et al., 2018).

The expression pattern of the DARPP-23 positive cells indicates that they are striatal projection neurons of the type normally generated by the lateral ganglionic eminence (LGE). This is further supported by their ability to establish axonal connections with downstream striatal targets, including globus pallidus and substantia nigra, accompanied by a gradual improvement in measures of sensorimotor performance on the side opposite to the excitotoxic lesion and transplantation (Ma et al., 2012; Adil et al., 2018; Besusso et al., 2020). Although the extent and functionality of the graft-derived connectivity need to be explored in greater detail, the recent study by Besusso et al. (2020), using a combination of immunohistochemistry and monosynaptic rabies virus tracing, has provided initial evidence that the hESC-derived striatal grafts can become well integrated into the lesioned host striatal circuitry.

The extent of functional recovery seen in studies using fetal GE grafts, as reviewed above, has been mostly obtained with grafts that contain precursors from both the lateral and medial parts of the ganglionic eminence. While the striatal projection neurons normally arise from the LGE, the MGE is the source of the complementary populations of GABAergic and cholinergic interneurons, and as a result, the fetal GE tissue grafts contain also these types of interneurons. Although striatal projection neurons are an essential component, a fully functional striatal transplant is likely to contain a complement of regulatory interneurons, and possibly also supportive glial cells, that can form a functional unit with the capacity to replace the damaged host striatum. The optimal composition of such grafts, mimicking the fetal GE graft preparations, has yet to be experimentally determined.

## Integration and Function of hESC-Derived DA Neuron Transplants

The graft-host connectivity of hESC-derived mDA neurons generated in the different versions of the floorplate protocols have been studied in a xenograft setting, either in 6-OHDA lesioned rats and mice in combination with immunosuppressive treatment, or immunodeficient rats and mice (without the need of immunosuppression), after transplantation either to the striatum (Kriks et al., 2011; Sundberg et al., 2013; Grealish et al., 2014; Steinbeck et al., 2015; Chen et al., 2016; Niclis et al., 2017) or to the substantia nigra (Grealish et al., 2014; Cardoso et al., 2018; Adler et al., 2019). Similar results have also

been obtained in MPTP-lesioned monkeys using hiPSC-derived, or primate iPSC-derived, DA neurons (Sundberg et al., 2013; Kikuchi et al., 2017). The performance of hESC-derived mDA neurons has been possible to compare head-to-head with that of the authentic mDA neuroblasts obtained from fetal human VM, showing that their survival, growth, and function, as well as their efferent connectivity pattern, match each other very closely (Grealish et al., 2014; Cardoso et al., 2018).

Transplants of human fetal or hPSC-derived mDA neurons mature slowly. As shown in **Figures 7B,C**, it takes, in rats, about 4–5 months before the connections become fully functional (compared to about 1 month in transplants of rat fetal VM, see **Figure 7A**), and it takes even longer, up to a year, in MPTP-lesioned monkeys where the growth distances are considerably larger (Kriks et al., 2011; Kirkeby et al., 2012; Sundberg et al., 2013; Lelos et al., 2016; Kikuchi et al., 2017). The axonal growth patterns from these transplants are highly specific and include innervation of developmentally appropriate targets of both A9 and A10 DA neurons (**Figures 7D,E**), indicating that the mDA neurons generated in the currently used floorplate-based protocols include the two major mDA neuron subtypes in proportions similar to the fetal VM grafts (Grealish et al., 2014; Niclis et al., 2017; Cardoso et al., 2018).

This is further supported by the observation that hESC-derived mDA neurons transplanted to the substantia nigra have the same capacity to extend axons along the nigrostriatal pathway and the MFB as the fetal VM grafts, and the same ability to reinnervate the appropriate A9 and A10 targets. Axonal tracing using human-specific antibodies (hNCAM) in combination with TH immunohistochemistry (Grealish et al., 2014), or use of a Pitx3-GFP expressing hES cell line (Niclis et al., 2017), has shown that the projections are derived from both DA and non-DA neurons. As in fetal VM grafts (see above) the widespread projections of the non-DA neuron component suggest that they are generated by the GABAergic neurons normally present in the VM, above all the GABAergic component of the VTA and the pars reticulata of the substantia nigra.

The functionality of the hPSC-derived mDA neuron grafts is further supported by experiments where the activity of the grafted cells is modulated using either optogenetics (Steinbeck et al., 2015) or chemogenetics (DREADDs; Chen et al., 2016). In these studies, which are summarized in **Figure 8**, the recovered motor performance was completely reversed by optogenetic or chemogenetic inhibition of the hPSC-derived mDA neurons, and in the Chen et al.'s (2016) study it was shown that the graft-induced motor
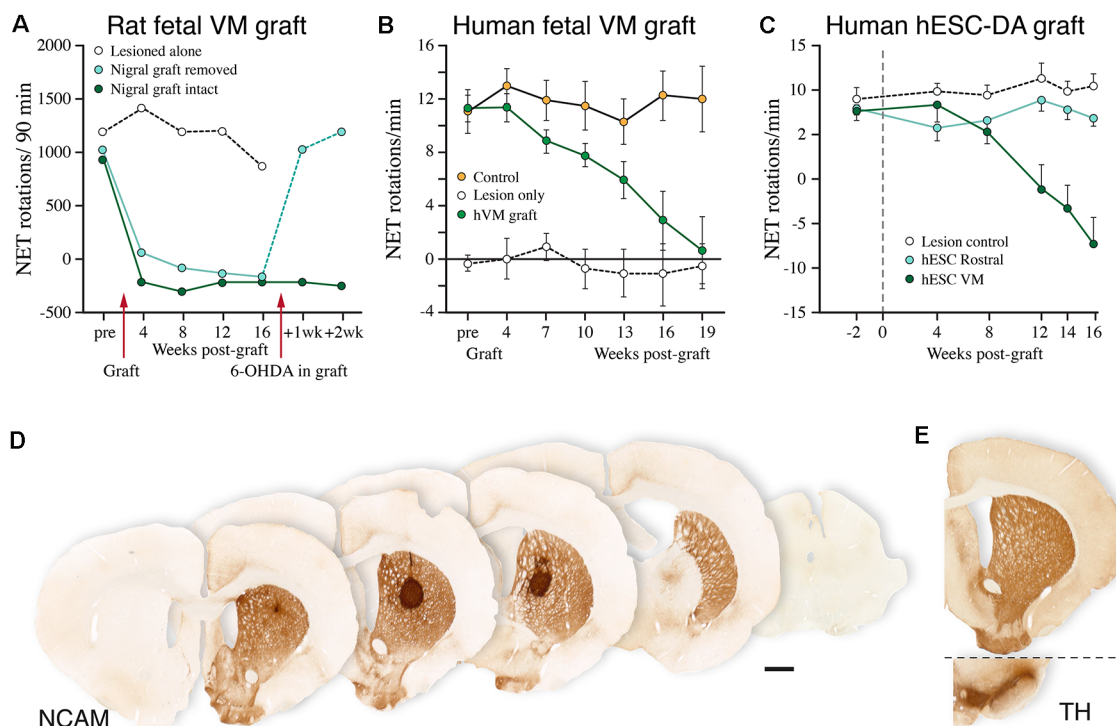


**FIGURE 7 |** Performance of human fetal and human embryonic stem cell (hESC)-derived DA neurons grafted to the striatum in the rat 6-OHDA lesion model. **(A–C)** Time-course of functional recovery in the amphetamine rotation test obtained with fetal rat VM transplants **(A)**, human fetal VM transplants **(B)**, and hESC-derived DA neuron transplants **(C)**. The time-course of recovery is notably similar for the fetal and hESC-derived human DA neurons, but much slower than that seen with rat DA neurons. In the experiment shown in panel **(A)**, the original functional deficit returned within a week after the graft had been removed with a second 6-OHDA lesion. **(D)** A single deposit of hESC-derived DA neurons is sufficient to reinnervate the entire striatum in the rat PD model, as visualized using a human-specific NCAM antibody. The graft-derived innervation pattern in **(D)** is notably similar to the distribution of the endogenous TH-positive innervation, as shown in **(E**; adapted from Nolbrant et al., 2017). Data compiled from Dunnett et al. (1988; **A**), Lelos et al. (2016; **B**), and Kirkeby et al. (2012; **C**).

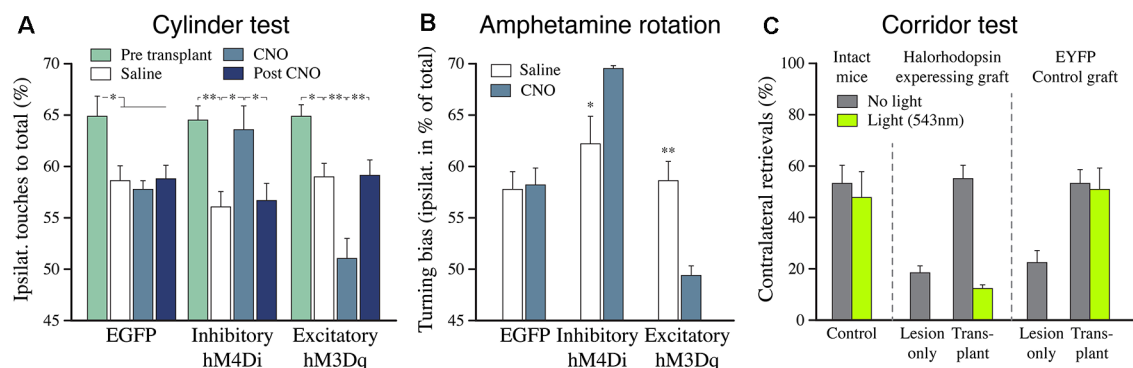**FIGURE 8 |** The functional impact of hESC-derived DA neuron grafts is tunable using chemo-and optogenetics. **(A)** The recovery of paw use in the cylinder test (contralateral to the 6-OHDA lesion) is abolished when the activity of the grafted DA neurons is inhibited by CNO (hM4Di), and it is further potentiated when the activity is increased by CNO (hM3Dq). **(B)** The effect of inhibition and activation of the grafted human DA neurons is similar in the amphetamine rotation test. The 6-OHDA-induced ipsilateral turning bias is abolished by the grafted DA neurons in all three groups (open bars). Activation of the inhibitory DREADD blocks the graft effect, seen as induction of an ipsilateral turning bias (similar to what is seen in lesioned controls), and activation of the excitatory DREADD potentiates the graft effect, seen as induction of turning in the direction away from the transplant. **(C)** The graft-induced recovery in sensorimotor performance seen in the corridor test (gray bars) is completely blocked when the activity of the grafted DA neurons is inhibited by light (green bars). Data in panels **(A,B)** are redrawn from Chen et al. (2016), data in panel **(C)** redrawn from Steinbeck et al. (2015). *$p < 0.05$, **$p < 0.01$.

improvement could be further enhanced by DREADD-induced stimulation of mDA neuron function. Using electrophysiological recording in slices they moved on to show that these effects were synaptically mediated *via* DA receptor activation and that the grafted neurons modulate host glutamatergic transmission onto striatal projection neurons in a manner that is reminiscent of DA neurotransmission in the intact striatum.

These studies have been performed on grafts placed in the striatum, i.e., that same ectopic location as used in the clinical trials. It is commonly assumed that the functional impact of ectopically placed DA neuron grafts is mediated by an autoregulated, tonic activity and that they may not need access to regulatory afferent inputs that are available to the mDA neurons in their normal location in the midbrain. Recent studies using monosynaptic rabies tracing have challenged this view, showing that intrastriatal hESC-derived dopaminergic grafts receive synaptic inputs from subtypes of cortical, striatal and pallidal neurons that are known to regulate the function of the endogenous nigral DA neurons (Grealish et al., 2015; Adler et al., 2019). These regulatory inputs are known to send collateral within the striatum, which makes it possible for the intrastriatal grafts to receive inputs from functionally appropriate subtypes of excitatory (cortical) and inhibitory (striatal and pallidal) neurons in the host. Indeed, in a dual tracer approach, Adler et al. (2019) could show that the host synaptic inputs to intrastriatal grafts are derived, at least in part, from the very same cortical, striatal and pallidal neurons that innervate the host substantia nigra.

## FUTURE PERSPECTIVES

The extent of graft-host connectivity obtained with intrastriatal grafts of fetal GE tissue is an intriguing example that more

complete functional circuitry repair is possible to achieve in the lesioned rodent brain. As discussed above, the GE grafts are remarkably effective in restoring both simple automatic and more complex motor behaviors of the type that normally depends on a well functioning cortico-striato-pallidal circuitry, as well as a functional DA input. In most of the behavioral tasks used in these studies, however, the recovery seen with grafts of rodent GE tissue is only partial and obtained with grafts that replace as little as 20–30% of the lost striatal projection neurons, and the graft-derived connectivity is mainly restricted to one of the principal output targets, the globus pallidus, while the substantia nigra is poorly re-innervated.

Grafting in the excitotoxic lesion model has so far been on the replacement of the lost DARPP32 expressing striatal projection neurons. Although the projection neurons are an essential component a fully functional striatal graft likely has to include also the different types of interneurons that are part of the striatal microcircuitry in the intact striatum. The hPSC-derived striatal graft preparations used so far been have been poorly characterized in this regard, and the current protocols likely need to be further developed to achieve a more optimal cellular composition that mimics the neuronal diversity seen in the intact striatum. In contrast to the mDA neuron grafts used in the PD model, the striatal grafts used in the striatal lesion model should be viewed not as simple single neuron replacement, but as a tool to restore a unit of local striatal microcircuitry, composed of both NSPs and regulatory interneurons, that is functionally integrated into the host basal ganglia circuitry.

Complete circuitry reconstruction is more challenging in the PD model since the normal location of the nigral DA neurons is at a relatively large distance from their striatal targets. Full circuitry reconstruction will require that the DA

neurons are placed in their normal location, i.e., the ventral midbrain. As reviewed above, in rodent models, the hESC- or fetal-derived human mDA neurons grafted to the nigra can extend their axons along the nigrostriatal pathway and the MFB and establish connections with all normally DA-innervated forebrain targets and receive abundant synaptic inputs from the host. The overall distribution of host-to-graft connections as revealed by rabies tracing matches well the endogenous nigral afferent circuitry, suggesting the possibility for more complete circuitry repair. Whether similar connectivity can be established in the much larger human brain remains to be investigated: the distance between substantia nigra and striatum is about 10-fold larger in humans than in rats, 3–3.5 mm in the rat compared to 3–3.5 cm in the human brain. Whether intranigral grafts are functionally superior to grafts placed in the common intrastriatal location needs also to be clarified. It seems possible that intranigral grafts could have a broader functional impact, e.g., on the recovery of more complex motor behavior and non-motor functions, than the intrastriatal ones, but that is so far not known.

To match the larger size of the human brain, and achieve connectivity over much larger distances, it will be important to find ways to improve the performance of the grafted cells, their survival, integration and growth capacity in particular, as well as their ability to evade the immune/inflammatory response of the host. The survival rate of grafted progenitors or neuroblasts is usually quite low, in the range of 5–20% for grafted fetal DA neurons (Castilho et al., 2000) and a similar range for grafted hESC-derived DA neuron progenitors (Kirkeby et al., 2017a; Niclis et al., 2017). Addition of growth factors (GDNF in particular) or cytoprotective agents, such as lazaroids and caspase inhibitors, can be used to increase this figure about 2-fold, a finding that has justified their use as additives in the cell preparations used in the clinical trials (Castilho et al., 2000).

In more recent years interesting progress has been made in the use of injectable biomaterial scaffolds to improve graft survival and provide a supportive microenvironment for integration and growth of the implanted cells, as well as protection from the host immune response. Such scaffolds can also be loaded with supportive growth factors, as illustrated by the recent studies of Moriarty et al. (2017, 2019a) showing a 4–5-fold increase in DA neuron survival and a 5-fold increase in host striatal reinnervation in 6-OHDA lesioned rats that had received fetal DA neuron grafts enclosed in a GDNF-loaded collagen hydrogel. Also, there is an increasing interest in the role and potential of extracellular matrix proteins, such as laminin and fibronectin, due to their ability to promote the survival, proliferation, and differentiation of fetal and hPSC-derived neural progenitors (Kirkeby et al., 2017b; Somaa et al., 2017; Zhang et al., 2017). For

a comprehensive overview of these promising developments, the reader is referred to the recent reviews by Bruggeman et al. (2019) and Moriarty et al. (2019b).

The brain repair field is now entering a new era where the development of new advanced tools for the study of brain connectomics, in combination with cell-specific optogenetic and chemogenetic methods, will provide new possibilities for detailed studies of circuitry dysfunction and cell-based repair. The new tools that have become available during recent years, and the rapidly expanding methods for *in vivo* reprogramming and trans-differentiation, open exciting new opportunities, not only for more refined experimental studies in rodent and primate models but in the longer perspective also for the exploration of circuitry repair in human neurodegenerative disease. Due to its accessibility for experimentation, its attractive anatomical and functional organization, and its clinical relevance, we anticipate that basal ganglia circuitry will remain at the forefront of this development. Exploratory clinical studies using intrastriatal transplants of hPSC-derived mDA neurons in PD patients are now underway (Barker et al., 2017), and similar studies using hPSC-derived striatal neurons in patients with Huntington's disease are likely to follow in the not too distant future. This development builds on the experiences gained from the clinical studies using fetal VM and GE tissue that have been performed over recent decades (Barker et al., 2013; Lindvall, 2015; Bachoud-Lévi, 2017). Further progress in this field will critically depend on a close interaction between experimental and clinical research where the experience gained from exploratory clinical trials will help to guide and inspire the experimental work, and conversely, where the continued development and refinement of methods and protocols that are investigated experimentally will help to drive the approaches explored in future clinical trials.

## AUTHOR CONTRIBUTIONS

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## REFERENCES

Adil, M. M., Gaj, T., Rao, A. T., Kulkarni, R. U., Fuentes, C. M., Ramadoss, G. N., et al. (2018). hPSC-derived striatal cells generated using a scalable 3D hydrogel promote recovery in a huntington disease mouse model. *Stem Cell Reports* 10, 1481–1491. doi: 10.1016/j.stemcr.2018.03.007

Adler, A. F., Cardoso, T., Nolbrant, S., Mattsson, B., Hoban, D. B., Jarl, U., et al. (2019). hESC-derived dopaminergic transplants integrate into basal ganglia circuitry in a preclinical model of Parkinson's disease. *Cell Rep.* 28, 3462.e5–3473.e5. doi: 10.1016/j.celrep.2019.08.058

Albin, R. L., Young, A. B., and Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci.* 12, 366–375. doi: 10.1016/0166-2236(89)90074-x

Annett, L. E., Torres, E. M., Ridley, R. M., Baker, H. F., and Dunnett, S. B. (1995). A comparison of the behavioural effects of embryonic nigral grafts in the caudate nucleus and in the putamen of marmosets with unilateral 6-OHDA lesions. *Exp. Brain Res.* 103, 355–371. doi: 10.1007/bf00241495

Arber, C., Precious, S. V., Cambray, S., Risner-Janiczek, J. R., Kelly, C., Noakes, Z., et al. (2015). Activin A directs striatal projection neuron differentiation of human pluripotent stem cells. *Development* 142, 1375–1386. doi: 10.1242/dev.117093

Bachoud-Lévi, A. C. (2017). From open to large-scale randomized cell transplantation trials in Huntington's disease: lessons from the multicentric intracerebral grafting in Huntington's disease trial (MIG-HD) and previous pilot studies. *Prog. Brain Res.* 230, 227–261. doi: 10.1016/bs.pbr.2016.12.011

Barker, R. A., Barrett, J., Mason, S. L., and Björklund, A. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. *Lancet Neurol.* 12, 84–91. doi: 10.1016/s1474-4422(12)70295-8

Barker, R. A., Parmar, M., Studer, L., and Takahashi, J. (2017). Human trials of stem cell-derived dopamine neurons for Parkinson's disease: dawn of a new era. *Cell Stem Cell* 21, 569–573. doi: 10.1016/j.stem.2017.09.014

Bentlage, C., Nikkhah, G., Cunningham, M. G., and Björklund, A. (1999). Reformation of the nigrostriatal pathway by fetal dopaminergic micrografts into the substantia nigra is critically dependent on the age of the host. *Exp. Neurol.* 159, 177–190. doi: 10.1006/exnr.1999.7110

Besusso, D., Schellino, R., Boido, M., Belloli, S., Parolisi, R., Conforti, P., et al. (2020). Stem cell-derived human striatal progenitors innervate striatal targets and alleviate sensorimotor deficit in a rat model of huntington disease. *Stem Cell Reports* doi: 10.1016/j.stemcr.2020.03.018 [Epub ahead of print].

Björklund, A., and Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends Neurosci.* 30, 194–202. doi: 10.1016/j.tins.2007.03.006

Björklund, A., and Dunnett, S. B. (2019). The amphetamine induced rotation test: a re-assessment of its use as a tool to monitor motor impairment and functional recovery in rodent models of Parkinson's disease. *J. Parkinsons Dis.* 9, 17–29. doi: 10.3233/jpd-181525

Bonilla, S., Hall, A. C., Pinto, L., Attardo, A., Götz, M., Huttner, W. B., et al. (2008). Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors. *Glia* 56, 809–820. doi: 10.1002/glia.20654

Brasted, P. J., Watts, C., Robbins, T. W., and Dunnett, S. B. (1999). Associative plasticity in striatal transplants. *Proc. Natl. Acad. Sci. U S A* 96, 10524–10529. doi: 10.1073/pnas.96.18.10524

Brasted, P. J., Watts, C., Torres, E. M., Robbins, T. W., and Dunnett, S. B. (2000). Behavioral recovery after transplantation into a rat model of Huntington's disease: dependence on anatomical connectivity and extensive postoperative training. *Behav. Neurosci.* 114, 431–436. doi: 10.1037/0735-7044.114.2.431

Breysse, N., Carlsson, T., Winkler, C., Björklund, A., and Kirik, D. (2007). The functional impact of the intrastriatal dopamine neuron grafts in parkinsonian rats is reduced with advancing disease. *J. Neurosci.* 27, 5849–5856. doi: 10.1523/JNEUROSCI.0626-07.2007

Bruggeman, K. F., Moriarty, N., Dowd, E., Nisbet, D. R., and Parish, C. L. (2019). Harnessing stem cells and biomaterials to promote neural repair. *Br. J. Pharmacol.* 176, 355–368. doi: 10.10.1111/bph.14545

Bye, C. R., Thompson, L. H., and Parish, C. L. (2012). Birth dating of midbrain dopamine neurons identifies A9 enriched tissue for transplantation into parkinsonian mice. *Exp. Neurol.* 236, 58–68. doi: 10.1016/j.expneurol.2012.04.002

Campbell, K., Wictorin, K., and Björklund, A. (1992). Differential regulation of neuropeptide mRNA expression in intrastriatal striatal transplants by host dopaminergic afferents. *Proc. Natl. Acad. Sci. U S A* 89, 10489–10493. doi: 10.1073/pnas.89.21.10489

Campbell, K., Wictorin, K., and Björklund, A. (1995). Neurotransmitter-related gene expression in intrastriatal striatal transplants—I. Phenotypical characterization of striatal and non-striatal graft regions. *Neuroscience* 64, 17–33. doi: 10.1016/0306-4522(94)00412-x

Cardoso, T., Adler, A. F., Mattsson, B., Hoban, D. B., Nolbrant, S., Wahlestedt, J. N., et al. (2018). Target-specific forebrain projections and appropriate synaptic inputs of hESC-derived dopamine neurons grafted to the midbrain of parkinsonian rats. *J. Comp. Neurol.* 526, 2133–2146. doi: 10.1002/cne.24500

Castilho, R. F., Hansson, O., and Brundin, P. (2000). Improving the survival of grafted embryonic dopamine neurons in rodent models of Parkinson's disease. *Prog. Brain Res.* 127, 203–231. doi: 10.1016/s0079-6123(00)27011-8

Cenci, M. A., Campbell, K., and Björklund, A. (1993). Neuropeptide messenger RNA expression in the 6-hydroxydopamine-lesioned rat striatum reinnervated by fetal dopaminergic transplants: differential effects of the grafts on preproenkephalin, preprotachykinin and prodynorphin messenger RNA levels. *Neuroscience* 57, 275–296. doi: 10.1016/0306-4522(93)90062-k

Cenci, M. A., Campbell, K., and Björklund, A. (1997). Glutamic acid decarboxylase gene expression in the dopamine-denervated striatum: effects of intrastriatal fetal nigral transplants or chronic apomorphine treatment. *Front. Pharmacol.* 48, 149–155. doi: 10.1016/s0169-328x(97)00130-7

Cenci, M. A., Kalén, P., Duan, W. M., and Björklund, A. (1994). Transmitter release from transplants of fetal ventral mesencephalon or locus coeruleus in the rat frontal cortex and nucleus accumbens: effects of pharmacological and behaviorally activating stimuli. *Brain Res.* 641, 225–248. doi: 10.1016/0006-8993(94)90150-3

Chen, Y., Xiong, M., Dong, Y., Haberman, A., Cao, J., Liu, H., et al. (2016). Chemical control of grafted human PSC-derived neurons in a mouse model of Parkinson's disease. *Cell Stem Cell* 18, 817–826. doi: 10.1016/j.stem.2016.03.014

Chevalier, G., and Deniau, J. M. (1990). Disinhibition as a basic process in the expression of striatal functions. *Trends Neurosci.* 13, 277–280. doi: 10.1016/0166-2236(90)90109-n

Chritin, M., Savasta, M., Mennicken, F., Bal, A., Abrous, D. N., Le Moal, M., et al. (1992). Intrastriatal dopamine-rich implants reverse the increase of dopamine D2 receptor mRNA levels caused by lesion of the nigrostriatal pathway: a quantitative in situ hybridization study. *Eur. J. Neurosci.* 4, 663–672. doi: 10.1111/j.1460-9568.1992.tb00175.x

Clarke, D. J., Brundin, P., Strecker, R. E., Nilsson, O. G., Björklund, A., and Lindvall, O. (1988a). Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: ultrastructural evidence for synapse formation using tyrosine hydroxylase immunocytochemistry. *Exp. Brain Res.* 73, 115–126. doi: 10.1007/bf00279666

Clarke, D. J., Dunnett, S. B., Isacson, O., Sirinathsinghji, D. J., and Björklund, A. (1988b). Striatal grafts in rats with unilateral neostriatal lesions—I. Ultrastructural evidence of afferent synaptic inputs from the host nigrostriatal pathway. *Neuroscience* 24, 791–801. doi: 10.1016/0306-4522(88)90067-x

Clarke, D. J., and Dunnett, S. B. (1993). Synaptic relationships between cortical and dopaminergic inputs and intrinsic GABAergic systems within intrastriatal striatal grafts. *J. Chem. Neuroanat.* 6, 147–158. doi: 10.1016/0891-0618(93)90024-x

Cooper, O., Hargus, G., Deleidi, M., Blak, A., Osborn, T., Marlow, E., et al. (2010). Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol. Cell. Neurosci.* 45, 258–266. doi: 10.1016/j.mcn.2010.06.017

Dawson, T. M., Dawson, V. L., Gage, F. H., Fisher, L. J., Hunt, M. A., and Wamsley, J. K. (1991). Functional recovery of supersensitive dopamine receptors after intrastriatal grafts of fetal substantia nigra. *Exp. Neurol.* 111, 282–292. doi: 10.1016/0014-4886(91)90095-t

Delli Carri, A., Onorati, M., Lelos, M. J., Castiglioni, V., Faedo, A., Menon, R., et al. (2013). Developmentally coordinated extrinsic signals drive human pluripotent stem cell differentiation toward authentic DARPP-32$^+$ medium-sized spiny neurons. *Development* 140, 301–312. doi: 10.1242/dev.084608

DeLong, M. R., and Wichmann, T. (2015). Basal ganglia circuits as targets for neuromodulation in Parkinson disease. *JAMA Neurol.* 72, 1354–1360. doi: 10.1001/jamaneurol.2015.2397

Di Loreto, S., Florio, T., Capozzo, A., Napolitano, A., Adorno, D., and Scarnati, E. (1996). Transplantation of mesencephalic cell suspension in dopamine-denervated striatum of the rat: I. Effects on spontaneous activity of striatal neurons. *Exp. Neurol.* 138, 318–326. doi: 10.1006/exnr.1996.0070

Döbrössy, M. D., and Dunnett, S. B. (2005). Training specificity, graft development and graft-mediated functional recovery in a rodent model of Huntington's

disease. *Neuroscience* 132, 543–552. doi: 10.1016/j.neuroscience.2005. 01.016

Doucet, G., Brundin, P., Descarries, L., and Björklund, A. (1990). Effect of prior dopamine denervation on survival and fiber outgrowth from intrastriatal fetal mesencephalic grafts. *Eur. J. Neurosci.* 2, 279–290. doi: 10.1111/j.1460-9568. 1990.tb00419.x

Doucet, G., Murata, Y., Brundin, P., Bosler, O., Mons, N., Geffard, M., et al. (1989). Host afferents into intrastriatal transplants of fetal ventral mesencephalon. *Exp. Neurol.* 106, 1–19. doi: 10.1016/0014-4886(89)90139-8

Dowd, E., and Dunnett, S. B. (2004). Deficits in a lateralized associative learning task in dopamine-depleted rats with functional recovery by dopamine-rich transplants. *Eur. J. Neurosci.* 20, 1953–1959. doi: 10.1111/j.1460-9568.2004. 03637.x

Dunnett, S. B., and Björklund, A. (2017). Mechanisms and use of neural transplants for brain repair. *Prog. Brain Res.* 230, 1–51. doi: 10.1016/bs.pbr. 2016.11.002

Dunnett, S. B., Hernandez, T. D., Summerfield, A., Jones, G. H., and Arbuthnott, G. (1988). Graft-derived recovery from 6-OHDA lesions: specificity of ventral mesencephalic graft tissues. *Exp. Brain Res.* 71, 411–424. doi: 10.1007/bf00247501

Dunnett, S. B., Nathwani, F., and Björklund, A. (2000). The integration and function of striatal grafts. *Prog. Brain Res.* 127, 345–380. doi: 10.1016/s0079-6123(00)27017-9

Dunnett, S. B., and White, A. (2006). Striatal grafts alleviate bilateral striatal lesion deficits in operant delayed alternation in the rat. *Exp. Neurol.* 199, 479–489. doi: 10.1016/j.expneurol.2006.01.013

Fasano, C. A., Chambers, S. M., Lee, G., Tomishima, M. J., and Studer, L. (2010). Efficient derivation of functional floor plate tissue from human embryonic stem cells. *Cell Stem Cell* 6, 336–347. doi: 10.1016/j.stem.2010.03.001

Fisher, L. J., Young, S. J., Tepper, J. M., Groves, P. M., and Gage, F. H. (1991). Electrophysiological characteristics of cells within mesencephalon suspension grafts. *Neuroscience* 40, 109–122. doi: 10.1016/0306-4522(91)90178-q

Freund, T. F., Bolam, J. P., Björklund, A., Stenevi, U., Dunnett, S. B., Powell, J. F., et al. (1985). Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host neostriatum: a tyrosine hydroxylase immunocytochemical study. *J. Neurosci.* 5, 603–616. doi: 10.1523/JNEUROSCI. 05-03-00603.1985

Fricker, R. A., Torres, E. M., and Dunnett, S. B. (1997). The effects of donor stage on the survival and function of embryonic striatal grafts in the adult rat brain: I. Morphological characteristics. *Neuroscience* 79, 695–710. doi: 10.1016/s0306-4522(96)00656-2

Gage, F. H., Björklund, A., Stenevi, U., and Dunnett, S. B. (1983). Intracerebral grafting of neuronal cell suspensions: VIII. Survival and growth of implants of nigral and septal cell suspensions in intact brains of aged rats. *Acta Physiol. Scand. Suppl.* 522, 67–75.

Gaillard, A., Decressac, M., Frappé, I., Fernagut, P. O., Prestoz, L., Besnard, S., et al. (2009). Anatomical and functional reconstruction of the nigrostriatal pathway by intranigral transplants. *Neurobiol. Dis.* 35, 477–488. doi: 10.1016/j.nbd.2009. 07.003

Graybiel, A. M., and Grafton, S. T. (2015). The striatum: where skills and habits meet. *Cold Spring Harb. Perspect. Biol.* 7:a021691. doi: 10.1101/cshperspect. a021691

Graybiel, A. M., Liu, F. C., and Dunnett, S. B. (1989). Intrastriatal grafts derived from fetal striatal primordia: I. Phenotypy and modular organization. *J. Neurosci.* 9, 3250–3271. doi: 10.1523/JNEUROSCI.09-09-03 250.1989

Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B., Heuer, A., Bramoulle, Y., et al. (2014). Human ESC-derived dopamine neurons show similar preclinical efficacy and potency to fetal neurons when grafted in a rat model of Parkinson's disease. *Cell Stem Cell* 15, 653–665. doi: 10.1016/j.stem.2014. 09.017

Grealish, S., Heuer, A., Cardoso, T., Kirkeby, A., Jonsson, M., Jöhansson, J., et al. (2015). Monosynaptic tracing using modified rabies virus reveals early and extensive circuit integration of human embryonic stem cell-derived neurons. *Stem Cell Reports* 4, 975–983. doi: 10.1016/j.stemcr.2015.04.011

Grealish, S., Jönsson, M. E., Li, M., Kirik, D., Björklund, A., and Thompson, L. H. (2010). The A9 dopamine neuron component in grafts of ventral mesencephalon is an important determinant for recovery of motor function in a rat model of Parkinson's disease. *Brain* 133, 482–495. doi: 10.1093/brain/awp328

Helm, G. A., Palmer, P. E., and Bennett, J. P. Jr. (1992). Choline acetyltransferase- and substance P-like immunoreactive elements in fetal striatal grafts in the rat: a correlated light and electron microscopic study. *Neuroscience* 47, 621–639. doi: 10.1016/0306-4522(92)90171-w

Isacson, O., Brundin, P., Gage, F. H., and Björklund, A. (1985). Neural grafting in a rat model of Huntington's disease: progressive neurochemical changes after neostriatal ibotenate lesions and striatal tissue grafting. *Neuroscience* 16, 799–817. doi: 10.1016/0306-4522(85)90095-8

Isacson, O., Brundin, P., Kelly, P. A., Gage, F. H., and Björklund, A. (1984). Functional neuronal replacement by grafted striatal neurones in the ibotenic acid-lesioned rat striatum. *Nature* 311, 458–460. doi: 10.1038/311458a0

Isacson, O., and Deacon, T. W. (1996). Specific axon guidance factors persist in the adult brain as demonstrated by pig neuroblasts transplanted to the rat. *Neuroscience* 75, 827–837. doi: 10.1016/0306-4522(96)00305-3

Isacson, O., Deacon, T. W., Pakzaban, P., Galpern, W. R., Dinsmore, J., and Burns, L. H. (1995). Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. *Nat. Med.* 1, 1189–1194. doi: 10.1038/nm1195-1189

Isacson, O., Dunnett, S. B., and Björklund, A. (1986). Graft-induced behavioral recovery in an animal model of Huntington disease. *Proc. Natl. Acad. Sci. U S A* 83, 2728–2732. doi: 10.1073/pnas.83.8.2728

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., et al. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 28, 31–40. doi: 10.1016/s0896-6273(00)00083-0

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., et al. (2017). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature* 548, 592–596. doi: 10.1038/nature 23664

Kim, J. H., Auerbach, J. M., Rodríguez-Gómez, J. A., Velasco, I., Gavin, D., Lumelsky, N., et al. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418, 50–56. doi: 10.1038/nature00900

Kirik, D., Winkler, C., and Björklund, A. (2001). Growth and functional efficacy of intrastriatal nigral transplants depend on the extent of nigrostriatal degeneration. *J. Neurosci.* 21, 2889–2896. doi: 10.1523/JNEUROSCI.21-08-02889.2001

Kirkeby, A., Nolbrant, S., Tiklova, K., Heuer, A., Kee, N., Cardoso, T., et al. (2017a). Predictive markers guide differentiation to improve graft outcome in clinical translation of hESC-based therapy for Parkinson's disease. *Cell Stem Cell* 20, 135–148. doi: 10.1016/j.stem.2016.09.004

Kirkeby, A., Parmar, M., and Barker, R. A. (2017b). Strategies for bringing stem cell-derived dopamine neurons to the clinic: a European approach (STEM-PD). *Prog. Brain Res.* 230, 165–190. doi: 10.1016/bs.pbr.2016.11.011

Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., et al. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. *Cell Rep.* 1, 703–714. doi: 10.1016/j.celrep.2012.04.009

Kish, S. J., Shannak, K., and Hornykiewicz, O. (1988). Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. *N. Engl. J. Med.* 318, 876–880. doi: 10.1056/nejm198804073181402

Klein, A., Lane, E. L., and Dunnett, S. B. (2013). Brain repair in a unilateral rat model of Huntington's disease: new insights into impairment and restoration of forelimb movement patterns. *Cell Transplant.* 22, 1735–1751. doi: 10.3727/096368912x657918

Knowlton, B. J., Mangels, J. A., and Squire, L. R. (1996). A neostriatal habit learning system in humans. *Science* 273, 1399–1402. doi: 10.1126/science.273. 5280.1399

Kriks, S., Shim, J. W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480, 547–551. doi: 10.1038/nature10648

Labandeira-Garcia, J. L., and Guerra, M. J. (1994). Cortical stimulation induces fos expression in intrastriatal striatal grafts. *Brain Res.* 652, 87–97. doi: 10.1016/0006-8993(94)90321-2

Labandeira-Garcia, J. L., Wictorin, K., Cunningham, E. T. Jr., and Björklund, A. (1991). Development of intrastriatal striatal grafts and their afferent innervation from the host. *Neuroscience* 42, 407–426. doi: 10.1016/0306-4522(91)90385-2

Lelos, M. J., Morgan, R. J., Kelly, C. M., Torres, E. M., Rosser, A. E., and Dunnett, S. B. (2016). Amelioration of non-motor dysfunctions after transplantation of human dopamine neurons in a model of Parkinson's disease. *Exp. Neurol.* 278, 54–61. doi: 10.1016/j.expneurol.2016.02.003

Li, M., and Rosser, A. E. (2017). Pluripotent stem cell-derived neurons for transplantation in Huntington's disease. *Prog. Brain Res.* 230, 263–281. doi: 10.1016/bs.pbr.2017.02.009

Lindvall, O. (2015). Treatment of Parkinson's disease using cell transplantation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140370. doi: 10.1098/rstb.2014.0370

Liu, F. C., Dunnett, S. B., and Graybiel, A. M. (1992). Influence of mesostriatal afferents on the development and transmitter regulation of intrastriatal grafts derived from embryonic striatal primordia. *J. Neurosci.* 12, 4281–4297. doi: 10.1523/JNEUROSCI.12-11-04281.1992

Liu, F. C., Dunnett, S. B., Robertson, H. A., and Graybiel, A. M. (1991). Intrastriatal grafts derived from fetal striatal primordia: III. Induction of modular patterns of fos-like immunoreactivity by cocaine. *Exp. Brain Res.* 85, 501–506. doi: 10.1007/bf00231733

Ma, L., Hu, B., Liu, Y., Vermilyea, S. C., Liu, H., Gao, L., et al. (2012). Human embryonic stem cell-derived GABA neurons correct locomotion deficits in quinolinic acid-lesioned mice. *Cell Stem Cell* 10, 455–464. doi: 10.1016/j.stem.2012.01.021

Mahalik, T. J., Finger, T. E., Stromberg, I., and Olson, L. (1985). Substantia nigra transplants into denervated striatum of the rat: ultrastructure of graft and host interconnections. *J. Comp. Neurol.* 240, 60–70. doi: 10.1002/cne.902400105

Mandel, R. J., Brundin, P., and Björklund, A. (1990). The importance of graft placement and task complexity for transplant-induced recovery of simple and complex sensorimotor deficits in dopamine denervated rats. *Eur. J. Neurosci.* 2, 888–894. doi: 10.1111/j.1460-9568.1990.tb00400.x

Mandel, R. J., Wictorin, K., Cenci, M. A., and Björklund, A. (1992). Fos expression in intrastriatal striatal grafts: regulation by host dopaminergic afferents. *Brain Res.* 583, 207–215. doi: 10.1016/s0006-8993(10)80026-5

Marsden, C. D. (1982). The mysterious motor function of the basal ganglia: the Robert Wartenberg Lecture. *Neurology* 32, 514–539. doi: 10.1212/wnl.32.5.514

Montoya, C. P., Astell, S., and Dunnett, S. B. (1990). Effects of nigral and striatal grafts on skilled forelimb use in the rat. *Prog. Brain Res.* 82, 459–466. doi: 10.1016/s0079-6123(08)62634-5

Moriarty, N., Cabré, S., Alamilla, V., Pandit, A., and Dowd, E. (2019a). Encapsulation of young donor age dopaminergic grafts in a GDNF loaded collagen hydrogel further increases their survival, reinnervation, and functional efficacy after intra-striatal transplantation in hemi-Parkinsonian rats. *Eur. J. Neurosci.* 49, 487–496. doi: 10.1111/ejn.14090

Moriarty, N., Parish, C. L., and Dowd, E. (2019b). Primary tissue for cellular brain repair in Parkinson's disease: promise, problems and the potential of biomaterials. *Eur. J. Neurosci.* 49, 472–486. doi: 10.1111/ejn.14051

Moriarty, N., Pandit, A., and Dowd, E. (2017). Encapsulation of primary dopaminergic neurons in a GDNF-loaded collagen hydrogel increases their survival, re-innervation and function after intra-striatal transplantation. *Sci. Rep.* 7:16033. doi: 10.1038/s41598-017-15970-w

Nakao, N., Grasbon-Frodl, E. M., Widner, H., and Brundin, P. (1996). DARPP-32-rich zones in grafts of lateral ganglionic eminence govern the extent of functional recovery in skilled paw reaching in an animal model of Huntington's disease. *Neuroscience* 74, 959–970. doi: 10.1016/0306-4522(96)00238-2

Nakao, N., Ogura, M., Nakai, K., and Itakura, T. (1999). Embryonic striatal grafts restore neuronal activity of the globus pallidus in a rodent model of Huntington's disease. *Neuroscience* 88, 469–477. doi: 10.1016/s0306-4522(98)00197-3

Niclis, J. C., Gantner, C. W., Hunt, C. P. J., Kauhausen, J. A., Durnall, J. C., Haynes, J. M., et al. (2017). A PITX3-EGFP reporter line reveals connectivity of dopamine and non-dopamine neuronal subtypes in grafts generated from human embryonic stem cells. *Stem Cell Reports* 9, 868–882. doi: 10.1016/j.stemcr.2017.08.002

Nicoleau, C., Varela, C., Bonnefond, C., Maury, Y., Bugi, A., Aubry, L., et al. (2013). Embryonic stem cells neural differentiation qualifies the role of Wnt/β-Catenin signals in human telencephalic specification and regionalization. *Stem Cells* 31, 1763–1774. doi: 10.1002/stem.1462

Nikkhah, G., Bentlage, C., Cunningham, M. G., and Björklund, A. (1994). Intranigral fetal dopamine grafts induce behavioral compensation in the rat Parkinson model. *J. Neurosci.* 14, 3449–3461. doi: 10.1523/JNEUROSCI.14-06-03449.1994

Nolbrant, S., Heuer, A., Parmar, M., and Kirkeby, A. (2017). Generation of high-purity human ventral midbrain dopaminergic progenitors for *in vivo* maturation and intracerebral transplantation. *Nat. Protoc.* 12, 1962–1979. doi: 10.1038/nprot.2017.078

Olsson, M., Campbell, K., Wictorin, K., and Björklund, A. (1995). Projection neurons in fetal striatal transplants are predominantly derived from the lateral ganglionic eminence. *Neuroscience* 69, 1169–1182. doi: 10.1016/0306-4522(95)00325-d

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., et al. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* 134, 3213–3225. doi: 10.1242/dev.02879

Packard, M. G., and Knowlton, B. J. (2002). Learning and memory functions of the Basal Ganglia. *Annu. Rev. Neurosci.* 25, 563–593. doi: 10.1146/annurev.neuro.25.112701.142937

Paille, V., Picconi, B., Bagetta, V., Ghiglieri, V., Sgobio, C., Di Filippo, M., et al. (2010). Distinct levels of dopamine denervation differentially alter striatal synaptic plasticity and NMDA receptor subunit composition. *J. Neurosci.* 30, 14182–14193. doi: 10.1523/JNEUROSCI.2149-10.2010

Pakzaban, P., Deacon, T. W., Burns, L. H., and Isacson, O. (1993). Increased proportion of acetylcholinesterase-rich zones and improved morphological integration in host striatum of fetal grafts derived from the lateral but not the medial ganglionic eminence. *Exp. Brain Res.* 97, 13–22. doi: 10.1007/bf00228813

Piccini, P., Pavese, N., Hagell, P., Reimer, J., Björklund, A., Oertel, W. H., et al. (2005). Factors affecting the clinical outcome after neural transplantation in Parkinson's disease. *Brain* 128, 2977–2986. doi: 10.1093/brain/awh649

Reading, P. J., and Dunnett, S. B. (1995). Embryonic striatal grafts reverse the disinhibitory effects of ibotenic acid lesions of the ventral striatum. *Exp. Brain Res.* 105, 76–86. doi: 10.1007/bf00242184

Reddington, A. E., Rosser, A. E., and Dunnett, S. B. (2014). Differentiation of pluripotent stem cells into striatal projection neurons: a pure MSN fate may not be sufficient. *Front. Cell. Neurosci.* 8:398. doi: 10.3389/fncel.2014.00398

Redgrave, P., Rodriguez, M., Smith, Y., Rodriguez-Oroz, M. C., Lehericy, S., Bergman, H., et al. (2010). Goal-directed and habitual control in the basal ganglia: implications for Parkinson's disease. *Nat. Rev. Neurosci.* 11, 760–772. doi: 10.1038/nrn2915

Rioux, L., Gaudin, D. P., Gagnon, C., Di Paolo, T., and Bedard, P. J. (1991). Decrease of behavioral and biochemical denervation supersensitivity of rat striatum by nigral transplants. *Neuroscience* 44, 75–83. doi: 10.1016/0306-4522(91)90251-i

Roberts, R. C., and Difiglia, M. (1988). Localization of immunoreactive GABA and enkephalin and NADPH-diaphorase-positive neurons in fetal striatal grafts in the quinolinic-acid-lesioned rat neostriatum. *J. Comp. Neurol.* 274, 406–421. doi: 10.1002/cne.902740309

Rodriguez-Oroz, M. C., Jahanshahi, M., Krack, P., Litvan, I., Macias, R., Bezard, E., et al. (2009). Initial clinical manifestations of Parkinson's disease: features and pathophysiological mechanisms. *Lancet Neurol.* 8, 1128–1139. doi: 10.1016/s1474-4422(09)70293-5

Rutherford, A., Garcia-Munoz, M., Dunnett, S. B., and Arbuthnott, G. W. (1987). Electrophysiological demonstration of host cortical inputs to striatal grafts. *Neurosci. Lett.* 83, 275–281. doi: 10.1016/0304-3940(87)90099-1

Rylander, D., Bagetta, V., Pendolino, V., Zianni, E., Grealish, S., Gardoni, F., et al. (2013). Region-specific restoration of striatal synaptic plasticity by dopamine grafts in experimental parkinsonism. *Proc. Natl. Acad. Sci. U S A* 110, E4375–E4384. doi: 10.1073/pnas.1311187110

Savasta, M., Mennicken, F., Chritin, M., Abrous, D. N., Feuerstein, C., Le Moal, M., et al. (1992). Intrastriatal dopamine-rich implants reverse the changes in dopamine D2 receptor densities caused by 6-hydroxydopamine lesion of the nigrostriatal pathway in rats: an autoradiographic study. *Neuroscience* 46, 729–738. doi: 10.1016/0306-4522(92)90159-y

Silberberg, G., and Bolam, J. P. (2015). Local and afferent synaptic pathways in the striatal microcircuitry. *Curr. Opin. Neurobiol.* 33, 182–187. doi: 10.1016/j.conb.2015.05.002

Smith, A. D., and Bolam, J. P. (1990). The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends Neurosci.* 13, 259–265. doi: 10.1016/0166-2236(90)90106-k

Somaa, F. A., Wang, T. Y., Niclis, J. C., Bruggeman, K. F., Kauhausen, J. A., Guo, H., et al. (2017). Peptide-based scaffolds support human cortical progenitor graft integration to reduce atrophy and promote functional repair in a model of stroke. *Cell Rep.* 20, 1964–1977. doi: 10.1016/j.celrep.2017.07.069

Sorensen, A. T., Thompson, L., Kirik, D., Björklund, A., Lindvall, O., and Kokaia, M. (2005). Functional properties and synaptic integration of genetically labelled dopaminergic neurons in intrastriatal grafts. *Eur. J. Neurosci.* 21, 2793–2799. doi: 10.1111/j.1460-9568.2005.04116.x

Steinbeck, J. A., Choi, S. J., Mrejeru, A., Ganat, Y., Deisseroth, K., Sulzer, D., et al. (2015). Optogenetics enables functional analysis of human embryonic stem cell-derived grafts in a Parkinson's disease model. *Nat. Biotechnol.* 33, 204–209. doi: 10.1038/nbt.3124

Strecker, R. E., Sharp, T., Brundin, P., Zetterström, T., Ungerstedt, U., and Björklund, A. (1987). Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. *Neuroscience* 22, 169–178. doi: 10.1016/0306-4522(87)90207-7

Strömberg, I., Kehr, J., Andbjer, B., and Fuxe, K. (2000). Fetal ventral mesencephalic grafts functionally reduce the dopamine D2 receptor supersensitivity in partially dopamine reinnervated host striatum. *Exp. Neurol.* 164, 154–165. doi: 10.1006/exnr.2000.7421

Studer, L. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic-The NYSTEM trial. *Prog. Brain Res.* 230, 191–212. doi: 10.1016/bs.pbr.2017.02.008

Sundberg, M., Bogetofte, H., Lawson, T., Jansson, J., Smith, G., Astradsson, A., et al. (2013). Improved cell therapy protocols for Parkinson's disease based on differentiation efficiency and safety of hESC-, hiPSC-, and non-human primate iPSC-derived dopaminergic neurons. *Stem Cells* 31, 1548–1562. doi: 10.1002/stem.1415

Takahashi, J. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic: the Kyoto trial. *Prog. Brain Res.* 230, 213–226. doi: 10.1016/bs.pbr.2016.11.003

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Takeshima, T., Johnston, J. M., and Commissiong, J. W. (1994). Mesencephalic type 1 astrocytes rescue dopaminergic neurons from death induced by serum deprivation. *J. Neurosci.* 14, 4769–4779. doi: 10.1523/JNEUROSCI.14-08-04769.1994

Taylor, S. R., Badurek, S., Dileone, R. J., Nashmi, R., Minichiello, L., and Picciotto, M. R. (2014). GABAergic and glutamatergic efferents of the mouse ventral tegmental area. *J. Comp. Neurol.* 522, 3308–3334. doi: 10.1002/cne.23603

Thompson, L. H., Andersson, E., Jensen, J. B., Barraud, P., Guillemot, F., Parmar, M., et al. (2006). Neurogenin2 identifies a transplantable dopamine neuron precursor in the developing ventral mesencephalon. *Exp. Neurol.* 198, 183–198. doi: 10.1016/j.expneurol.2005.11.025

Thompson, L., Barraud, P., Andersson, E., Kirik, D., and Björklund, A. (2005). Identification of dopaminergic neurons of nigral and ventral tegmental area subtypes in grafts of fetal ventral mesencephalon based on cell morphology, protein expression, and efferent projections. *J. Neurosci.* 25, 6467–6477. doi: 10.1523/JNEUROSCI.1676-05.2005

Thompson, L., and Björklund, A. (2012). Survival, differentiation, and connectivity of ventral mesencephalic dopamine neurons following transplantation. *Prog. Brain Res.* 200, 61–95. doi: 10.1016/b978-0-444-59575-1.00004-1

Thompson, L. H., and Björklund, A. (2015). Reconstruction of brain circuitry by neural transplants generated from pluripotent stem cells. *Neurobiol. Dis.* 79, 28–40. doi: 10.1016/j.nbd.2015.04.003

Thompson, L. H., Grealish, S., Kirik, D., and Björklund, A. (2009). Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain. *Eur. J. Neurosci.* 30, 625–638. doi: 10.1111/j.1460-9568.2009.06878.x

Thompson, L. H., Kirik, D., and Björklund, A. (2008). Non-dopaminergic neurons in ventral mesencephalic transplants make widespread axonal connections in the host brain. *Exp. Neurol.* 213, 220–228. doi: 10.1016/j.expneurol.2008.06.005

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. doi: 10.1126/science.282.5391.1145

Whishaw, I. Q., O'Connor, W. T., and Dunnett, S. B. (1986). The contributions of motor cortex, nigrostriatal dopamine and caudate-putamen to skilled forelimb use in the rat. *Brain* 109, 805–843. doi: 10.1093/brain/109.5.805

Wickersham, I. R., Lyon, D. C., Barnard, R. J., Mori, T., Finke, S., Conzelmann, K. K., et al. (2007). Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron* 53, 639–647. doi: 10.1016/j.neuron.2007.01.033

Wictorin, K., and Björklund, A. (1989). Connectivity of striatal grafts implanted into the ibotenic acid-lesioned striatum—II. Cortical afferents. *Neuroscience* 30, 297–311. doi: 10.1016/0306-4522(89)90255-8

Wictorin, K., Brundin, P., Gustavii, B., Lindvall, O., and Björklund, A. (1990). Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. *Nature* 347, 556–558. doi: 10.1038/347556a0

Wictorin, K., Brundin, P., Sauer, H., Lindvall, O., and Björklund, A. (1992). Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. *J. Comp. Neurol.* 323, 475–494. doi: 10.1002/cne.903230403

Wictorin, K., Clarke, D. J., Bolam, J. P., and Björklund, A. (1989a). Host corticostriatal fibres establish synaptic connections with grafted striatal neurons in the ibotenic acid lesioned striatum. *Eur. J. Neurosci.* 1, 189–195. doi: 10.1111/j.1460-9568.1989.tb00787.x

Wictorin, K., Ouimet, C. C., and Björklund, A. (1989b). Intrinsic organization and connectivity of intrastriatal striatal transplants in rats as revealed by DARPP-32 immunohistochemistry: specificity of connections with the lesioned host brain. *Eur. J. Neurosci.* 1, 690–701. doi: 10.1111/j.1460-9568.1989.tb00375.x

Wictorin, K., Simerly, R. B., Isacson, O., Swanson, L. W., and Björklund, A. (1989c). Connectivity of striatal grafts implanted into the ibotenic acid-lesioned striatum—III. Efferent projecting graft neurons and their relation to host afferents within the grafts. *Neuroscience* 30, 313–330. doi: 10.1016/0306-4522(89)90256-x

Wictorin, K., Isacson, O., Fischer, W., Nothias, F., Peschanski, M., and Björklund, A. (1988). Connectivity of striatal grafts implanted into the ibotenic acid-lesioned striatum—I. Subcortical afferents. *Neuroscience* 27, 547–562. doi: 10.1016/0306-4522(88)90288-6

Wictorin, K., Lagenaur, C. F., Lund, R. D., and Björklund, A. (1991). Efferent projections to the host brain from intrastriatal striatal mouse-to-rat grafts: time course and tissue-type specificity as revealed by a mouse specific neuronal marker. *Eur. J. Neurosci.* 3, 86–101. doi: 10.1111/j.1460-9568.1991.tb00814.x

Winkler, C., Bentlage, C., Cenci, M. A., Nikkhah, G., and Björklund, A. (2003). Regulation of neuropeptide mRNA expression in the basal ganglia by intrastriatal and intranigral transplants in the rat Parkinson model. *Neuroscience* 118, 1063–1077. doi: 10.1016/s0306-4522(03)00007-1

Winkler, C., Bentlage, C., Nikkhah, G., Samii, M., and Björklund, A. (1999). Intranigral transplants of GABA-rich striatal tissue induce behavioral recovery in the rat Parkinson model and promote the effects obtained by intrastriatal dopaminergic transplants. *Exp. Neurol.* 155, 165–186. doi: 10.1006/exnr.1998.6916

Winkler, C., Kirik, D., Björklund, A., and Dunnett, S. B. (2000). Transplantation in the rat model of Parkinson's disease: ectopic versus homotopic graft

placement. *Prog. Brain Res.* 127, 233–265. doi: 10.1016/s0079-6123(00)27012-x

Wu, M., Zhang, D., Bi, C., Mi, T., Zhu, W., Xia, L., et al. (2018). A chemical recipe for generation of clinical-grade striatal neurons from hESCs. *Stem Cell Reports* 11, 635–650. doi: 10.1016/j.stemcr.2018.08.005

Xu, Z. C., Wilson, C. J., and Emson, P. C. (1991). Restoration of thalamostriatal projections in rat neostriatal grafts: an electron microscopic analysis. *J. Comp. Neurol.* 303, 22–34. doi: 10.1002/cne.903030104

Xu, C., Zhao, J., Liu, Y., Zeng, X., Jia, Y., Wang, Y., et al. (2012). Dopaminergic axons preferentially innervate dendritic spines with hyperactive glutamatergic synapses in the rat striatum. *Brain Res.* 1486, 92–102. doi: 10.1016/j.brainres.2012.09.032

Zetterström, T., Brundin, P., Gage, F. H., Sharp, T., Isacson, O., Dunnett, S. B., et al. (1986). *In vivo* measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. *Brain Res.* 362, 344–349. doi: 10.1016/0006-8993(86)90460-9

Zhang, D., Yang, S., Toledo, E. M., Gyllborg, D., Saltó, C., Carlos Villaescusa, J., et al. (2017). Niche-derived laminin-511 promotes midbrain dopaminergic neuron survival and differentiation through YAP. *Sci. Signal.* 10:eaal4165. doi: 10.1126/scisignal.aal4165

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# From Synaptic Dysfunction to Neuroprotective Strategies in Genetic Parkinson's Disease: Lessons From LRRK2

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The pathogenesis of Parkinson's disease (PD) is thought to rely on a complex interaction between the patient's genetic background and a variety of largely unknown environmental factors. In this scenario, the investigation of the genetic bases underlying familial PD could unveil key molecular pathways to be targeted by new disease-modifying therapies, still currently unavailable. Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are responsible for the majority of inherited familial PD cases and can also be found in sporadic PD, but the pathophysiological functions of LRRK2 have not yet been fully elucidated. Here, we will review the evidence obtained in transgenic LRRK2 experimental models, characterized by altered striatal synaptic transmission, mitochondrial dysfunction, and α-synuclein aggregation. Interestingly, the processes triggered by mutant LRRK2 might represent early pathological phenomena in the pathogenesis of PD, anticipating the typical neurodegenerative features characterizing the late phases of the disease. A comprehensive view of LRRK2 neuronal pathophysiology will support the possible clinical application of pharmacological compounds targeting this protein, with potential therapeutic implications for patients suffering from both familial and sporadic PD.

Keywords: Parkinson's disease, LRRK2, synaptic dysfunction, mitochondrial dysfunction, α-synuclein, neuroprotection

## INTRODUCTION

Parkinson's disease (PD) represents one of the most common neurodegenerative disorders of the central nervous system (CNS; Dorsey et al., 2007; Kalia and Lang, 2015; Ascherio and Schwarzschild, 2016). The prevalence of PD has been reported to be higher in Europe and Northern America, with respect to African, Asian, and Arabic countries (Kalia and Lang, 2015). Overall, PD is thought to affect a number of people ranging from 66 to 1,500 per 100,000 in Europe (von Campenhausen et al., 2005)

and from 111 to 329 per 100,000 in Northern America (Strickland and Bertoni, 2004). The incidence of PD is strictly dependent on demographic factors, with an exponential increase after 80 years of age (Driver et al., 2009), a male-to-female ratio of 3:2 (de Lau and Breteler, 2006), and a higher number of cases among Hispanics and non-Hispanics white Americans (Van Den Eeden et al., 2003). Taking into account the expected progressive population aging, the number of patients suffering from PD is thought to significantly increase in the next decades, making this disease one of the main health issues to be faced in the future. Unfortunately, despite the availability of various symptomatic treatments (Connolly and Lang, 2014), effective disease-modifying therapies aimed at blocking or slowing down the progression of the disease are still lacking.

In this scenario, the development of new effective therapeutic strategies requires a better understanding of the pathogenetic processes leading to PD. The main histopathological features of PD are represented by the progressive loss of dopaminergic (DAergic) neurons in the midbrain substantia nigra pars compacta (SNpc) and the accumulation of intraneuronal insoluble protein aggregates named Lewy bodies (LBs; Kalia and Lang, 2015; Poewe et al., 2017). Since their discovery, the pathologic pathways leading to the formation of LBs have been considered crucial processes to be decrypted in order to unveil the pathogenesis of PD. The effort dedicated to the investigation of the molecular composition of LBs led to the identification of an abnormally folded protein as their main component, α-synuclein (α-syn; Goedert et al., 2013). The physiological functions of α-syn, which are still under investigation, include a wide range of neuronal homeostatic processes, such as synaptic vesicle dynamics and mitochondrial activity regulation (Vekrellis et al., 2011; Wales et al., 2013; Burré, 2015).

The basal ganglia network was traditionally depicted as divided in two structurally and functionally separated pathways, one favoring (direct) and one inhibiting (indirect) locomotor activation and movements (Calabresi et al., 2014). The projections arising from the SNpc can modulate the activation of the direct and indirect pathway and, specifically, dopamine (DA) can favor the movement through the activation of DA $D_1$ receptor ($D_1$R) mainly expressed by striatal spiny projections neurons (SPNs) of the direct pathway. Conversely, SPNs of the indirect pathway preferentially express DA $D_2$ receptor ($D_2$R), which exerts a neuronal inhibitory effect (Calabresi et al., 2014). It should be noted that recent findings have suggested a more complex and less simplistic view of the basal ganglia network, in which the DAergic regulation of striatal synaptic plastic properties is crucial to maintain a physiological motor function (Calabresi et al., 2014). Based on this, the loss of the regulatory role played by the DAergic nigral projections to the nucleus striatum causes a dysfunction of the whole basal ganglia neural circuit, paralleled by the occurrence of the typical parkinsonian motor syndrome.

The characteristic PD clinical picture was described by James Parkinson almost 200 years ago and includes bradykinesia, muscular rigidity, resting tremor, and postural and gait impairment (Przedborski, 2017). Over time, it became clear that PD patients are also characterized by a multitude of nonmotor features, which can precede the onset of motor symptoms by several years, such as depression, hyposmia, constipation, and sleep disorders (Kalia and Lang, 2015; Schapira et al., 2017). In this context, it should be noted that, among PD-related nonmotor features, the presence of cognitive impairment and autonomic dysfunction could have a dramatic impact on patients' quality of life (Poewe et al., 2017; Schapira et al., 2017). These observations have radically changed the pathogenic view of PD, suggesting an involvement of different brain areas at different times during the course of the disease, even before the loss of nigral neurons. While as previously mentioned the classical avenue of research has focused on the hallmark degeneration of the SNpc, the identification of early nonmotor features suggests a functional impairment of different brain areas at different times during the course of the disease, anticipating degeneration by several decades. Indeed, aberrant neuronal specific functions such as synaptic efficacy and neurotransmission represent an early temporal window that can be exploited for therapeutics benefit. In addition, the loss of DAergic cells was reported not to be complete at the onset of the parkinsonian motor syndrome (Kordower et al., 2013), also suggesting the presence of a therapeutic window in which the administration of neuroprotective drugs could significantly ameliorate the prognosis of patients suffering from the premotor and early motor PD phases. In this scenario, the identification of the mechanisms triggering PD-related neurodegenerative process is mandatory.

After almost two centuries of research in this field, PD is considered a multifactorial disease in which genetic and environmental factors synergistically trigger the disruption of multiple cellular processes, such as mitochondrial activity, synaptic transmission, and protein degradation pathways (Kalia and Lang, 2015). Several environmental toxins, herbicides, or pesticides have initially catalyzed the attention of the scientific community as possible etiologic factors, leading to the development of multiple toxin-based experimental models of PD (Goldman, 2014). However, the intense investigation of the genetic abnormalities underlying PD development has changed the etiologic view of the disease.

Genome-wide complex trait analysis suggested that the potential heritable factors leading to PD account for 30% of the total risk, with 28 identified genetic loci and many still unknown abnormalities that remain to be discovered/identified (Keller et al., 2012; Wood et al., 2013). The genetic abnormalities associated with a high risk of disease development, underlying almost 5% to 10% of all PD cases, are often called monogenic or causative mutations (Keller et al., 2012). By now, the list of genes involved in PD pathogenesis includes genes responsible for autosomal dominant PD (such as PARK1/SNCA, LRRK2, VPS35, EIF4G1, DNAJC13, and CHCHD2), autosomal recessive PD (such as Parkin, PINK1 and DJ-1) or less typical parkinsonian syndromes (for instance, PLA2G6 or ATP13A2; Kalia and Lang, 2015). Among these, PARK1/SNCA gene encoding α-syn was the first to be identified as responsible for autosomal dominant PD in Italian and Greek families (Polymeropoulos et al., 1997; Verstraeten et al., 2015). Point missense mutations (including the A30P, A53T and E46K), duplication, or triplication of this

gene confer high risk of PD development (Singleton et al., 2003; Vekrellis et al., 2011; Kara et al., 2014; Verstraeten et al., 2015), reinforcing the idea that α-syn aggregation plays a crucial role in the pathogenesis of the disease (Surmeier et al., 2017).

A particular interest has grown around the gene encoding leucine-rich repeat kinase (LRRK2), localized in the PARK8 locus, because its mutations can account for approximately 4% of all familial PD cases, representing the most frequent genetic cause of PD, and can be also identified in approximately 1% of sporadic cases (Healy et al., 2008). LRRK2 is a large protein, weighing 280 kDa with more than 2,500 amino acids, characterized by different domains such as leucine-rich repeats, WD40, Ras of complex-carboxy terminal of Roc (Roc-COR) GTPase, and serine–threonine kinase domains (Mata et al., 2006; Cookson, 2010). LRRK2 is expressed throughout the body and the brain, in many cell types; it is enriched in axonal and dendritic processes of cortical and striatal neurons, with a lower expression in DAergic nigral cell bodies (Melrose et al., 2006, 2007; Lee et al., 2010).

The functions of this protein have been extensively investigated, suggesting its involvement in a wide range of physiological processes including synaptogenesis and immune system modulation (Saha et al., 2009; Cookson, 2010, 2012; Piccoli et al., 2011; Dzamko and Halliday, 2012; Lee S. et al., 2012; Sanna et al., 2012; Russo et al., 2014; Taymans et al., 2015; Wallings et al., 2015; Roosen and Cookson, 2016; Rassu et al., 2017; Price et al., 2018). A temporal increase of LRRK2 levels in both primary culture and tissues (Biskup et al., 2006; Piccoli et al., 2011; Beccano-Kelly et al., 2014) illustrates a probable role in neuronal development and neurite outgrowth, also supported by the evidence obtained in knockout (KO) or mutant LRRK2 neurons (Sepulveda et al., 2013). However, the specific roles of this fascinating protein still need to be fully defined. In this review, we will focus on the evidence pointing toward LRRK2-dependent modulation of striatal synaptic transmission, mitochondrial activity, and α-syn aggregation in both physiological and pathological conditions. Taking into account the frequency of LRRK2 gene abnormalities in familial and sporadic PD, the investigation of the molecular pathways influenced by mutant LRRK2 is particularly intriguing, and will potentially lead to the identification of effective neuroprotective therapies suitable for a large number of patients.

## FROM LRRK2 GENE DISCOVERY TO DEVELOPMENT OF EXPERIMENTAL MODELS

In 2002, the research team headed by Funayama performed a genome-wide linkage analysis of a Japanese family from Sagamihara region presenting familial parkinsonism with autosomal dominant transmission (Funayama et al., 2002). Patients from the "Sagamihara family" exhibited clinical features resembling classical PD, with an average onset of symptoms at 50 years of age (Funayama et al., 2002). A pathological study performed in this family showed that all cases had evidence

of nigral degeneration at autopsy with varying amounts of LB pathology, ranging from completely undetected (at time of autopsy) to present and similar to conventional PD (Hasegawa et al., 2009). In one case, the pathological α-syn accumulation in glial cells was more widespread than what is usually observed in multiple systemic atrophy, an atypical parkinsonism (Hasegawa et al., 2009). The affected genomic locus was identified in the centromeric region of chromosome 12 (12p11.2-q13.1) and named PARK8 (Funayama et al., 2002). Interestingly, this haplotype was found not only in all the family members presenting a parkinsonian syndrome, but also in some unaffected carriers. This evidence suggested an incomplete penetrance of the mutation, with other genetic or environmental factors influencing the development of the disease (Funayama et al., 2002). A few years later, the linkage between PARK8 locus and PD was confirmed by a broad analysis of 21 Caucasian families with suspected autosomal dominant PD. This study showed the involvement of PARK8 in one family with German–Canadian kindred and one family from Western Nebraska (Zimprich et al., 2004b). The autoptic analysis of patients coming from these families confirmed the pleomorphic pathological picture of PARK8-related PD, ranging from a diffuse LB disease to a form of pure nigral degeneration (Zimprich et al., 2004a). Interestingly, the identification of PARK8 mutation in four Basque families, characterized by multiple cases of clinically typical PD with a mean age of 55 years at disease onset, suggested that this could be a commonly affected locus worldwide (Paisán-Ruíz et al., 2004; Paisàn-Ruìz et al., 2005). The specific gene was identified, thanks to two contemporary studies, published in the same *Journal*, and named LRRK2 or *dardarin* from the Basque term meaning "tremor" (Paisán-Ruíz et al., 2004; Zimprich et al., 2004a).

These advancements represented the beginning of LRRK2-centered research, which has led to the identification of 6 pathogenic LRRK2 mutations and more than 30 potentially pathogenic variants likely playing a key role in both familial and sporadic PD (Cookson, 2010; Monfrini and Di Fonzo, 2017). Among LRRK2 mutations, the one leading to the glycine-to-serine substitution G2019S in the LRRK2 protein was identified with unexpected high frequency (Di Fonzo et al., 2005; Gilks et al., 2005; Kachergus et al., 2005; Nichols et al., 2005). Indeed, the G2019S mutation was detected in approximately 5% to 6% of large familial European and American PD cohorts (Di Fonzo et al., 2005; Nichols et al., 2005) and in approximately 1% to 2% of sporadic PD from the United Kingdom (Gilks et al., 2005). Moreover, a very high frequency of G2019S mutation was identified in North African descent (up to 37%) and Ashkenazi Jewish (23%) familial and sporadic PD cases (Lesage et al., 2006; Ozelius et al., 2006). Overall, even taking into account the differences between various ethnicities, the G2019S LRRK2 mutation has arisen as the most frequent genetic determinant of PD (Monfrini and Di Fonzo, 2017). R1441 is the second most common pathogenic residue involved, with three known nonsynonymous (R1441C, R1441G, and R1441H) substitutions identified in several families worldwide (Puschmann, 2013; Monfrini and Di Fonzo, 2017). Of note, members of the Sagamihara family

were found to carry a mutation in the I2020 residue of LRRK2 (I2020T), which is located in the kinase domain of the protein (Funayama et al., 2005).

Most LRRK2 mutations were found to be located within the catalytic core domains of LRRK2, specifically Roc-GTPase and kinase domains (Cookson, 2010; Benson et al., 2018; Outeiro et al., 2019). Indeed, the common site of mutation R1441 (G/C/H) is located in the Roc-GTPase domain, whereas G2019S mutation involves the kinase domain itself, increasing its activity by twofold to threefold (West et al., 2005; Greggio et al., 2006; Jaleel et al., 2007; Nichols et al., 2010; Steger et al., 2016). Of note, the activity of the Roc-GTPase domain is essential for intramolecular activation of LRRK2 serine–threonine kinase, as the binding with GTP leads to its autophosphorylation with the subsequent activation of downstream cell signaling pathways (Guo et al., 2007; Outeiro et al., 2019). Conversely, the hydrolysis of GTP is able to induce LRRK2 inactivation (Guo et al., 2007; Outeiro et al., 2019). Whether LRRK2 acts as a homodimer, interacting through its Roc-COR domains, or as a monomer is still under debate (Klein et al., 2009; Ito and Iwatsubo, 2012; Terheyden et al., 2015; Nixon-Abell et al., 2016). It has been hypothesized that the cytosolic protein could be mainly represented by a monomeric and kinase-inactive form, whereas the dimeric form is kinase-active and mainly found in association with cellular membranous structures (Berger et al., 2010; James et al., 2012). The evidence that LRRK2 mainly acts in a dimeric membrane-bound form suggested that its physiological functions could be primarily represented by the regulation of cellular processes involving membranes or vesicular dynamics. Moreover, considerig that pathogenic mutations were found to alter LRRK2's active sites, the deregulation of the processes influenced by LRRK2 kinase activity could be crucial in PD development. Accordingly, several transgenic animal models, with different behavioral and neuropathological features, have been developed to unveil the pathophysiological consequences of abnormal LRRK2 function (Dawson et al., 2010; Blesa and Przedborski, 2014; Volta and Melrose, 2017).

The first transgenic mouse models were developed through the insertion of bacterial artificial chromosomes (BACs) carrying human or murine, mutant or wild-type LRRK2. These models were able to partially resemble the physiological specie-specific endogenous pattern of LRRK2 expression within the CNS, probably due to inappropriate regulation of gene expression induced by the insertion of exogenous human regulatory elements (Volta and Melrose, 2017). Overall, the BAC models expressing G2019S or R1441G/C exhibit mild abnormalities in striatal DAergic transmission, without significant nigral degeneration or LB accumulation (Li et al., 2009, 2010; Melrose et al., 2010; Sanchez et al., 2014; Walker et al., 2014; Beccano-Kelly et al., 2015; Volta et al., 2015; Sloan et al., 2016). From a behavioral point of view, BAC transgenic mice showed different phenotypes. Specifically, human BAC R1441G-LRRK2 mouse models showed a progressive and age-dependent hypokinesia reminiscent of PD, responsive to pharmacological treatments with L-DOPA (Li et al., 2009; Bichler et al., 2013), which could evolve to a state of immobility similar to late PD akinesia,

as assessed through home cage activity analysis, open field, and cylinder tests (Li et al., 2009). Conversely, BAC human G2019S-LRRK2 animals were characterized by a paradoxical mild hyperactivity during young/juvenile age and a subsequent progressive mild motor impairment with late cognitive deficits, not fully resembling a PD-like behavior (Melrose et al., 2010; Volta et al., 2015). Other authors showed that rats expressing human G2019S and R1441C LRRK2 developed an age-dependent and L-DOPA-responsive motor impairment (Sloan et al., 2016). It should be highlighted that the evidence obtained from BAC LRRK2 models could have been limited by the utilization of different background strains, by the possibility that endogenous LRRK2 expression could influence the phenotype of the animals, and by the fact that BAC models are produced through the random insertion of human or murine transgene with variable integration site and copy number (Volta and Melrose, 2017). While the error in copy number can be low, if it does occur the expression level will change accordingly making comparisons harder (Chandler et al., 2007). Moreover, the discussed studies have mainly compared control mice to mice overexpressing either wild-type or mutant LRRK2, because comparisons among animals overexpressing different genetic LRRK2 variants could be altered by many confounding factors. Collectively, these aspects could represent potential limitations of BAC transgenic model use in studies aimed at investigating the pathological and functional consequences of a specific LRRK2 mutation.

A different genetic strategy to study the role of LRRK2 was represented by the overexpression of LRRK2 through complementary DNA (cDNA) under the control of specific promoters. With this technique, different research groups developed transgenic models overexpressing mutant or wild-type LRRK2 in the whole brain or selectively in DAergic neurons (Ramonet et al., 2011; Zhou et al., 2011; Chen et al., 2012; Maekawa et al., 2012; Chou et al., 2014; Liu et al., 2015; Weng et al., 2016). The investigation of these genetic models led to a wide variety of results. For instance, it has been shown that the expression of human G2019S LRRK2 in the whole brain, including SNpc, is accompanied by a progressive loss of tyrosine hydroxylase–positive (TH$^+$) DAergic neurons with respect to nontransgenic littermates (Ramonet et al., 2011). Interestingly, the loss of TH$^+$ nigral cells was paralleled by a reduction of Nissl$^+$ nigral neurons, suggesting a neuronal degeneration, similar to that observed in LRRK2-related and idiopathic PD, rather than a loss of DAergic phenotype (Ramonet et al., 2011). This observation is further supported by the evidence that transgenic mice expressing human G2019S LRRK2 were characterized by a significant reduction in the number of SNpc DAergic neurons, whereas age-matched transgenic mice expressing human wild-type LRRK2 did not (Chen et al., 2012). Recently, a study investigating a tetracycline-inducible conditional transgenic mouse model, specifically expressing G2019S LRRK2 in DAergic neurons under the control of TH promoter, found an age- and kinase-dependent degeneration of neurons producing DA and norepinephrine (Xiong et al., 2018). Conversely, transgenic mice overexpressing R1441C LRRK2 were characterized by signs of neuronal suffering and cytopathological abnormalities such as enlarged vacuolar structures resembling autophagic vacuoles

and condensed aggregated mitochondria in the cerebral cortex, without degeneration of the nigrostriatal DAergic pathway probably due to the observed lack of transgene expression in the SNpc of the investigated mice (Ramonet et al., 2011). Of note, the mentioned cortical cytopathological abnormalities were more pronounced in mice overexpressing G2019S LRRK2 (Ramonet et al., 2011). Interestingly, even if there were no signs of neuronal loss in the midbrain, R1441C transgenic mice displayed an impairment of locomotor activity, which was related to the observed cortical involvement (Ramonet et al., 2011). In line with the previous study, conditional transgenic mice that selectively expressed human R1441C LRRK2 in DAergic neurons, under the control of the endogenous murine ROSA26 promoter, displayed abnormalities only at the nuclear envelope of nigral cells, without evidence of neuronal loss (Tsika et al., 2014). Significant loss of SNpc DAergic neurons in R1441C transgenic mice was reported in a different study, in which the expression of human R1441C LRRK2 was controlled by CMV enhancer and a platelet-derived growth factor β promoter (Weng et al., 2016). This conflicting observation could rely on the utilization of different murine strains (C57BL/6J vs. FVB/N mice) with different susceptibility to neurodegeneration and different gene promoters influencing the temporal patterns and/or the levels of neuronal transgene expression (Tsika et al., 2014; Weng et al., 2016). Similarly, the analysis of striatal DA levels in these models revealed partially conflicting results. Some authors found no differences in striatal DA concentration in G2019S and R1441C LRRK2 mouse models compared to nontransgenic littermates (Ramonet et al., 2011), whereas others showed a reduction of evoked DA levels in the R1441C model compared to wild-type mice (Weng et al., 2016). Conversely, a reduction in striatal DA content was found in mice with conditional overexpression of human G2019S LRRK2, selectively expressing the transgene in midbrain DAergic neurons, with respect to nontransgenic littermates and transgenic mice expressing human wild-type LRRK2 (Liu et al., 2015).

It should be noted that the absence of significant nigral degeneration should not be strictly interpreted as a limitation. Indeed, these models can be useful to understand the pathogenetic events occurring in the disease phases preceding neurodegeneration, potentially unveiling the early stages of LRRK2-related PD progression. Accordingly, an increased presence of high-molecular-weight species of α-syn, indicative of aggregation, was found in the striatum of a tetracycline-inducible transgenic human G2019S LRRK2 mouse model, using a CAMKIIα promoter and conditionally expressing the transgene in the forebrain, compared to nontransgenic animals and animals expressing the double-mutant, kinase-dead, G2019S/D1994A LRRK2 as functionally negative control (Xiong et al., 2017). Of note, in G2019S LRRK2 transgenic mice, the striatal accumulation of insoluble α-syn was accompanied by the presence of subtle behavioral deficits compared to nontransgenic and kinase-dead mice, even if the authors did not find significant loss of DAergic neurons in the midbrain (Xiong et al., 2017). Overall, the studies using BAC and cDNA models suggest that LRRK2 plays a crucial role in the modulation of the nigrostriatal pathway and specifically of striatal DA release. Moreover, it

should be mentioned that LRRK2 KO mice were extensively investigated, showing no abnormalities in the DAergic striatal transmission (Tong et al., 2010; Herzig et al., 2011; Hinkle et al., 2012; Tozzi et al., 2018b). This observation suggests that: (i) the lack of LRRK2 could be balanced by compensatory mechanisms; or (ii) the abnormalities of the nigrostriatal pathway observed in overexpressing LRRK2 models are mediated by a gain of function of the protein. In this context, the investigation of genetically modified knock-in (KI) mice could offer a more informative background than BAC- or c-DNA–based models, focusing on the effects of specific mutations without the confounding factors represented by the overexpression of altered LRRK2 together with endogenous LRRK2. Various G2019S and R1441C/G KI models have been developed on different murine genetic backgrounds (as reviewed in Volta and Melrose, 2017). Thanks to the studies performed in these transgenic models, it has been hypothesized that LRRK2 can modulate mitochondrial activity and corticostriatal synaptic transmission in an age-dependent manner, during both physiological and pathological conditions (Beccano-Kelly et al., 2015; Yue et al., 2015; Matikainen-Ankney et al., 2018).

Altogether, transgenic LRRK2 models can display a wide range of pathological features and functional abnormalities in the nigrostriatal pathway, which could help to unravel the pathogenetic events taking place before the irreversible loss of DAergic midbrain neurons.

# LRRK2 INVOLVEMENT IN STRIATAL SYNAPTIC TRANSMISSION

## Synaptogenesis and Synaptic Function

Possible LRRK2-induced abnormalities in corticostriatal synaptic transmission are suggested by the key role this protein plays in synaptogenesis and synaptic function (Esteves et al., 2014; Benson et al., 2018; Chen et al., 2018; **Figure 1**). Several studies, some of which investigating synaptic fraction preparations (Biskup et al., 2006; Piccoli et al., 2011), showed that LRRK2 is highly expressed in cerebral cortex and dorsal striatum compared to other brain areas (Taymans et al., 2006; Westerlund et al., 2008; Mandemakers et al., 2012; Davies et al., 2013; Giesert et al., 2013; West et al., 2014). Interestingly, the analysis of the LRRK2 gene expression patterns revealed a progressive temporal increase during *in vitro* neuronal development (Piccoli et al., 2011) and during postnatal development (Beccano-Kelly et al., 2014), reaching a maximum during the experience-dependent shaping of these connections (Benson et al., 2018). In line with this observation, some studies have shown that wild-type LRRK2 can modulate neurite outgrowth in developing neurons, because the LRRK2 KO was associated with abnormal elongation of neuronal processes (MacLeod et al., 2006; Parisiadou et al., 2009). Moreover, the G2019S LRRK2 mutation was found to be associated with a significant decrease in neurite length (Plowey et al., 2008). This effect, however, was found to be transient, overcome with time, and shown to be a function of velocity (Sepulveda et al., 2013). While the neurite outgrowth effect has been shown to be transient, it is no less important and illustrates another link to its potential in development of neurons.
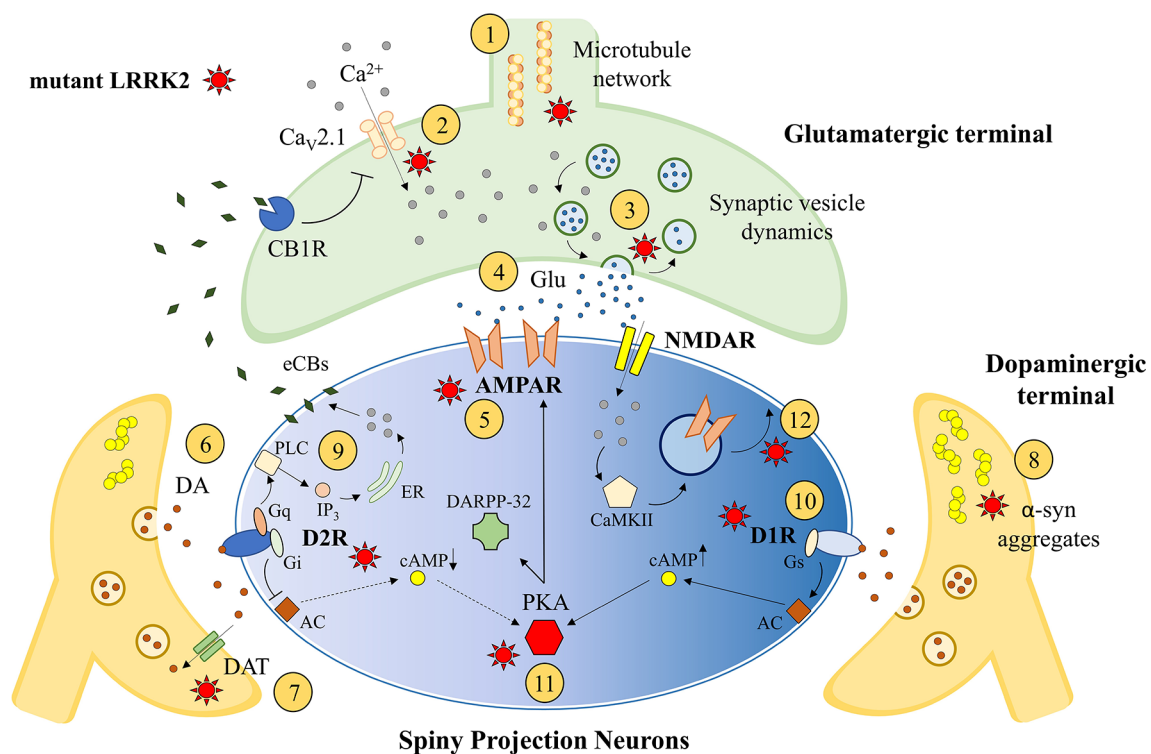
**FIGURE 1 |** Striatal synaptic effects of leucine-rich repeat kinase 2 (LRRK2). LRRK2 is thought to influence striatal synaptogenesis interacting with cytoskeleton and microtubules ①. The modulation of presynaptic voltage-gated $Ca^{2+}$ channels ($Ca_V2.1$; ②), as well as the regulation of synaptic vesicle exocytosis, endocytosis, and recycling ③, could influence the release of glutamate (Glu) from the corticostriatal excitatory terminal ④. Moreover, the postsynaptic expression of glutamatergic AMPAR could be influenced by LRRK2 activity ⑤. LRRK2 could alter striatal DAergic transmission ⑥ inducing midbrain neuronal loss or DAergic terminals abnormalities, including abnormal DAT activity ⑦ and pathological α-synuclein aggregation ⑧. The postsynaptic expression and function of DA receptors can be influenced by mutant LRRK2, including altered $D_2R$-dependent postsynaptic synthesis of endocannabinoids (eCBs; ⑨) and $D_1R$ expression/internalization ⑩. Finally, the dysfunction of PKA and DARPP32 pathways ⑪ and the impairment of intracellular AMPAR exocytosis ⑫ may result in altered synaptic long-term changes. AC, adenylate cyclase; CaMKII, $Ca^{2+}$-calmodulin dependent protein kinase II; cAMP, 3′,5′-cyclic adenosine monophosphate; CB1R, cannabinoid receptor type 1; ER, endoplasmic reticulum; IP3, inositol trisphosphate; PLC, phospholipase C.

The regulation of neurite outgrowth and synaptogenesis could rely on an LRRK2-dependent regulation of microtubule dynamics. Indeed, it has been reported that PD-causing mutations of LRRK2 can induce its abnormal binding to microtubules (Godena et al., 2014) and its aggregation into filamentous structures associated with the cytoskeleton in a well-ordered and periodic fashion (Kett et al., 2012).

Beyond synaptogenesis and cytoskeleton modulation, LRRK2 can exert additional roles at mature synaptic sites. Specifically, many studies have highlighted abnormal synaptic vesicle trafficking in transgenic LRRK2 models (Shin et al., 2008; Piccoli et al., 2011; Matta et al., 2012; Arranz et al., 2015; Belluzzi et al., 2016; Pan et al., 2017). LRRK2 was found to be associated with synaptic vesicle membranes where it could interact with vesicular proteins involved in exocytosis, endocytosis, and recycling dynamics, such as SNARE-complex proteins VAMP2, SNAP25, dynamin 1 and synaptophysin (Biskup et al., 2006; Piccoli et al., 2011, 2014). For instance, LRRK2 was found to interact with *N*-ethylmaleimide–sensitive fusion protein (NSF), which is a hexameric ATPase allowing the disassembling of SNARE

proteins during synaptic vesicle exocytosis (Belluzzi et al., 2016). Mutations of LRRK2 associated with an increased kinase activity may impair synaptic vesicle dynamics through aberrant phosphorylation of NSF, potentially leading to altered neurotransmitter release (Belluzzi et al., 2016).

It may not be only exocytosis that is altered by mutant LRRK2, because vesicle endocytosis was also found to be abnormal in both G2019S and R1441C/G LRRK2 mutants (Shin et al., 2008; Pan et al., 2017; Nguyen and Krainc, 2018; Nguyen et al., 2019). In this context, the specific endocytic pathway modulated by LRRK2 has not yet been identified, but possible kinase substrates are represented by Rab proteins, Synaptojanin1, or EndoA, an evolutionary, conserved protein critically involved in synaptic vesicle endocytosis (Shin et al., 2008; Matta et al., 2012; Pan et al., 2017; Nguyen et al., 2019). Interestingly, G2019S LRRK2 mutation was found to be associated with impaired synaptic vesicle endocytosis in ventral midbrain neurons, including DAergic neurons, but not in neurons from the neocortex or the hippocampus, suggesting a region-specific effect (Pan et al., 2017). The same study showed that pharmacological inhibition of LRRK2 kinase activity

rescued the observed endocytic defect in G2019S-expressing neurons, highlighting the involvement of the kinase domain in the modulation of synaptic dynamics (Pan et al., 2017). Lastly, it should be mentioned that LRRK2 could alter synaptic transmission not only by affecting exocytic/endocytic mechanisms, but also through interaction with voltage-gated calcium ($Ca^{2+}$) channels ($Ca_V2.1$ channels; Bedford et al., 2016). Indeed, LRRK2-dependent modulation of $Ca^{2+}$ entrance at the presynaptic site could dramatically influence neurotransmitter vesicle release (Bedford et al., 2016). Furthermore, increased $Ca^{2+}$ flux of this sort may influence $Ca^{2+}$ stores in organelles affecting their function. Mitochondrial $Ca^{2+}$ content, for instance, may influence mitochondrial membrane potential and ATP production (via dehydrogenase enzymes; Duchen, 2000; Denton, 2009). As mentioned earlier, ATP levels influence vesicle recycling and thus neurotransmitter release (Belluzzi et al., 2016), providing another route for altered neurotransmission. Overall, accumulating evidence suggests that LRRK2 PD-linked mutations could alter synaptic vesicle trafficking, potentially leading to abnormalities in striatal synaptic transmission, as well as to toxic effects contributing to the neurodegenerative process leading to PD (Nguyen et al., 2019).

## Glutamatergic Synaptic Transmission

The disruption of cortical glutamatergic inputs to the nucleus striatum could result in a severe alteration of the whole basal ganglia network. As such, possible alterations of striatal glutamatergic neurotransmission have been investigated in several KI LRRK2 experimental models. As discussed, the levels of LRRK2 are comparatively higher in both striatal and cortical regions (Melrose et al., 2006, 2007; Lee et al., 2010), making these areas worthy of investigation. Increase of spontaneous glutamatergic activity was observed in striatal neurons of G2019S LRRK2 KI mice during the postnatal period (Matikainen-Ankney et al., 2016; Volta et al., 2017), as well as in G2019S LRRK2 KI cortical neuronal cultures (Beccano-Kelly et al., 2014). Specifically, glutamate release was found to be markedly elevated in 3-week-old G2019S KI cortical neuronal cultures, without changes in synapse density. This observation suggested that the enhanced release could depend on increased vesicle release probability due to altered presynaptic regulatory protein profile (Beccano-Kelly et al., 2014; Piccoli et al., 2014). In acute corticostriatal slices, obtained from less than 1-month postnatal G2019S LRRK2 KI mice, spontaneous glutamatergic activity onto SPNs was significantly increased, both in the direct and indirect basal ganglia pathway (Matikainen-Ankney et al., 2016; Volta et al., 2017). The acute in vitro exposure to LRRK2 kinase inhibitors, as well as the isolation of the striatum from the overlying neocortex, were able to normalize the excitatory transmission in G2019S mutants, supporting an LRRK2 kinase-dependent alteration of corticostriatal function (Matikainen-Ankney et al., 2016). The hypothesis that LRRK2 kinase hyperactivity is required to induce synaptic changes is further supported by the evidence that subtle synaptic abnormalities were found in wild-type LRRK2 overexpressing ($\sim3\times$) neurons (Beccano-Kelly et al., 2014).

The effects of G2019S LRRK2 mutation on glutamatergic transmission appear to be age-dependent, prominent in young mice and progressively declining with age (Matikainen-Ankney et al., 2016; Volta et al., 2017). This is in line with the hypothesized involvement of LRRK2 in shaping neural connections during the postnatal development of striatal circuits, with potential permanent consequences (Matikainen-Ankney et al., 2016). The age-dependent effects of LRRK2 kinase hyperactivity on synaptic transmission are paralleled by the presence of behavioral abnormalities in young mice, such as an increased spontaneous exploration, which progressively normalize with time (Volta et al., 2017). Accordingly, different authors showed normal spontaneous glutamatergic transmission in adult LRRK2 KI mice (Matikainen-Ankney et al., 2016; Volta et al., 2017; Tozzi et al., 2018a) and adult BAC mice overexpressing human wild-type LRRK2 (Beccano-Kelly et al., 2015) compared to nontransgenic animals. Of interest, it has been proposed that the effects of LRRK2 kinase hyperactivity on glutamatergic corticostriatal transmission could still be present but more subtle during the adult age, unveiled only during specific tasks or by the activation of the DAergic receptors (Beccano-Kelly et al., 2015; Tozzi et al., 2018a). Specifically, the pharmacological stimulation of DA $D_2R$ was able to induce an enhanced reduction of glutamatergic transmission in 6-month-old G2019S LRRK2 KI mice compared to age-matched wild-type mice, and this effect was hypothesized to be dependent on a greater release of retrograde messengers from the SPNs (Tozzi et al., 2018a). It should be noted that the acute in vitro inhibition of LRRK2 kinase was not able to reverse the observed effect of $D_2R$ activation, suggesting that the constitutive LRRK2 kinase activation in striatal SPNs could permanently shape striatal connections (Tozzi et al., 2018a). The involvement of $D_2R$ in the LRRK2-dependent modulation of excitatory transmission could be an intriguing field of research, because the increased glutamate release observed in young G2019S KI mice was not influenced by the pharmacological agonism of $D_2R$ (Volta et al., 2017). Thus, the loss of the $D_2R$-dependent physiological inhibitory effect on striatal excitatory transmission may contribute to the enhanced glutamate release observed in young G2019S KI mice, with a subsequent age-dependent recovery leading to enhanced inhibition in adult mice.

Lastly, it has been shown that glutamatergic transmission could be influenced by LRRK2-induced changes to postsynaptic glutamatergic receptors, such as AMPA receptor (AMPAR) subunit expression. Indeed, an increase in amplitude of spontaneous excitatory postsynaptic currents was shown in LRRK2 KO mice compared to wild-type animals, potentially due to increased expression of GluR1 AMPAR subunit at the synaptic site (Parisiadou et al., 2014). Moreover, mice expressing G2019S LRRK2 KI mutation lacked functional calcium-permeable AMPARs in SPNs of the nucleus accumbens (Matikainen-Ankney et al., 2018).

## Dopaminergic Synaptic Transmission

As previously discussed, the various existing transgenic LRRK2 experimental models are characterized by different degrees of DA depletion within the nucleus striatum.

Considering that LRRK2 transgenic models investigated so far are characterized by various levels and spatial/temporal patterns of LRRK2 expression (mutant or wild-type), it is difficult to conclude if LRRK2-related PD is associated with a primary damage of DAergic cells in the midbrain or with an isolated dysfunction of striatal DAergic terminals. Indeed, transgenic LRRK2 models based on the use of the cDNA usually displayed nigral DAergic neuronal loss (Ramonet et al., 2011; Chen et al., 2012; Weng et al., 2016; Xiong et al., 2018), whereas the transgenic BAC mice were not characterized by neurodegenerative features in the midbrain (Li et al., 2009, 2010; Melrose et al., 2010; Sanchez et al., 2014; Walker et al., 2014; Beccano-Kelly et al., 2015; Volta et al., 2015; Sloan et al., 2016).

Focusing on the effects of increased LRRK2 activity upon DA transmission in mutants, an age-dependent reduction of basal striatal extracellular DA levels has been shown in G2019S KI mice, which was hypothesized to be dependent on a latent impairment of synaptic DA release (Yue et al., 2015). A subsequent study suggested that striatal DA loss in G2019S KI mice could represent the consequence of an altered regulation of DA release and/or nigral burst firing patterns *in vivo*, rather than impaired single synapse release or DA transporter (DAT) activity (Volta et al., 2017). Moreover, slices from young G2019S KI mice displayed enhanced DA release upon repeated stimulation compared to wild-type animals. This effect was no longer evident in old animals, suggesting that DAergic transmission could be modulated by LRRK2 in an age-dependent manner similarly to glutamatergic transmission (Volta et al., 2017). Considering these results, the authors suggested that one possible explanation would be that the hyperactivation of LRRK2 could induce a premature aging of DAergic terminals (Volta et al., 2017). Another such hypothesis would be the acute-cum-chronic compensation, which occurs due to $D_2R$ insensitivity at young ages and results in an unsustainable situation and synaptic stress.

The hypothesis of a specific DAergic terminal vulnerability is further supported by the results obtained by other groups, showing that G2019S mutation is associated with lower DA striatal levels in old mice (Tozzi et al., 2018b) and is able to progressively alter DAT activity together with α-syn accumulation at striatal DAergic terminals (Longo et al., 2017). Accordingly, BAC transgenic mice overexpressing G2019S LRRK2 showed age-dependent decrease of striatal DA content, release, and uptake, with possible selective DAergic terminal damage because no nigral cell loss was detected (Li et al., 2010). Finally, a transgenic model selectively expressing the G2019S LRRK2 in midbrain DAergic neurons displayed no substantial SNpc neuronal loss (Liu et al., 2015). However, it was possible to detect a reduction of striatal DA content and release, coincident with the degeneration of DAergic axonal terminals and with the reduction of the enzymatic machinery responsible for DA synthesis, transport, and degradation (Liu et al., 2015). Overall, despite the previously discussed limitations of existing LRRK2 experimental models, accumulating evidence suggests that the expression of mutant G2019S LRRK2 could induce a selective damage of DAergic axon terminals in the nucleus striatum, potentially preceding midbrain neuronal loss. This is further supported by the observation that G2019S

LRRK2 expressing mice were characterized by early-phase dysfunction of SNpc DAergic neurons, including a reduction in striatal evoked DA release, several months before the irreversible degeneration of these cells (Chou et al., 2014), as observed also in BAC mice overexpressing human wild-type LRRK2 (Beccano-Kelly et al., 2015).

Mutant LRRK2 does not alter only DAergic projections, as reported by different studies describing the presence of abnormal DA receptor expression and function in transgenic LRRK2 models. For instance, it has been demonstrated that transfection of SH-SY5Y cells with G2019S or R1441G LRRK2 increased the expression of DA $D_1R$, an effect confirmed by Western blot analysis of striatal membrane fractions obtained from transgenic mice overexpressing G2019S LRRK2, showing increased $D_1R$ expression with respect to nontransgenic animals (Migheli et al., 2013). In addition, mutant G2019S LRRK2 could impair the internalization of $D_1R$, which should take place after its sustained activation, prolonging the activation of its signaling transduction pathway and increasing intraneuronal production of cAMP (Rassu et al., 2017). The $D_1R$ transduction pathway could be influenced by LRRK2 activity itself, because the protein kinase A (PKA)-dependent phosphorylation of synaptic AMPAR subunit GluR1 was abnormally enhanced after treatment with a $D_1R$ agonist in a mouse model lacking LRRK2 (Parisiadou et al., 2014). Collectively, these effects could contribute to the abnormal striatal synaptogenesis and transmission observed in LRRK2 transgenic models. Moreover, G2019S LRRK2 was also able to influence the physiological turnover of $D_2R$ by decreasing the rate of its trafficking from the Golgi complex to the cell membrane (Rassu et al., 2017). In apparent contrast with this observation, other studies highlighted the presence of unaltered $D_2R$ expression in transgenic LRRK2 mouse models (Li et al., 2010; Melrose et al., 2010).

Overall, it has been hypothesized that LRRK2 overexpression could influence $D_2R$ surface expression, with variations depending on the analyzed model, whereas the mutations leading to LRRK2 hyperactivation could influence the downstream $D_2R$ signaling pathway (Volta and Melrose, 2017). In line with this hypothesis, abnormal $D_2R$ function was identified in young G2019S KI mice, where the physiological $D_2R$-dependent negative regulation of glutamatergic transmission was absent (Volta et al., 2017), whereas an increased inhibitory effect following $D_2R$ activation was found in adult G2019S KI mice (Tozzi et al., 2018a). In this last work, it has been shown that the enhanced inhibition of excitatory transmission was mediated by the postsynaptic release of endocannabinoids (eCBs), produced after phospholipase C (PLC) activation, which act as retrograde messengers on the presynaptic cannabinoid receptor type 1 (CB1R; Tozzi et al., 2018a). Because the function of CB1R was not itself altered, an increased activation of the $D_2R$/PLC/eCBs pathway in SPNs of adult transgenic KI mice has been hypothesized (Tozzi et al., 2018a). Lastly, another interesting report has shown the presence of altered $D_2R$ signaling in a transgenic model overexpressing LRRK2 (Beccano-Kelly et al., 2015). Specifically, the authors showed an alteration of another postsynaptic $D_2R$-dependent transduction

pathway, involving the PKA-regulated phosphoprotein DARPP-32 (Beccano-Kelly et al., 2015).

The effects of LRRK2 activity on DA receptor expression and function deserve further investigation. Considering that LRRK2 is poorly expressed in DAergic nigral cells but highly expressed in the striatum (Melrose et al., 2006, 2007; Lee et al., 2010), abnormalities of striatal DA transmission could be a result of postsynaptic rather than presynaptic alterations. In this scenario, a more in-depth characterization of the LRRK2-depedent modulation of DA receptors could help in understanding the mechanisms leading to the striatal synaptic dysfunction occurring in PD (Calabresi et al., 2007, 2014; Schirinzi et al., 2016).

## Striatal Synaptic Plasticity

Considering that LRRK2 activity influences both glutamatergic and DAergic striatal synaptic transmission, it is reasonable to hypothesize the presence of alterations of synaptic long-lasting changes. Interestingly, recent work has shown that both $D_1R$- and $D_2R$-expressing SPNs were unable to express synaptic long-term potentiation (LTP) at corticostriatal synapses in G2019S LRRK2 KI mice, probably because of an LRRK2-dependent impairment of AMPAR trafficking (Matikainen-Ankney et al., 2018). Of note, $D_2R$-expressing SPNs exhibited synaptic long-term depression (LTD) after the stimulation protocol able to induce LTP in wild-type mice (Matikainen-Ankney et al., 2018). This observation is in line with the previously discussed enhancement of $D_2R$-dependent eCB release observed in G2019S LRRK2 mice (Tozzi et al., 2018a). Moreover, it should be considered that the activation of DA $D_2Rs$ normally exerts a negative control on the induction of $N$-methyl-D-aspartate receptor (NMDAR)-dependent LTP, and the induction of LTD is thought to require a weaker DAergic input because of the higher affinity of $D_2R$ for DA compared to $D_1R$ (Jaber et al., 1996; Calabresi et al., 2007). The presence of reduced DA levels in the striatum of LRRK2 G2019S KI mice (Liu et al., 2015; Tozzi et al., 2018b), together with an enhanced $D_2R$ signaling, could favor the induction of an eCB-dependent LTD in $D_2R$-expressing SPNs. This hypothesis deserves further investigations, since other authors have shown an impairment of striatal LTD induction, together with reduced evoked striatal DA release, in transgenic mice overexpressing human G2019S LRRK2 (Chou et al., 2014).

In physiological conditions, the molecular mechanisms leading to LTP induction involve the activation of $Ca^{2+}$-calmodulin-dependent protein kinase II (CaMKII), which can increase the number of AMPARs expressed at the postsynaptic membrane through the exocytosis of intracellular vesicular AMPAR pools or through lateral diffusion of extrasynaptic receptors (Choquet and Triller, 2003; Malenka and Bear, 2004; Opazo et al., 2012; Nicoll, 2017). In this scenario, an LRRK2-induced alteration of vesicle trafficking (discussed earlier) could play a critical role. LRRK2 could influence the LTP-dependent AMPAR synaptic expression through interaction with Rab8a (Steger et al., 2016), a small vesicular transport protein acting as a critical component of the molecular pathway leading to AMPARs insertion into synapses (Gerges et al., 2004).

Another possible mechanism explaining the aberrant synaptic plasticity is the dysregulation of PKA/DARPP32 pathway. In the striatum, $D_1R$ and $D_2R$ exert opposite effects on PKA activity, stimulating and inhibiting its function (Calabresi et al., 2007). Once activated, PKA plays a key role in the modulation of LTP and LTD induction, mediating the synaptic incorporation of AMPARs through the phosphorylation of GluR4 and GluR1 subunits (Esteban et al., 2003) and activating the DA- and cAMP-regulated DARPP32 protein, which acts as an inhibitor of protein phosphatase 1 (Greengard et al., 1999; Calabresi et al., 2007). Interestingly, as previously discussed, LRRK2 interacts with both PKA and DARPP32 (Parisiadou et al., 2014; Beccano-Kelly et al., 2015; Greggio et al., 2017; Tozzi et al., 2018b) and a better understanding of the effects induced by LRRK2 mutants on these proteins could explain the observed alterations of striatal synaptic plasticity.

Of note, the effects of LRRK2 kinase activity on synaptic plasticity could go beyond corticostriatal connections, because the induction of synaptic LTD was impaired in the hippocampus of BAC transgenic mice overexpressing G2019S LRRK2 (Sweet et al., 2015). Also in this case, an impairment of AMPAR trafficking behind the synaptic defect was hypothesized, because its internalization during LTD induction could be impaired by LRRK2 hyperactivation (Sweet et al., 2015). Collectively, the mechanisms leading to synaptic long-term changes could be disrupted in the presence of abnormal LRRK2 kinase activity, and this synaptic dysfunction could take place long before the progressive loss of DAergic nigral cells, accompanied by the presence of clinical symptoms (dystonia, goal-directed movement dysfunction), which are thought to rely on such functions (Mink, 2018). The identification of the molecular pathways involved in this process could unveil new therapeutic strategies aimed at preserving neural network activity earlier in PD progression.

## LRRK2 INVOLVEMENT IN MITOCHONDRIAL FUNCTION

Mitochondrial dysfunction is considered a crucial pathogenic mechanism in the neurodegenerative process leading to PD (Schapira, 2007; Bose and Beal, 2016). Epidemiological studies highlighted a possible association between PD development and exposure to environmental toxic agents targeting mitochondrial activity, such as pesticides or herbicides (Kalia and Lang, 2015). Moreover, the recreational use of a meperidine analog, 1-methyl-4-phenyl-4-propionoxypiperidine, was associated in some individuals with the development of a parkinsonian syndrome (Langston et al., 1983; Ballard et al., 1985). The pathogenesis of this syndrome was found to be caused by the presence of a contaminant molecule, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which could be converted in a compound targeting mitochondrial respiratory chain complex I (Nicklas et al., 1985). Subsequently, the activity of mitochondrial complex I was found to be reduced in several tissues isolated from PD patients (Schapira, 2007; Bose and Beal, 2016), and mitochondrial complex I inhibitors, such as MPTP or rotenone, were found to be able to lead to the somewhat specific death

of catecholaminergic neurons including nigral DA cells and have been widely employed to induce experimental PD models (Cicchetti et al., 2009; Bezard and Przedborski, 2011).

The discovery and the study of the genetic abnormalities linked to PD further supported the importance of mitochondrial dysfunction in the pathogenetic process leading to the development of the disease. Many of the proteins encoded by genes causing recessive, atypical forms of PD, such as parkin, PINK1, and DJ-1, are involved in mitochondrial homeostatic processes (Lin and Beal, 2006; McCoy and Cookson, 2012; Kalia and Lang, 2015; Bose and Beal, 2016). The investigation of the pathophysiological consequences of LRRK2 mutations also unveiled a mitochondrial regulatory role for this protein (Esteves et al., 2014; Singh et al., 2019). Indeed, the presence of LRRK2 mutations has been linked to abnormalities in mitochondrial ATP and reactive oxygen species (ROS) production, mitochondrial fusion and fission dynamics, mitophagy, mitochondrial DNA (mtDNA) damage, and calcium homeostasis (**Figure 2**). For instance, analysis of mitochondrial function and morphology in skin biopsies obtained from LRRK2 mutant patients revealed the presence of altered mitochondrial membrane potential, reduced ATP levels, mitochondrial elongation, and increased mitochondrial interconnectivity in the G2019S mutation carriers (Mortiboys et al., 2010).
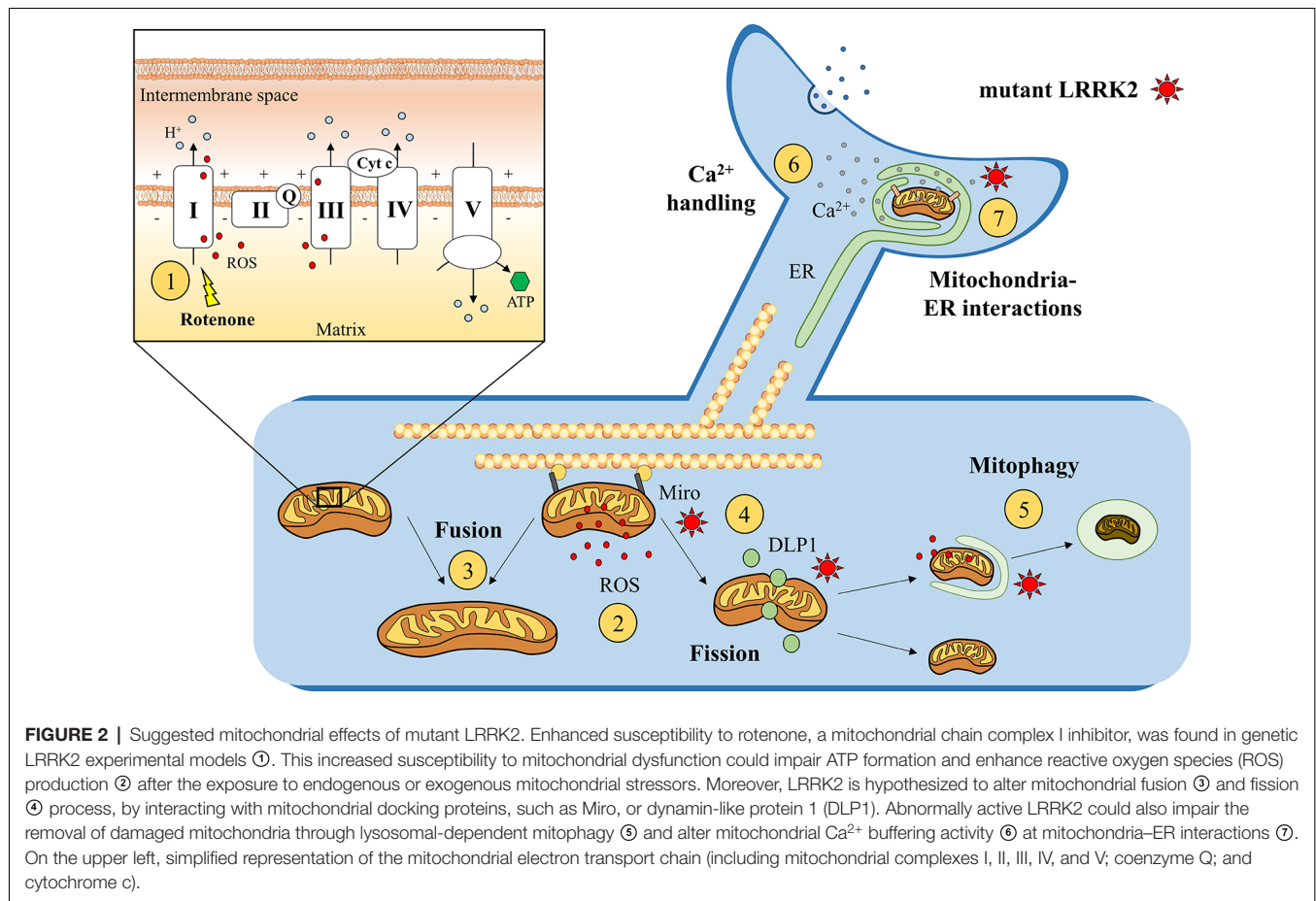
A mitochondrial regulatory role for LRRK2 is also supported by the evidence that, in LRRK2 overexpressing models, approximately 10% of the protein was found in the mitochondrial cell fractions, with immunohistochemical and biochemical studies suggesting a mitochondrial localization (West et al., 2005; Biskup et al., 2006). The preferential association of LRRK2 with a variety of cellular membrane and vesicular structures suggests an affinity of LRRK2 for lipids or lipid-associated proteins and a potential localization in mitochondrial outer membrane (West et al., 2005; Biskup et al., 2006). Such localization could influence mitochondrial fusion and fission processes, crucial for the maintenance of a functional mitochondrial network along the neuron and the axon (Cho et al., 2010; Su et al., 2010; Bertholet et al., 2016). It should be noted that the large amount of evidence for LRRK2 effects on mitochondria is not matched by a corresponding amount of data supporting a physical interaction (from Berwick et al., 2019). Indeed, a direct association between LRRK2 and mitochondrial membranes was not confirmed by subsequent studies, and the utilization of tagged LRRK2 suggested an association with different cellular structures such as endosomes/endoplasmic reticulum (ER; Gómez-Suaga et al., 2014; Schreij et al., 2015).

The fusion and fission of neuronal mitochondria are highly regulated processes, which could be disrupted by mutant LRRK2. Indeed, aged LRRK2 G2019S KI mice were characterized by profound mitochondrial abnormalities in the striatum, consistent with arrested fission (Yue et al., 2015). Other authors suggested that LRRK2 regulates mitochondrial dynamics through direct interaction with a fission dynamin-like protein 1 (DLP1 or DRP1), because LRRK2 overexpression was associated with mitochondrial fragmentation together with increased DLP1 expression (Niu et al., 2012; Wang et al.,

2012). The role of DLP1 in mitochondrial fission is well established (Chang and Blackstone, 2010), and considering that mitochondrial fragmentation is thought to precede the elimination of dysfunctional mitochondria, a physiological DLP1 activity could facilitate mitochondrial elimination after a toxic stimulus (Arnoult et al., 2005). Disruption of this process could lead to abnormal mitochondrial fragmentation in physiological conditions and/or reduced mitochondrial elimination after exposure to environmental toxic agents. Both these processes could facilitate the development of PD. Interestingly, abnormal LRRK2 kinase activity was able to alter the DLP1-regulated biological processes (Wang et al., 2012). Specifically, LRRK2 G2019S mutation was found to enhance the translocation of DLP1 from cytosol to mitochondria leading to enhanced mitochondrial fission (Niu et al., 2012). In line with this hypothesis, fragmented and dysfunctional mitochondria were found in fibroblasts obtained from G2019S carriers (Grünewald et al., 2014), and treatment with a pharmacological inhibitor of DLP1 was able to reduce mitochondrial fragmentation in LRRK2 G2019S-expressing cells and PD patient fibroblasts (Su and Qi, 2013). Overall, the functional interactions between LRRK2 and DLP1 seem to be involved in the regulation of mitochondrial dynamics, as further suggested by the evidence that a recently identified LRRK2 variant, E193K, was able to alter the possible LRRK2/DLP1 binding, leading to an abnormal mitochondrial fission after a metabolic insult (Perez Carrion et al., 2018).

Other groups also found alterations in mitochondrial reaction to toxic agents in LRRK2 models. Indeed, it has been shown that G2019S LRRK2-overexpressing SHSY5Ycells were characterized by abnormally highly fragmented mitochondrial network after exposure to rotenone, a mitochondrial complex I inhibitor (Tozzi et al., 2018b). In this case, the potential involvement of DLP1 was not investigated, but the pharmacological activation of $D_2R$ was able to counteract the abnormal mitochondrial fragmentation, suggesting the involvement of additional pathways linking LRRK2 activity and mitochondrial dynamics that deserve further investigation (Tozzi et al., 2018b). In this context, it should be noted that increased mitochondrial fragmentation after toxic stimuli could represent a neuronal attempt to remove dysfunctional mitochondria, thus meaning an enhanced mitochondrial vulnerability to environmental toxic injuries in the presence of abnormal LRRK2 kinase activity. Moreover, mitochondria that underwent fission process should be subsequently eliminated through autophagic mechanisms, but an alteration at this level could increase the content of uncleared fragmented mitochondria. For now, it is difficult to have a single answer to these questions because available studies support both hypotheses.

An investigation performed in *Caenorhabditis elegans* showed that human wild-type LRRK2 reduced the toxic effect of mitochondrial toxins, such as rotenone or paraquat, but this protective effect was lost in G2019S LRRK2-expressing nematodes, with a rapid loss of DAergic markers (DAT:GFP fluorescence and dopamine levels; Saha et al., 2009). Indeed, increased susceptibility to rotenone-induced toxicity has been described in transgenic *Drosophila*-expressing mutant LRRK2,

**FIGURE 2 |** Suggested mitochondrial effects of mutant LRRK2. Enhanced susceptibility to rotenone, a mitochondrial chain complex I inhibitor, was found in genetic LRRK2 experimental models ①. This increased susceptibility to mitochondrial dysfunction could impair ATP formation and enhance reactive oxygen species (ROS) production ② after the exposure to endogenous or exogenous mitochondrial stressors. Moreover, LRRK2 is hypothesized to alter mitochondrial fusion ③ and fission ④ process, by interacting with mitochondrial docking proteins, such as Miro, or dynamin-like protein 1 (DLP1). Abnormally active LRRK2 could also impair the removal of damaged mitochondria through lysosomal-dependent mitophagy ⑤ and alter mitochondrial $Ca^{2+}$ buffering activity ⑥ at mitochondria–ER interactions ⑦. On the upper left, simplified representation of the mitochondrial electron transport chain (including mitochondrial complexes I, II, III, IV, and V; coenzyme Q; and cytochrome c).

including G2019S (Ng et al., 2009). A similar enhanced cellular susceptibility to mitochondrial dysfunction was described in neural cells generated from induced pluripotent stem cells (iPSCs) obtained from LRRK2 PD patients (Cooper et al., 2012) and DAergic neurons derived from iPSCs of patients carrying the G2019S mutation (Nguyen et al., 2011). Accordingly, transgenic G2019S mice seem to be more vulnerable to the detrimental effect of mitochondrial toxins (Tozzi et al., 2018b). Specifically, it has been shown that the neurotoxic effect induced by rotenone exposure was enhanced in corticostriatal slices obtained from G2019S KI mice, relative to wild-type, LRRK2 kinase-dead, and LRRK2 KO mice, suggesting that the sustained activation of LRRK2 kinase domain was involved (Tozzi et al., 2018b). Furthermore, the rotenone-dependent reduction of cellular ATP synthesis, associated with increased ROS production, was significantly enhanced in SHSY5Y cells overexpressing G2019S LRRK2 compared to control cells (Tozzi et al., 2018b). Of note, the pharmacological activation of $D_2R$ reduced rotenone toxicity in G2019S LRRK2 KI mice, with potential involvement of the cAMP/PKA pathway because the pharmacological inhibition of PKA was able to mimic the $D_2R$-dependent protective effect in G2019S KI mice, whereas the exposure to a cAMP analog enhanced rotenone toxicity in the striatum of wild-type mice (Tozzi

et al., 2018b). In this scenario, the possible involvement of PKA pathway in mitochondrial homeostasis should be further investigated to be better understood (Valsecchi et al., 2013; Di Benedetto et al., 2018), considering that PKA-dependent phosphorylation of mitochondrial proteins may enhance mitochondrial ROS production (Prabu et al., 2006; Fang et al., 2007). Interestingly, the induced expression in cultured cortical neurons of both wild-type and G2019S LRRK2 was associated with increased cellular ROS production, an effect not seen with the kinase-dead mutant LRRK2 (Niu et al., 2012), and LRRK2 kinase hyperactivity could reduce the antioxidant mitochondrial defense through interaction with peroxiredoxin-3, the most important mitochondrial scavenger of hydrogen peroxide (Angeles et al., 2014). The presence of increased ROS production could trigger a vicious cycle through mtDNA damage, leading to irreversible mitochondrial dysfunction. Accordingly, LRRK2 G2019S patient-derived lymphoblastoid cell lines (LCLs) and iPSC-derived neural cells exhibited increased mtDNA damage (Sanders et al., 2014; Howlett et al., 2017), and treatment with an LRRK2 kinase inhibitor (Howlett et al., 2017) or the zinc finger nuclease-mediated gene correction of G2019S mutation (Sanders et al., 2014) was able to prevent or to restore it.

During physiological conditions, dysfunctional and damaged mitochondria are removed through lysosomal-dependent mitophagy. Several studies suggested that LRRK2 could modulate this cellular process (Ferree et al., 2012; Beilina et al., 2014; Schapansky et al., 2014; Hsieh et al., 2016; Wallings et al., 2019). Experiments through protein–protein interaction arrays revealed a possible link among LRRK2 and BCL2-associated athanogene 5, Rab7L1 (RAB7, member RAS oncogene family-like 1), and cyclin-G-associated kinase, all of which are involved in the autophagy–lysosome system (Beilina et al., 2014). A role in the regulation of autophagy was suggested by the evidence that silencing endogenous LRRK2 expression, or its kinase activity inhibition, resulted in deficits of the autophagic processes in immune cells (Schapansky et al., 2014), as well as by a transcriptome analysis of human brain, human blood cells, and *C. elegans* expressing human wild-type LRRK2 (Ferree et al., 2012). LRRK2 was shown to significantly contribute to autophagosome-lysosome fusion and lysosomal pH. This is achieved *via* direct binding of LRRK2 to the vacuolar-type H$^+$-ATPase pump a1 (Wallings et al., 2019). Overall, it can be hypothesized that some of LRRK2 PD–related mutations may alter the neuronal ability to degrade damaged intracellular organelles. Interestingly, a possible molecular mechanism linking LRRK2 and mitophagy has been suggested by a work showing in iPSC-derived neurons that wild-type LRRK2 promotes the removal of a mitochondrial docking protein, Miro, as an early step in dysfunctional mitochondria clearance (Hsieh et al., 2016). The presence of G2019S mutation disrupted this physiological LRRK2 function, delaying the arrest of damaged mitochondria with subsequent impairment of mitophagy (Hsieh et al., 2016). Of note, Miro degradation and mitochondrial motility were also found to be impaired in fibroblasts obtained from sporadic PD patients (Hsieh et al., 2016), suggesting that this pathway could be commonly involved during the development of familial and idiopathic PD. Thus, the emerging picture seems extremely complex, with LRRK2 potentially influencing mitochondrial chain complex activity, susceptibility to oxidative stress, and mitochondrial removal pathways. Overall, the presence of LRRK2 mutations could influence the ability of DAergic neurons to cope with exposure to environmental or endogenous mitochondrial stressors, acting as a strong predisposing factor for PD. This observation could explain the frequency of LRRK2 abnormalities in familial and sporadic PD, thus increasing the potential impact of LRRK2-centered neuroprotective strategies.

As a concluding remark, it should be considered that LRRK2 could also alter physiological mitochondrial Ca$^{2+}$ buffering activity. The entrance of Ca$^{2+}$ into the mitochondrial matrix through mitochondrial Ca$^{2+}$ uniporter is made possible by the electrochemical proton gradient created by the electron transport chain. Thanks to this property, mitochondria can dynamically uptake and release Ca$^{2+}$, influencing the concentration of this ion in the whole cellular cytosol or in a specific subcompartment, such as presynaptic and postsynaptic terminals (Rizzuto et al., 2012). Dysregulation of this process could affect neuronal synaptic transmission *via* microdomain Ca$^{2+}$ release and/or trigger cellular death, through the apoptotic

or necrotic pathways. In this context, it has been shown that murine cortical neurons expressing mutant G2019S or R1441C LRRK2 were characterized by neuronal Ca$^{2+}$ imbalance (Cherra et al., 2013). Also, LRRK2 G2019S iPSC-derived sensory neurons displayed altered Ca$^{2+}$ dynamics, observed through live-cell Ca$^{2+}$ imaging, which was counteracted by LRRK2 inhibitors (Schwab and Ebert, 2015).

Interestingly, in order to facilitate Ca$^{2+}$ buffering, it has been shown that mitochondria are frequently located in proximity of specific cellular microdomains with local high Ca$^{2+}$ concentration, such as the synaptic terminals, Ca$^{2+}$ channels at the plasma membrane (David et al., 1998; Glitsch et al., 2002; Young et al., 2008), and the ER, with which the mitochondria closely interact (Rizzuto et al., 1998; Csordás et al., 2006, 2010). In this context, the structurally tethered ER–mitochondria interactions, named mitochondria-associated membranes (MAMs), can facilitate Ca$^{2+}$ exchange and regulate local Ca$^{2+}$ concentration, influencing various cellular processes including ATP production, autophagy, apoptosis, and synaptic transmission when located at the presynaptic sites (Simmen et al., 2010; Rizzuto et al., 2012; Rowland and Voeltz, 2012; Hamasaki et al., 2013; Kornmann, 2013; Marchi et al., 2014; Devine and Kittler, 2018). A possible structural and/or functional disruption of the MAMs is thought to occur during the development of various neurodegenerative diseases, including PD (Paillusson et al., 2016; Devine and Kittler, 2018). Interestingly, a recent publication showed that LRRK2 is involved in the regulation of ER–mitochondria interactions, with the evidence that the G2019S LRRK2 mutation could lead to the ubiquitin-mediated degradation of ER–mitochondrial tethering proteins (Toyofuku et al., 2020). Of note, it has been shown that also α-syn can localize at the level of MAMs, and its genetic abnormalities are associated with reduced mitochondria–ER apposition and abnormal Ca$^{2+}$ exchange (Guardia-Laguarta et al., 2014; Paillusson et al., 2017). Overall, mitochondria–ER interactions represent an interesting avenue to be investigated, potentially unveiling new molecular pathogenic pathways linking LRRK2, α-syn, and mitochondrial homeostasis.

## LRRK2 AND α-SYNUCLEIN AGGREGATION

The investigation of the molecular pathways linking LRRK2 and α-syn has attracted a lot of attention (Esteves et al., 2014; Schapansky et al., 2015; Cresto et al., 2019; Outeiro et al., 2019). A possible role for LRRK2 in the formation of abnormally folded α-syn aggregates was suggested by histopathological studies showing that LRRK2 could be found in the context of LBs. Specifically, immunohistochemical analysis of brain samples obtained from patients with confirmed PD and LB dementia revealed that 20% to 100% (mean, 60%) of α-syn–positive LBs contained LRRK2 (Perry et al., 2008). Interestingly, other authors showed that the presence of LRRK2 in the core of LBs was higher in the SNpc than in the locus coeruleus of brains obtained from sporadic PD patients, but the percentage of LBs with detectable LRRK2 was significantly higher in both the brain areas of patients carrying the G2019S LRRK2 mutation (Vitte et al., 2010). Accordingly, it has been shown that LRRK2 levels

are positively correlated to pathological α-syn aggregation in the affected brain regions, colocalizing with neurons and LBs (Guerreiro et al., 2013).

Other clues on the possible relationship between LRRK2 and α-syn have been given by preclinical studies, highlighting molecular interactions between the two proteins in a cell culture model of α-syn inclusion formation (Guerreiro et al., 2013). Moreover, LRRK2 overexpression significantly accelerated the progression of α-syn aggregation in PD-related A53T SNCA transgenic mice, whereas the genetic ablation of LRRK2 was able to delay it (Lin et al., 2009).

It has been suggested that the abnormal LRRK2-induced α-syn aggregation and somatic accumulation could rely on altered microtubule dynamics and ubiquitin–proteasome pathway activity (Lin et al., 2009), which is linked to pathological α-syn expression (Bentea et al., 2015). Interestingly, LRRK2 kinase hyperactivation could be involved in this detrimental process, because an increased presence of the pathological phosphorylated form of α-syn, the pSer129 α-syn, was found in the striatal dopaminergic terminals of 12-month-old G2019S LRRK2 transgenic mice (Longo et al., 2017), and G2019S LRRK2 expression in cultured neurons, or in rat midbrain, was able to increase the recruitment of endogenous α-syn into pathological inclusions (Volpicelli-Daley et al., 2016). This last evidence led to the hypothesis that LRRK2 could facilitate, through its kinase activity, the progression of α-syn pathology by creating a pool of α-syn more susceptible to aggregates (Volpicelli-Daley et al., 2016), as also shown in G2019S KI mice (MacIsaac et al., 2020) and in hIPSC expressing G2019S LRRK2 (Bieri et al., 2019). In line with this, transgenic mice with a conditional expression of G2019S LRRK2 in the forebrain were characterized by kinase-dependent behavioral deficits associated with α-syn pathology in the CNS (Xiong et al., 2017), whereas cortical neurons from G2019S LRRK2 transgenic mice showed endogenous insoluble α-syn aggregates that could be reduced by the pharmacological inhibition of LRRK2 kinase activity (Schapansky et al., 2018). Moreover, a twofold higher load of pSer129 α-syn compared to wild-type animals was found in 12-month-old G2019S KI mice injected with a viral vector overexpressing human mutant A53T α-syn (Novello et al., 2018). LRRK2, as well as fragments containing its kinase domain, was hypothesized to phosphorylate recombinant α-syn at serine 129, especially in the presence of G2019S mutation (Qing et al., 2009).

However, it should be noted that subsequent studies have criticized the possible pathological interaction between LRRK2 and α-syn. Indeed, the coexpression of LRRK2 and α-syn genes was not followed by changes in the extent of the α-synucleinopathy or α-syn phosphorylation state (Herzig et al., 2012), and the overexpression of human G2019S LRRK2 did not modify the α-synucleinopathy characterizing A53T α-syn transgenic mice (Daher et al., 2012). Since the tissutal and temporal expression of LRRK2 could vary among the various experimental models analyzed, some authors have hypothesized that the LRRK2-mediated exacerbation of α-syn pathology could be cell type- and brain region-dependent (Herzig et al., 2012).

It should also be considered that LRRK2 could influence α-syn aggregation through indirect pathways. In this scenario, the

Rab GTPases have been proposed as possible mediators, because they represent one of the main endogenous LRRK2 substrates and were found to be involved in LRRK2-dependent α-synucleinopathy propagation (Bae et al., 2018). In addition, the interaction between LRRK2 and Rab proteins could also influence the physiological trafficking of autophagosomes and lysosomes, which plays a key role in the removal of pathological α-syn aggregates (Dinter et al., 2016; Bellomo et al., 2020). Indeed, different authors suggested that mutant LRRK2 could impair the mechanisms leading to the clearance of pathological α-syn aggregates, such as the neuronal chaperone-mediated autophagy (Cuervo et al., 2004; Vogiatzi et al., 2008; Tong et al., 2010; Orenstein et al., 2013) or the immune-dependent clearance of α-syn aggregates (Schapansky et al., 2015). Specifically, a recent report showed that G2019S mutant LRRK2 could influence lysosomal acidification, decreasing the autophagic processes and increasing the accumulation of neuronal insoluble α-syn aggregates, which could be subsequently released in the extracellular space (Schapansky et al., 2018). Moreover, pharmacologic inhibition of LRRK2 kinase activity was able to reverse this pathological pathway (Schapansky et al., 2018).

In this scenario, of particular interest is the possibility that LRRK2 could influence the inflammatory and microglial response to progressive α-synucleinopathy within the CNS. Different studies have reported that LRRK2 acts as a regulator of microglial activation (Gillardon et al., 2012; Moehle et al., 2012; Russo et al., 2015), and the hyperactivation of its kinase domain could amplify phagocytic activity and/or proinflammatory microglial response (Kim et al., 2012, 2018; Moehle et al., 2015). An exaggerated LRRK2-dependent inflammatory response to α-syn aggregation could worsen the neuronal oxidative stress and the neurodegenerative process leading to PD (Cresto et al., 2019). Accordingly, double-transgenic G2019S/A53T mice were characterized by the presence of microgliosis and enhanced DAergic neuronal loss (Lin et al., 2009), and rats expressing G2019S LRRK2 showed an exacerbated inflammatory response to α-syn overexpression, which was reduced by LRRK2 kinase inhibition (Daher et al., 2015). Other authors have reported that microglial cells obtained from LRRK2 KO mice showed increased α-syn uptake and clearance (Maekawa et al., 2016) and that nigral or striatal microglial activation was not significantly different between transgenic G2019S LRRK2 and wild-type mice injected with a AAV–α-syn (Novello et al., 2018), suggesting the need of further investigations on the theme.

Lastly, another intriguing hypothesis that could explain the link between LRRK2 and α-syn is the possibility that mutant LRRK2 may enhance neuronal spreading of α-syn within the CNS. Indeed, cell-to-cell transmission of α-syn was investigated in G2019S LRRK2-expressing neuroblastoma cells, showing enhanced α-syn release into extracellular media (Kondo et al., 2011).

In conclusion, different studies suggested that LRRK2 and α-syn may interact in various ways during the progressive loss of striatal DAergic innervation characterizing PD. Mutant LRRK2 could influence the development of PD-related α-synucleinopathy at different time points, altering its phosphorylation, aggregation, propagation, or clearance.

This could have particular relevance during the earlier phases of the disease, in which the abnormalities of striatal synaptic transmission are thought to play a crucial pathogenic role (Schirinzi et al., 2016). Indeed, it has been demonstrated that exposure to pathological α-syn oligomers is able to disrupt the expression of synaptic LTP in striatal cholinergic interneurons (Tozzi et al., 2016) and SPNs (Durante et al., 2019), through an interaction with different subunits of postsynaptic NMDAR, such as GluN2D and GluN2A, respectively. The pathological consequences of α-syn aggregates on synaptic plasticity could worsen the synaptic abnormalities uncovered in LRRK2 genetic models, leading to a diffuse disruption of striatal network functioning even before the loss of DAergic nigral cells. Despite all this, it is important to note that α-syn pathology is not present in all LRRK2 cases. Histopathological studies have shown that that a high percentage (∼43%) of cases had no LB inclusions (Poulopoulos et al., 2012). Intriguingly, this seemed to be in a higher proportion in non-G2019S mutations. These human patient data make the LRRK–α-syn interaction even more complex and warrants further study with multiple mutations.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Approximately two decades ago, the characterization of the "Sagamihara family" unveiled a new unexpected and extremely intriguing field of research in neuroscience. What was initially considered a rare genetic cause of parkinsonism turned out to be a crucial PD-related pathogenic protein, potentially involved in familial and sporadic PD. Thanks to the investigation performed in genetic experimental models, the previously unknown pathophysiological functions of LRRK2 started to be understood. LRRK2 mutation seems to influence striatal synaptic transmission in an age-dependent way, through the regulation of both presynaptic vesicle release and postsynaptic receptor activity, contributing to impairment of basal ganglia network during the course of PD. The study of transgenic LRRK2 mouse models has reinforced the idea that early PD phases are characterized by diffuse, and potentially reversible, striatal synaptopathy, preceding the progressive loss of nigral cells. Indeed, the dysfunction of presynaptic DAergic terminals triggered by LRRK2 through different mechanisms, such as vesicle trafficking deregulation, mitochondrial dysfunction, and disrupted $Ca^{2+}$ homeostasis, could be slowly followed by the degeneration of the DAergic neuronal bodies located in the SNpc. This process could be further sustained by the enhanced susceptibility to ROS and environmental stressors seen in LRRK2 mutant models. Moreover, LRRK2 is thought to favor pathological α-syn phosphorylation, aggregation, and interneuronal propagation, which could worsen itself mitochondrial activity sustaining the detrimental vicious cycle leading to nigral degeneration (Ordonez et al., 2018; Bastioli et al., 2019). Accordingly, LRRK2 could represent a molecular target for strategies counteracting the progressive α-synucleinopathy and mitochondrial impairment characterizing PD.

Efforts have been made to identify pharmacological, brain-penetrant inhibitors of LRRK2 usable as potential disease-modifying strategies. The first compounds identified as LRRK2 inhibitors were nonselective kinase inhibitors with multiple targets and potential adverse effects (Vancraenenbroeck et al., 2011; Lee B. D. et al., 2012; Taymans and Greggio, 2016; West, 2017; Chen et al., 2018). More recently, new generation LRRK2 kinase inhibitors have been developed and tested in vitro or in vivo, with improved potency, better selectivity, and/or long-term efficacy (Taymans and Greggio, 2016; West, 2017; Chen et al., 2018). It should be considered that, even if the in vivo or in vitro inhibition of LRRK2 kinase activity was able to rescue many of the detrimental neuronal effects triggered by mutant LRRK2, the systemic consequences of chronic LRRK2 inhibition should be carefully considered. For example, studies performed in LRRK2 KO models showed that the loss of LRRK2 activity could impair cellular lysosomal pathways in different organs, such as kidneys, lungs, and liver (Tong et al., 2010; Herzig et al., 2011; Baptista et al., 2013; Ness et al., 2013). The effects of LRRK2 inhibition could be tissue- and age-dependent, so the observations made in transgenic LRRK2 KO models may not reflect the complete picture of a pharmacological LRRK2 inhibition during adult age. Moreover, the phenotype of LRRK2 KO models could rely on the loss of the global protein function, beyond its kinase activity (Taymans and Greggio, 2016), reinforcing the need of a global safety/efficacy assessment of these compounds in preclinical transgenic KI models of PD. Indeed, it has been noted that not all LRRK2 PD-related mutations cause an increase in kinase activity (Rudenko et al., 2012), importantly implying that kinase function alone may not be the key (Cookson, 2015); thus, reduction of kinase in all cases could actually be detrimental.

Furthermore, the possible application in humans should be carefully planned. Indeed, many questions should be answered for an adequate evaluation of the potential benefits of these molecules in a clinical setting. First, reliable biomarkers reflecting in vivo LRRK2 kinase activity beyond total LRRK2 protein levels are needed. This could be crucial in identifying patients with high LRRK2 activity, independently from the genetic testing for known LRRK2 mutation, as well as to assess the efficacy of the drug and its therapeutic range in a longitudinal clinical context. In this scenario, hypothetical LRRK2 kinase substrates, such as the suggested pSer1292-LRRK2 or pRabs, could be analyzed in white blood cells and in purified exosome fractions from cerebrospinal fluid and urine to provide a surrogate measure of LRRK2 activity (West, 2017; Zhao and Dzamko, 2019). Specifically, the determination of Rab10 Thr73 phosphoepitope in neutrophils obtained from patients' blood samples has been proposed to assess LRRK2 kinase activity in vivo (Thirstrup et al., 2017; Fan et al., 2018), even if with some limitations (Atashrazm et al., 2019). Moreover, other authors have proposed as LRRK2 kinase activity biomarker the analysis of centrosomal cohesion deficit in peripheral blood mononuclear cell–derived LCLs (Fernández et al., 2019), because it is dependent on phospho-Rab8 and phospho-Rab10 and can be reverted by LRRK2 inhibition (Lara Ordónez et al., 2019). Such biomarkers would allow to treat an appropriate cohort of patients,

considering that not only mutant LRRK2 carriers but also a subpopulation of sporadic PD patients could benefit from LRRK2 pharmacological inhibition. Lastly, it should be defined which strategy of LRRK2 inhibitors administration could be associated with the best efficacy. Even if the administration of the drug could be started during the presymptomatic phase in mutant LRRK2 carriers, or during the early symptomatic phase in sporadic PD patients, many studies have suggested that the biological functions of LRRK2 are age-dependent and the pathological long-term changes of striatal network could be triggered very early during the postnatal life. Pharmacological LRRK2 inhibition could have minor effects if the pathogenic events leading to PD, such as progressive α-synucleinopathy or ROS-induced mitochondrial dysfunction, have been already started. To date, there is poor evidence to exclude the presence of an early, reversible, LRRK2-dependent and a late, irreversible, LRRK2-independent pathogenic phase in PD development.

Overall, the investigation of LRRK2-related PD has brought unexpected results, improving our understanding of PD pathogenesis with potential implications for a large number of patients. For now, there is the need for a differentiated research effort to reach multiple objectives and clarify this promising pathological pathway. The molecular mechanisms linking LRRK2 function, striatal synaptopathy, mitochondrial dysfunction, and progressive α-synucleinopathy should be better understood, as well as their timing and mutual relationships. The efficacy/safety ratio of LRRK2 inhibition should be clarified in transgenic models resembling human LRRK2 expression pattern and function, during both physiological and pathological

conditions. Reliable biomarkers reflecting LRRK2 *in vivo* activity should be developed to identify all PD patients that could benefit from an anti-LRRK2 therapy. Solving these issues surely does not represent an easy project, but LRRK2 appears as one of the more promising targets for a neuroprotective therapy counteracting the multiple pathogenic processes underlying PD development. The chances that the observations identified in the "Sagamihara family" could turn into an effective therapy for millions of people worldwide may be small, but we should not miss this opportunity.

## AUTHOR CONTRIBUTIONS

AMa, AT, and PC conceived the review. AMa, AT, and DB-K performed literature review. AMa wrote the manuscript draft and prepared the figures. AT, DB-K, MDF, and PC reviewed the manuscript draft and the figures. All authors read and approved the final manuscript.

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## REFERENCES

Angeles, D. C., Ho, P., Chua, L. L., Wang, C., Yap, Y. W., Ng, C., et al. (2014). Thiol peroxidases ameliorate LRRK2 mutant-induced mitochondrial and dopaminergic neuronal degeneration in *Drosophila*. *Hum. Mol. Genet.* 23, 3157–3165. doi: 10.1093/hmg/ddu026

Arnoult, D., Rismanchi, N., Grodet, A., Roberts, R. G., Seeburg, D. P., Estaquier, J., et al. (2005). Bax/Bak-dependent release of DDP/TIMM8a promotes Drp1-mediated mitochondrial fission and mitoptosis during programmed cell death. *Curr. Biol.* 15, 2112–2118. doi: 10.1016/j.cub.2005.10.041

Arranz, A. M., Delbroek, L., Van Kolen, K., Guimarães, M. R., Mandemakers, W., Daneels, G., et al. (2015). LRRK2 functions in synaptic vesicle endocytosis through a kinase-dependent mechanism. *J. Cell Sci.* 128, 541–552. doi: 10.1242/jcs.158196

Ascherio, A., and Schwarzschild, M. A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. *Lancet Neurol.* 15, 1257–1272. doi: 10.1016/S1474-4422(16)30230-7

Atashrazm, F., Hammond, D., Perera, G., Bolliger, M. F., Matar, E., Halliday, G. M., et al. (2019). LRRK2-mediated Rab10 phosphorylation in immune cells from Parkinson's disease patients. *Mov. Disord.* 34, 406–415. doi: 10.1002/mds.27601

Bae, E.-J., Kim, D.-K., Kim, C., Mante, M., Adame, A., Rockenstein, E., et al. (2018). LRRK2 kinase regulates α-synuclein propagation *via* RAB35 phosphorylation. *Nat. Commun.* 9:3465. doi: 10.1038/s41467-018-05958-z

Ballard, P. A., Tetrud, J. W., and Langston, J. W. (1985). Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): seven cases. *Neurology* 35, 949–956. doi: 10.1212/wnl.35.7.949

Baptista, M. A. S., Dave, K. D., Frasier, M. A., Sherer, T. B., Greeley, M., Beck, M. J., et al. (2013). Loss of leucine-rich repeat kinase 2 (LRRK2) in rats leads to progressive abnormal phenotypes in peripheral organs. *PLoS One* 8:e80705. doi: 10.1371/journal.pone.0080705

Bastioli, G., Piccirillo, S., Castaldo, P., Magi, S., Tozzi, A., Amoroso, S., et al. (2019). Selective inhibition of mitochondrial sodium-calcium exchanger protects striatal neurons from α-synuclein plus rotenone induced toxicity. *Cell Death Dis.* 10:80. doi: 10.1038/s41419-018-1290-6

Beccano-Kelly, D. A., Kuhlmann, N., Tatarnikov, I., Volta, M., Munsie, L. N., Chou, P., et al. (2014). Synaptic function is modulated by LRRK2 and glutamate release is increased in cortical neurons of G2019S LRRK2 knock-in mice. *Front. Cell. Neurosci.* 8:301. doi: 10.3389/fncel.2014.00301

Beccano-Kelly, D. A., Volta, M., Munsie, L. N., Paschall, S. A., Tatarnikov, I., Co, K., et al. (2015). LRRK2 overexpression alters glutamatergic presynaptic plasticity, striatal dopamine tone, postsynaptic signal transduction, motor activity and memory. *Hum. Mol. Genet.* 24, 1336–1349. doi: 10.1093/hmg/ddu543

Bedford, C., Sears, C., Perez-Carrion, M., Piccoli, G., and Condliffe, S. B. (2016). LRRK2 regulates voltage-gated calcium channel function. *Front. Mol. Neurosci.* 9:35. doi: 10.3389/fnmol.2016.00035

Beilina, A., Rudenko, I. N., Kaganovich, A., Civiero, L., Chau, H., Kalia, S. K., et al. (2014). Unbiased screen for interactors of leucine-rich repeat kinase 2 supports a common pathway for sporadic and familial Parkinson disease. *Proc. Natl. Acad. Sci. U S A* 111, 2626–2631. doi: 10.1073/pnas.1318306111

Bellomo, G., Paciotti, S., Gatticchi, L., and Parnetti, L. (2020). The vicious cycle between α-synuclein aggregation and autophagic-lysosomal dysfunction. *Mov. Disord.* 35, 34–44. doi: 10.1002/mds.27895

Belluzzi, E., Gonnelli, A., Cirnaru, M.-D., Marte, A., Plotegher, N., Russo, I., et al. (2016). LRRK2 phosphorylates pre-synaptic N-ethylmaleimide sensitive fusion (NSF) protein enhancing its ATPase activity and SNARE complex

disassembling rate. *Mol. Neurodegener.* 11:1. doi: 10.1186/s13024-015-0066-z

Benson, D. L., Matikainen-Ankney, B. A., Hussein, A., and Huntley, G. W. (2018). Functional and behavioral consequences of Parkinson's disease-associated LRRK2–G2019S mutation. *Biochem. Soc. Trans.* 46, 1697–1705. doi: 10.1042/BST20180468

Bentea, E., Van der Perren, A., Van Liefferinge, J., El Arfani, A., Albertini, G., Demuyser, T., et al. (2015). Nigral proteasome inhibition in mice leads to motor and non-motor deficits and increased expression of Ser129 phosphorylated α-synuclein. *Front. Behav. Neurosci.* 9:68. doi: 10.3389/fnbeh.2015.00068

Berger, Z., Smith, K. A., and Lavoie, M. J. (2010). Membrane localization of LRRK2 is associated with increased formation of the highly active LRRK2 dimer and changes in its phosphorylation. *Biochemistry* 49, 5511–5523. doi: 10.1021/bi100157u

Bertholet, A. M., Delerue, T., Millet, A. M., Moulis, M. F., David, C., Daloyau, M., et al. (2016). Mitochondrial fusion/fission dynamics in neurodegeneration and neuronal plasticity. *Neurobiol. Dis.* 90, 3–19. doi: 10.1016/j.nbd.2015.10.011

Berwick, D. C., Heaton, G. R., Azeggagh, S., and Harvey, K. (2019). LRRK2 Biology from structure to dysfunction: research progresses, but the themes remain the same. *Mol. Neurodegener.* 14:49. doi: 10.1186/s13024-019-0344-2

Bezard, E., and Przedborski, S. (2011). A tale on animal models of Parkinson's disease. *Mov. Disord.* 26, 993–1002. doi: 10.1002/mds.23696

Bichler, Z., Lim, H. C., Zeng, L., and Tan, E. K. (2013). Non-motor and motor features in LRRK2 transgenic mice. *PLoS One* 8:e70249. doi: 10.1371/journal.pone.0070249

Bieri, G., Brahic, M., Bousset, L., Couthouis, J., Kramer, N. J., Ma, R., et al. (2019). LRRK2 modifies α-syn pathology and spread in mouse models and human neurons. *Acta Neuropathol.* 137, 961–980. doi: 10.1007/s00401-019-01995-0

Biskup, S., Moore, D. J., Celsi, F., Higashi, S., West, A. B., Andrabi, S. A., et al. (2006). Localization of LRRK2 to membranous and vesicular structures in mammalian brain. *Ann. Neurol.* 60, 557–569. doi: 10.1002/ana.21019

Blesa, J., and Przedborski, S. (2014). Parkinson's disease: animal models and dopaminergic cell vulnerability. *Front. Neuroanat.* 8:155. doi: 10.3389/fnana.2014.00155

Bose, A., and Beal, M. F. (2016). Mitochondrial dysfunction in Parkinson's disease. *J. Neurochem.* 139, 216–231. doi: 10.1111/jnc.13731

Burré, J. (2015). The synaptic function of α-synuclein. *J. Parkinsons Dis.* 5, 699–713. doi: 10.3233/JPD-150642

Calabresi, P., Picconi, B., Tozzi, A., and Di Filippo, M. (2007). Dopamine-mediated regulation of corticostriatal synaptic plasticity. *Trends Neurosci.* 30, 211–219. doi: 10.1016/j.tins.2007.03.001

Calabresi, P., Picconi, B., Tozzi, A., Ghiglieri, V., and Di Filippo, M. (2014). Direct and indirect pathways of basal ganglia: a critical reappraisal. *Nat. Neurosci.* 17, 1022–1030. doi: 10.1038/nn.3743

Chandler, K. J., Chandler, R. L., Broeckelmann, E. M., Hou, Y., Southard-Smith, E. M., and Mortlock, D. P. (2007). Relevance of BAC transgene copy number in mice: transgene copy number variation across multiple transgenic lines and correlations with transgene integrity and expression. *Mamm. Genome* 18, 693–708. doi: 10.1007/s00335-007-9056-y

Chang, C.-R., and Blackstone, C. (2010). Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. *Ann. N Y Acad. Sci.* 1201, 34–39. doi: 10.1111/j.1749-6632.2010.05629.x

Chen, J., Chen, Y., and Pu, J. (2018). Leucine-rich repeat kinase 2 in Parkinson's disease: updated from pathogenesis to potential therapeutic target. *Eur. Neurol.* 79, 256–265. doi: 10.1159/000488938

Chen, C.-Y., Weng, Y.-H., Chien, K.-Y., Lin, K.-J., Yeh, T.-H., Cheng, Y.-P., et al. (2012). (G2019S) LRRK2 activates MKK4-JNK pathway and causes degeneration of SN dopaminergic neurons in a transgenic mouse model of PD. *Cell Death Differ.* 19, 1623–1633. doi: 10.1038/cdd.2012.42

Cherra, S. J., Steer, E., Gusdon, A. M., Kiselyov, K., and Chu, C. T. (2013). Mutant LRRK2 elicits calcium imbalance and depletion of dendritic mitochondria in neurons. *Am. J. Pathol.* 182, 474–484. doi: 10.1016/j.ajpath.2012.10.027

Cho, D.-H., Nakamura, T., and Lipton, S. A. (2010). Mitochondrial dynamics in cell death and neurodegeneration. *Cell. Mol. Life Sci.* 67, 3435–3447. doi: 10.1007/s00018-010-0435-2

Choquet, D., and Triller, A. (2003). The role of receptor diffusion in the organization of the postsynaptic membrane. *Nat. Rev. Neurosci.* 4, 251–265. doi: 10.1038/nrn1077

Chou, J.-S., Chen, C.-Y., Chen, Y.-L., Weng, Y.-H., Yeh, T.-H., Lu, C.-S., et al. (2014). (G2019S) LRRK2 causes early-phase dysfunction of SNpc dopaminergic neurons and impairment of corticostriatal long-term depression in the PD transgenic mouse. *Neurobiol. Dis.* 68, 190–199. doi: 10.1016/j.nbd.2014.04.021

Cicchetti, F., Drouin-Ouellet, J., and Gross, R. E. (2009). Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? *Trends Pharmacol. Sci.* 30, 475–483. doi: 10.1016/j.tips.2009.06.00

Connolly, B. S., and Lang, A. E. (2014). Pharmacological treatment of Parkinson disease: a review. *JAMA* 311, 1670–1683. doi: 10.1001/jama.2014.3654

Cookson, M. R. (2010). The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. *Nat. Rev. Neurosci.* 11, 791–797. doi: 10.1038/nrn2935

Cookson, M. R. (2012). Cellular effects of LRRK2 mutations. *Biochem. Soc. Trans.* 40, 1070–1073. doi: 10.1042/bst20120165

Cookson, M. R. (2015). LRRK2 pathways leading to neurodegeneration. *Curr. Neurol. Neurosci. Rep.* 15:42. doi: 10.1007/s11910-015-0564-y

Cooper, O., Seo, H., Andrabi, S., Guardia-Laguarta, C., Graziotto, J., Sundberg, M., et al. (2012). Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. *Sci. Transl. Med.* 4:141ra90. doi: 10.1126/scitranslmed.3003985

Cresto, N., Gardier, C., Gubinelli, F., Gaillard, M.-C., Liot, G., West, A. B., et al. (2019). The unlikely partnership between LRRK2 and α-synuclein in Parkinson's disease. *Eur. J. Neurosci.* 49, 339–363. doi: 10.1111/ejn.14182

Csordás, G., Renken, C., Várnai, P., Walter, L., Weaver, D., Buttle, K. F., et al. (2006). Structural and functional features and significance of the physical linkage between ER and mitochondria. *J. Cell Biol.* 174, 915–921. doi: 10.1083/jcb.200604016

Csordás, G., Várnai, P., Golenár, T., Roy, S., Purkins, G., Schneider, T. G., et al. (2010). Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol. Cell* 39, 121–132. doi: 10.1016/j.molcel.2010.06.029

Cuervo, A. M., Stefanis, L., Fredenburg, R., Lansbury, P. T., and Sulzer, D. (2004). Impaired degradation of mutant α-synuclein by chaperone-mediated autophagy. *Science* 305, 1292–1295. doi: 10.1126/science.1101738

Daher, J. P. L., Abdelmotilib, H. A., Hu, X., Volpicelli-Daley, L. A., Moehle, M. S., Fraser, K. B., et al. (2015). Leucine-rich repeat kinase 2 (LRRK2) pharmacological inhibition Abates α-synuclein gene-induced neurodegeneration. *J. Biol. Chem.* 290, 19433–19444. doi: 10.1074/jbc.M115.660001

Daher, J. P. L., Pletnikova, O., Biskup, S., Musso, A., Gellhaar, S., Galter, D., et al. (2012). Neurodegenerative phenotypes in an A53T α-synuclein transgenic mouse model are independent of LRRK2. *Hum. Mol. Genet.* 21, 2420–2431. doi: 10.1093/hmg/dds057

David, G., Barrett, J. N., and Barrett, E. F. (1998). Evidence that mitochondria buffer physiological $Ca^{2+}$ loads in lizard motor nerve terminals. *J. Physiol.* 509, 59–65. doi: 10.1111/j.1469-7793.1998.059bo.x

Davies, P., Hinkle, K. M., Sukar, N. N., Sepulveda, B., Mesias, R., Serrano, G., et al. (2013). Comprehensive characterization and optimization of anti-LRRK2 (leucine-rich repeat kinase 2) monoclonal antibodies. *Biochem. J.* 453, 101–113. doi: 10.1042/bj20121742

Dawson, T. M., Ko, H. S., and Dawson, V. L. (2010). Genetic animal models of Parkinson's disease. *Neuron* 66, 646–661. doi: 10.1016/j.neuron.2010.04.034

de Lau, L. M. L., and Breteler, M. M. B. (2006). Epidemiology of Parkinson's disease. *Lancet Neurol.* 5, 525–535. doi: 10.1016/S1474-4422(06)70471-9

Denton, R. M. (2009). Regulation of mitochondrial dehydrogenases by calcium ions. *Biochim. Biophys. Acta* 1787, 1309–1316. doi: 10.1016/j.bbabio.2009.01.005

Devine, M. J., and Kittler, J. T. (2018). Mitochondria at the neuronal presynapse in health and disease. *Nat. Rev. Neurosci.* 19, 63–80. doi: 10.1038/nrn.2017.170

Di Benedetto, G., Gerbino, A., and Lefkimmiatis, K. (2018). Shaping mitochondrial dynamics: the role of cAMP signalling. *Biochem. Biophys. Res. Commun.* 500, 65–74. doi: 10.1016/j.bbrc.2017.05.041

Di Fonzo, A., Rohé, C. F., Ferreira, J., Chien, H. F., Vacca, L., Stocchi, F., et al. (2005). A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease. *Lancet* 365, 412–415. doi: 10.1016/s0140-6736(05)17829-5

Dinter, E., Saridaki, T., Nippold, M., Plum, S., Diederichs, L., Komnig, D., et al. (2016). Rab7 induces clearance of α-synuclein aggregates. *J. Neurochem.* 138, 758–774. doi: 10.1111/jnc.13712

Dorsey, E. R., Constantinescu, R., Thompson, J. P., Biglan, K. M., Holloway, R. G., Kieburtz, K., et al. (2007). Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology* 68, 384–386. doi: 10.1212/01.wnl.0000247740.47667.03

Driver, J. A., Logroscino, G., Gaziano, J. M., and Kurth, T. (2009). Incidence and remaining lifetime risk of Parkinson disease in advanced age. *Neurology* 72, 432–438. doi: 10.1212/01.wnl.0000341769.50075.bb

Duchen, M. R. (2000). Mitochondria and calcium: from cell signalling to cell death. *J. Physiol.* 529, 57–68. doi: 10.1111/j.1469-7793.2000.00057.x

Durante, V., de Iure, A., Loffredo, V., Vaikath, N., De Risi, M., Paciotti, S., et al. (2019). α-synuclein targets GluN2A NMDA receptor subunit causing striatal synaptic dysfunction and visuospatial memory alteration. *Brain* 142, 1365–1385. doi: 10.1093/brain/awz065

Dzamko, N., and Halliday, G. M. (2012). An emerging role for LRRK2 in the immune system. *Biochem. Soc. Trans.* 40, 1134–1139. doi: 10.1042/bst2012 0119

Esteban, J. A., Shi, S.-H., Wilson, C., Nuriya, M., Huganir, R. L., and Malinow, R. (2003). PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat. Neurosci.* 6, 136–143. doi: 10.1038/nn997

Esteves, A. R., Swerdlow, R. H., and Cardoso, S. M. (2014). LRRK2, a puzzling protein: insights into Parkinson's disease pathogenesis. *Exp. Neurol.* 261, 206–216. doi: 10.1016/j.expneurol.2014.05.025

Fan, Y., Howden, A. J. M., Sarhan, A. R., Lis, P., Ito, G., Martinez, T. N., et al. (2018). Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10 phosphorylation in human neutrophils. *Biochem. J.* 475, 23–44. doi: 10.1042/BCJ20170803

Fang, J.-K., Prabu, S. K., Sepuri, N. B., Raza, H., Anandatheerthavarada, H. K., Galati, D., et al. (2007). Site specific phosphorylation of cytochrome c oxidase subunits I, IVi1 and Vb in rabbit hearts subjected to ischemia/reperfusion. *FEBS Lett.* 581, 1302–1310. doi: 10.1016/j.febslet.2007.02.042

Fernández, B., Lara Ordóñez, A. J., Fdez, E., Mutez, E., Comptdaer, T., Leghay, C., et al. (2019). Centrosomal cohesion deficits as cellular biomarker in lymphoblastoid cell lines from LRRK2 Parkinson's disease patients. *Biochem. J.* 476, 2797–2813. doi: 10.1042/bcj20190315

Ferree, A., Guillily, M., Li, H., Smith, K., Takashima, A., Squillace, R., et al. (2012). Regulation of physiologic actions of LRRK2: focus on autophagy. *Neurodegener. Dis.* 10, 238–241. doi: 10.1159/000332599

Funayama, M., Hasegawa, K., Kowa, H., Saito, M., Tsuji, S., and Obata, F. (2002). A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2–q13.1. *Ann. Neurol.* 51, 296–301. doi: 10.1002/ana.10113

Funayama, M., Hasegawa, K., Ohta, E., Kawashima, N., Komiyama, M., Kowa, H., et al. (2005). AnLRRK2 mutation as a cause for the parkinsonism in the originalPARK8 family. *Ann. Neurol.* 57, 918–921. doi: 10.1002/ana.20484

Gerges, N. Z., Backos, D. S., and Esteban, J. A. (2004). Local control of AMPA receptor trafficking at the postsynaptic terminal by a small GTPase of the Rab family. *J. Biol. Chem.* 279, 43870–43878. doi: 10.1074/jbc.m404982200

Giesert, F., Hofmann, A., Bürger, A., Zerle, J., Kloos, K., Hafen, U., et al. (2013). Expression analysis of Lrrk1, Lrrk2 and Lrrk2 splice variants in mice. *PLoS One* 8:e63778. doi: 10.1371/journal.pone.0063778

Gilks, W. P., Abou-Sleiman, P. M., Gandhi, S., Jain, S., Singleton, A., Lees, A. J., et al. (2005). A common LRRK2 mutation in idiopathic Parkinson's disease. *Lancet* 365, 415–416. doi: 10.1016/S0140-6736(05)17830-1

Gillardon, F., Schmid, R., and Draheim, H. (2012). Parkinson's disease-linked leucine-rich repeat kinase 2(R1441G) mutation increases proinflammatory cytokine release from activated primary microglial cells and resultant neurotoxicity. *Neuroscience* 208, 41–48. doi: 10.1016/j.neuroscience.2012.02.001

Glitsch, M. D., Bakowski, D., and Parekh, A. B. (2002). Store-operated $Ca^{2+}$ entry depends on mitochondrial $Ca^{2+}$ uptake. *EMBO J.* 21, 6744–6754. doi: 10.1093/emboj/cdf675

Godena, V. K., Brookes-Hocking, N., Moller, A., Shaw, G., Oswald, M., Sancho, R. M., et al. (2014). Increasing microtubule acetylation rescues axonal transport and locomotor deficits caused by LRRK2 Roc-COR domain mutations. *Nat. Commun.* 5:5245. doi: 10.1038/ncomms6245

Goedert, M., Spillantini, M. G., Del Tredici, K., and Braak, H. (2013). 100 years of Lewy pathology. *Nat. Rev. Neurol.* 9, 13–24. doi: 10.1038/nrneurol.2012.242

Goldman, S. M. (2014). Environmental toxins and Parkinson's disease. *Annu. Rev. Pharmacol. Toxicol.* 54, 141–164. doi: 10.1146/annurev-pharmtox-011613-135937

Gómez-Suaga, P., Rivero-Ríos, P., Fdez, E., Blanca Ramírez, M., Ferrer, I., Aiastui, A., et al. (2014). LRRK2 delays degradative receptor trafficking by impeding late endosomal budding through decreasing Rab7 activity. *Hum. Mol. Genet.* 23, 6779–6796. doi: 10.1093/hmg/ddu395

Greengard, P., Allen, P. B., and Nairn, A. C. (1999). Beyond the dopamine receptor. *Neuron* 23, 435–447. doi: 10.1016/s0896-6273(00)80798-9

Greggio, E., Bubacco, L., and Russo, I. (2017). Cross-talk between LRRK2 and PKA: implication for Parkinson's disease? *Biochem. Soc. Trans.* 45, 261–267. doi: 10.1042/bst20160396

Greggio, E., Jain, S., Kingsbury, A., Bandopadhyay, R., Lewis, P., Kaganovich, A., et al. (2006). Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol. Dis.* 23, 329–341. doi: 10.1016/j.nbd.2006.04.001

Grünewald, A., Arns, B., Meier, B., Brockmann, K., Tadic, V., and Klein, C. (2014). Does uncoupling protein 2 expression qualify as marker of disease status in LRRK2-associated Parkinson's disease? *Antioxid. Redox Signal.* 20, 1955–1960. doi: 10.1089/ars.2013.5737

Guardia-Laguarta, C., Area-Gomez, E., Rüb, C., Liu, Y., Magrané, J., Becker, D., et al. (2014). α-Synuclein is localized to mitochondria-associated ER membranes. *J. Neurosci.* 34, 249–259. doi: 10.1523/JNEUROSCI.2507-13.2014

Guerreiro, P. S., Huang, Y., Gysbers, A., Cheng, D., Gai, W. P., Outeiro, T. F., et al. (2013). LRRK2 interactions with α-synuclein in Parkinson's disease brains and in cell models. *J. Mol. Med.* 91, 513–522. doi: 10.1007/s00109-012-0984-y

Guo, L., Gandhi, P. N., Wang, W., Petersen, R. B., Wilson-Delfosse, A. L., and Chen, S. G. (2007). The Parkinson's disease-associated protein, leucine-rich repeat kinase 2 (LRRK2), is an authentic GTPase that stimulates kinase activity. *Exp. Cell Res.* 313, 3658–3670. doi: 10.1016/j.yexcr.2007.07.007

Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., et al. (2013). Autophagosomes form at ER-mitochondria contact sites. *Nature* 495, 389–393. doi: 10.1038/nature11910

Hasegawa, K., Stoessl, A. J., Yokoyama, T., Kowa, H., Wszolek, Z. K., and Yagishita, S. (2009). Familial parkinsonism: Study of original Sagamihara PARK8 (I2020T) kindred with variable clinicopathologic outcomes. *Parkinsonism Relat. Disord.* 15, 300–306. doi: 10.1016/j.parkreldis.2008.07.010

Healy, D. G., Falchi, M., O'Sullivan, S. S., Bonifati, V., Durr, A., Bressman, S., et al. (2008). Phenotype, genotype and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *Lancet Neurol.* 7, 583–590. doi: 10.1016/S1474-4422(08)70117-0

Herzig, M. C., Bidinosti, M., Schweizer, T., Hafner, T., Stemmelen, C., Weiss, A., et al. (2012). High LRRK2 levels fail to induce or exacerbate neuronal α-synucleinopathy in mouse brain. *PLoS One* 7:e36581. doi: 10.1371/journal.pone.0036581

Herzig, M. C., Kolly, C., Persohn, E., Theil, D., Schweizer, T., Hafner, T., et al. (2011). LRRK2 protein levels are determined by kinase function and are crucial for kidney and lung homeostasis in mice. *Hum. Mol. Genet.* 20, 4209–4223. doi: 10.1093/hmg/ddr348

Hinkle, K. M., Yue, M., Behrouz, B., Dächsel, J. C., Lincoln, S. J., Bowles, E. E., et al. (2012). LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors. *Mol. Neurodegener.* 7:25. doi: 10.1186/1750-1326-7-25

Howlett, E. H., Jensen, N., Belmonte, F., Zafar, F., Hu, X., Kluss, J., et al. (2017). LRRK2 G2019S-induced mitochondrial DNA damage is LRRK2 kinase dependent and inhibition restores mtDNA integrity in Parkinson's disease. *Hum. Mol. Genet.* 26, 4340–4351. doi: 10.1093/hmg/ddx320

Hsieh, C.-H., Shaltouki, A., Gonzalez, A. E., Bettencourt da Cruz, A., Burbulla, L. F., St Lawrence, E., et al. (2016). Functional impairment in

miro degradation and mitophagy is a shared feature in familial and sporadic Parkinson's disease. *Cell Stem Cell* 19, 709–724. doi: 10.1016/j.stem.2016.08.002

Ito, G., and Iwatsubo, T. (2012). Re-examination of the dimerization state of leucine-rich repeat kinase 2: predominance of the monomeric form. *Biochem. J.* 441, 987–994. doi: 10.1042/bj20111215

Jaber, M., Robinson, S. W., Missale, C., and Caron, M. G. (1996). Dopamine receptors and brain function. *Neuropharmacology* 35, 1503–1519. doi: 10.1016/s0028-3908(96)00100-1

Jaleel, M., Nichols, R. J., Deak, M., Campbell, D. G., Gillardon, F., Knebel, A., et al. (2007). LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. *Biochem. J.* 405, 307–317. doi: 10.1042/bj20070209

James, N. G., Digman, M. A., Gratton, E., Barylko, B., Ding, X., Albanesi, J. P., et al. (2012). Number and brightness analysis of LRRK2 oligomerization in live cells. *Biophys. J.* 102, L41–L43. doi: 10.1016/j.bpj.2012.04.046

Kachergus, J., Mata, I. F., Hulihan, M., Taylor, J. P., Lincoln, S., Aasly, J., et al. (2005). Identification of a novel LRRK2 mutation linked to autosomal dominant Parkinsonism: evidence of a common founder across european populations. *Am. J. Hum. Genet.* 76, 672–680. doi: 10.1086/429256

Kalia, L. V., and Lang, A. E. (2015). Parkinson's disease. *Lancet* 386, 896–912. doi: 10.1016/S0140-6736(14)61393-3

Kara, E., Kiely, A. P., Proukakis, C., Giffin, N., Love, S., Hehir, J., et al. (2014). A 6.4 Mb duplication of the α-synuclein locus causing frontotemporal dementia and Parkinsonism. *JAMA Neurol.* 71, 1162–1171. doi: 10.1001/jamaneurol.2014.994

Keller, M. F., Saad, M., Bras, J., Bettella, F., Nicolaou, N., Simón-Sánchez, J., et al. (2012). Using genome-wide complex trait analysis to quantify "missing heritability" in Parkinson's disease. *Hum. Mol. Genet.* 21, 4996–5009. doi: 10.1093/hmg/dds335

Kett, L. R., Boassa, D., Ho, C. C.-Y., Rideout, H. J., Hu, J., Terada, M., et al. (2012). LRRK2 Parkinson disease mutations enhance its microtubule association. *Hum. Mol. Genet.* 21, 890–899. doi: 10.1093/hmg/ddr526

Kim, K. S., Marcogliese, P. C., Yang, J., Callaghan, S. M., Resende, V., Abdel-Messih, E., et al. (2018). Regulation of myeloid cell phagocytosis by LRRK2 *via* WAVE2 complex stabilization is altered in Parkinson's disease. *Proc. Natl. Acad. Sci. U S A* 115, E5164–E5173. doi: 10.1073/pnas.1718946115

Kim, B., Yang, M.-S., Choi, D., Kim, J.-H., Kim, H.-S., Seol, W., et al. (2012). Impaired inflammatory responses in murine Lrrk2-knockdown brain microglia. *PLoS One* 7:e34693. doi: 10.1371/journal.pone.0034693

Klein, C. L., Rovelli, G., Springer, W., Schall, C., Gasser, T., and Kahle, P. J. (2009). Homo- and heterodimerization of ROCO kinases: LRRK2 kinase inhibition by the LRRK2 ROCO fragment. *J. Neurochem.* 111, 703–715. doi: 10.1111/j.1471-4159.2009.06358.x

Kondo, K., Obitsu, S., and Teshima, R. (2011). α-synuclein aggregation and transmission are enhanced by leucine-rich repeat kinase 2 in human neuroblastoma SH-SY5Y cells. *Biol. Pharm. Bull.* 34, 1078–1083. doi: 10.1248/bpb.34.1078

Kordower, J. H., Olanow, C. W., Dodiya, H. B., Chu, Y., Beach, T. G., Adler, C. H., et al. (2013). Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain* 136, 2419–2431. doi: 10.1093/brain/awt192

Kornmann, B. (2013). The molecular hug between the ER and the mitochondria. *Curr. Opin. Cell Biol.* 25, 443–448. doi: 10.1016/j.ceb.2013.02.010

Langston, J., Ballard, P., Tetrud, J., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 219, 979–980. doi: 10.1126/science.6823561

Lara Ordónez, A. J., Fernández, B., Fdez, E., Romo-Lozano, M., Madero-Pérez, J., Lobbestael, E., et al. (2019). RAB8, RAB10 and RILPL1 contribute to both LRRK2 kinase-mediated centrosomal cohesion and ciliogenesis deficits. *Hum. Mol. Genet.* 28, 3552–3568. doi: 10.1093/hmg/ddz201

Lee, B. D., Dawson, V. L., and Dawson, T. M. (2012). Leucine-rich repeat kinase 2 (LRRK2) as a potential therapeutic target in Parkinson's disease. *Trends Pharmacol. Sci.* 33, 365–373. doi: 10.1016/j.tips.2012.04.001

Lee, S., Imai, Y., Gehrke, S., Liu, S., and Lu, B. (2012). The synaptic function of LRRK2. *Biochem. Soc. Trans.* 40, 1047–1051. doi: 10.1042/bst20120113

Lee, H., Melrose, H. L., Yue, M., Pare, J.-F., Farrer, M. J., and Smith, Y. (2010). Lrrk2 localization in the primate basal ganglia and thalamus: a light and electron microscopic analysis in monkeys. *Exp. Neurol.* 224, 438–447. doi: 10.1016/j.expneurol.2010.05.004

Lesage, S., Dürr, A., Tazir, M., Lohmann, E., Leutenegger, A.-L., Janin, S., et al. (2006). LRRK2 G2019S as a cause of Parkinson's disease in north african arabs. *N. Engl. J. Med.* 354, 422–423. doi: 10.1056/NEJMc055540

Li, Y., Liu, W., Oo, T. F., Wang, L., Tang, Y., Jackson-Lewis, V., et al. (2009). Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. *Nat. Neurosci.* 12, 826–828. doi: 10.1038/nn.2349

Li, X., Patel, J. C., Wang, J., Avshalumov, M. V., Nicholson, C., Buxbaum, J. D., et al. (2010). Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. *J. Neurosci.* 30, 1788–1797. doi: 10.1523/jneurosci.5604-09.2010

Lin, M. T., and Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 443, 787–795. doi: 10.1038/nature05292

Lin, X., Parisiadou, L., Gu, X.-L., Wang, L., Shim, H., Sun, L., et al. (2009). Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant α-synuclein. *Neuron* 64, 807–827. doi: 10.1016/j.neuron.2009.11.006

Liu, G., Sgobio, C., Gu, X., Sun, L., Lin, X., Yu, J., et al. (2015). Selective expression of Parkinson's disease-related Leucine-rich repeat kinase 2 G2019S missense mutation in midbrain dopaminergic neurons impairs dopamine release and dopaminergic gene expression. *Hum. Mol. Genet.* 24, 5299–5312. doi: 10.1093/hmg/ddv249

Longo, F., Mercatelli, D., Novello, S., Arcuri, L., Brugnoli, A., Vincenzi, F., et al. (2017). Age-dependent dopamine transporter dysfunction and Serine129 phospho-α-synuclein overload in G2019S LRRK2 mice. *Acta Neuropathol. Commun.* 5:22. doi: 10.1186/s40478-017-0426-8

MacIsaac, S., Quevedo Melo, T., Zhang, Y., Volta, M., Farrer, M. J., and Milnerwood, A. J. (2020). Neuron-autonomous susceptibility to induced synuclein aggregation is exacerbated by endogenous Lrrk2 mutations and ameliorated by Lrrk2 genetic knock-out. *Brain Commun.* 2:fcz052. doi: 10.1093/braincomms/fcz052

MacLeod, D., Dowman, J., Hammond, R., Leete, T., Inoue, K., and Abeliovich, A. (2006). The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* 52, 587–593. doi: 10.1016/j.neuron.2006.10.008

Maekawa, T., Mori, S., Sasaki, Y., Miyajima, T., Azuma, S., Ohta, E., et al. (2012). The I2020T Leucine-rich repeat kinase 2 transgenic mouse exhibits impaired locomotive ability accompanied by dopaminergic neuron abnormalities. *Mol. Neurodegener.* 7:15. doi: 10.1186/1750-1326-7-15

Maekawa, T., Sasaoka, T., Azuma, S., Ichikawa, T., Melrose, H. L., Farrer, M. J., et al. (2016). Leucine-rich repeat kinase 2 (LRRK2) regulates α-synuclein clearance in microglia. *BMC Neurosci.* 17:77. doi: 10.1186/s12868-016-0315-2

Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5–21. doi: 10.1016/j.neuron.2004.09.012

Mandemakers, W., Snellinx, A., O'Neill, M. J., and de Strooper, B. (2012). LRRK2 expression is enriched in the striosomal compartment of mouse striatum. *Neurobiol. Dis.* 48, 582–593. doi: 10.1016/j.nbd.2012.07.017

Marchi, S., Patergnani, S., and Pinton, P. (2014). The endoplasmic reticulum-mitochondria connection: one touch, multiple functions. *Biochim. Biophys. Acta* 1837, 461–469. doi: 10.1016/j.bbabio.2013.10.015

Mata, I. F., Wedemeyer, W. J., Farrer, M. J., Taylor, J. P., and Gallo, K. A. (2006). LRRK2 in Parkinson's disease: protein domains and functional insights. *Trends Neurosci.* 29, 286–293. doi: 10.1016/j.tins.2006.03.006

Matikainen-Ankney, B. A., Kezunovic, N., Menard, C., Flanigan, M. E., Zhong, Y., Russo, S. J., et al. (2018). Parkinson's disease-linked LRRK2–G2019S mutation alters synaptic plasticity and promotes resilience to chronic social stress in young adulthood. *J. Neurosci.* 38, 9700–9711. doi: 10.1523/JNEUROSCI.1457-18.2018

Matikainen-Ankney, B. A., Kezunovic, N., Mesias, R. E., Tian, Y., Williams, F. M., Huntley, G. W., et al. (2016). Altered development of synapse structure and function in striatum caused by Parkinson's disease-linked LRRK2–G2019S mutation. *J. Neurosci.* 36, 7128–7141. doi: 10.1523/jneurosci.3314-15.2016

Matta, S., Van Kolen, K., da Cunha, R., van den Bogaart, G., Mandemakers, W., Miskiewicz, K., et al. (2012). LRRK2 controls an EndoA phosphorylation cycle in synaptic endocytosis. *Neuron* 75, 1008–1021. doi: 10.1016/j.neuron.2012.08.022

McCoy, M. K., and Cookson, M. R. (2012). Mitochondrial quality control and dynamics in Parkinson's disease. *Antioxid. Redox Signal.* 16, 869–882. doi: 10.1089/ars.2011.4019

Melrose, H. L., Dächsel, J. C., Behrouz, B., Lincoln, S. J., Yue, M., Hinkle, K. M., et al. (2010). Impaired dopaminergic neurotransmission and microtubule-associated protein tau alterations in human LRRK2 transgenic mice. *Neurobiol. Dis.* 40, 503–517. doi: 10.1016/j.nbd.2010.07.010

Melrose, H. L., Kent, C. B., Taylor, J. P., Dachsel, J. C., Hinkle, K. M., Lincoln, S. J., et al. (2007). A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. *Neuroscience* 147, 1047–1058. doi: 10.1016/j.neuroscience.2007.05.027

Melrose, H., Lincoln, S., Tyndall, G., Dickson, D., and Farrer, M. (2006). Anatomical localization of leucine-rich repeat kinase 2 in mouse brain. *Neuroscience* 139, 791–794. doi: 10.1016/j.neuroscience.2006.01.017

Migheli, R., Del Giudice, M. G., Spissu, Y., Sanna, G., Xiong, Y., Dawson, T. M., et al. (2013). LRRK2 affects vesicle trafficking, neurotransmitter extracellular level and membrane receptor localization. *PLoS One* 8:e77198. doi: 10.1371/journal.pone.0077198

Mink, J. W. (2018). Basal ganglia mechanisms in action selection, plasticity, and dystonia. *Eur. J. Paediatr. Neurol.* 22, 225–229. doi: 10.1016/j.ejpn.2018.01.005

Moehle, M. S., Daher, J. P. L., Hull, T. D., Boddu, R., Abdelmotilib, H. A., Mobley, J., et al. (2015). The G2019S LRRK2 mutation increases myeloid cell chemotactic responses and enhances LRRK2 binding to actin-regulatory proteins. *Hum. Mol. Genet.* 24, 4250–4267. doi: 10.1093/hmg/ddv157

Moehle, M. S., Webber, P. J., Tse, T., Sukar, N., Standaert, D. G., DeSilva, T. M., et al. (2012). LRRK2 inhibition attenuates microglial inflammatory responses. *J. Neurosci.* 32, 1602–1611. doi: 10.1523/JNEUROSCI.5601-11.2012

Monfrini, E., and Di Fonzo, A. (2017). "Leucine-rich repeat kinase (LRRK2) genetics and Parkinson's disease," in *Advances in Neurobiology*, ed. H. Rideout (Cham: Springer), 3–30.

Mortiboys, H., Johansen, K. K., Aasly, J. O., and Bandmann, O. (2010). Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. *Neurology* 75, 2017–2020. doi: 10.1212/wnl.0b013e3181ff9685

Ness, D., Ren, Z., Gardai, S., Sharpnack, D., Johnson, V. J., Brennan, R. J., et al. (2013). Leucine-rich repeat kinase 2 (LRRK2)-deficient rats exhibit renal tubule injury and perturbations in metabolic and immunological homeostasis. *PLoS One* 8:e66164. doi: 10.1371/journal.pone.0066164

Ng, C.-H., Mok, S. Z. S., Koh, C., Ouyang, X., Fivaz, M. L., Tan, E.-K., et al. (2009). Parkin protects against LRRK2 G2019S mutant-induced dopaminergic neurodegeneration in *Drosophila*. *J. Neurosci.* 29, 11257–11262. doi: 10.1523/jneurosci.2375-09.2009

Nguyen, H. N., Byers, B., Cord, B., Shcheglovitov, A., Byrne, J., Gujar, P., et al. (2011). LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. *Cell Stem Cell* 8, 267–280. doi: 10.1016/j.stem.2011.01.013

Nguyen, M., and Krainc, D. (2018). LRRK2 phosphorylation of auxilin mediates synaptic defects in dopaminergic neurons from patients with Parkinson's disease. *Proc. Natl. Acad. Sci. U S A* 115, 5576–5581. doi: 10.1073/pnas.1717590115

Nguyen, M., Wong, Y. C., Ysselstein, D., Severino, A., and Krainc, D. (2019). Synaptic, mitochondrial and lysosomal dysfunction in Parkinson's disease. *Trends Neurosci.* 42, 140–149. doi: 10.1016/j.tins.2018.11.001

Nichols, R. J., Dzamko, N., Morrice, N. A., Campbell, D. G., Deak, M., Ordureau, A., et al. (2010). 14-3-3 binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization. *Biochem. J.* 430, 393–404. doi: 10.1042/BJ20100483

Nichols, W. C., Pankratz, N., Hernandez, D., Paisán-Ruíz, C., Jain, S., Halter, C. A., et al. (2005). Genetic screening for a single common LRRK2 mutation in familial Parkinson's disease. *Lancet* 365, 410–412. doi: 10.1016/s0140-6736(05)17828-3

Nicklas, W. J., Vyas, I., and Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci.* 36, 2503–2508. doi: 10.1016/0024-3205(85)90146-8

Nicoll, R. A. (2017). A brief history of long-term potentiation. *Neuron* 93, 281–290. doi: 10.1016/j.neuron.2016.12.015

Niu, J., Yu, M., Wang, C., and Xu, Z. (2012). Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics *via* Dynamin-like protein. *J. Neurochem.* 122, 650–658. doi: 10.1111/j.1471-4159.2012.07809.x

Nixon-Abell, J., Berwick, D. C., Grannó, S., Spain, V. A., Blackstone, C., and Harvey, K. (2016). Protective LRRK2 R1398H variant enhances GTPase and Wnt signaling activity. *Front. Mol. Neurosci.* 9:18. doi: 10.3389/fnmol.2016.00018

Novello, S., Arcuri, L., Dovero, S., Dutheil, N., Shimshek, D. R., Bezard, E., et al. (2018). G2019S LRRK2 mutation facilitates α-synuclein neuropathology in aged mice. *Neurobiol. Dis.* 120, 21–33. doi: 10.1016/j.nbd.2018.08.018

Opazo, P., Sainlos, M., and Choquet, D. (2012). Regulation of AMPA receptor surface diffusion by PSD-95 slots. *Curr. Opin. Neurobiol.* 22, 453–460. doi: 10.1016/j.conb.2011.10.010

Ordonez, D. G., Lee, M. K., and Feany, M. B. (2018). α-synuclein induces mitochondrial dysfunction through spectrin and the actin cytoskeleton. *Neuron* 97, 108.e6–124.e6. doi: 10.1016/j.neuron.2017.11.036

Orenstein, S. J., Kuo, S.-H., Tasset, I., Arias, E., Koga, H., Fernandez-Carasa, I., et al. (2013). Interplay of LRRK2 with chaperone-mediated autophagy. *Nat. Neurosci.* 16, 394–406. doi: 10.1038/nn.3350

Outeiro, T. F., Harvey, K., Dominguez-Meijide, A., and Gerhardt, E. (2019). LRRK2, α-synuclein and tau: partners in crime or unfortunate bystanders? *Biochem. Soc. Trans.* 47, 827–838. doi: 10.1042/bst20180466

Ozelius, L. J., Senthil, G., Saunders-Pullman, R., Ohmann, E., Deligtisch, A., Tagliati, M., et al. (2006). LRRK2 G2019S as a cause of Parkinson's disease in ashkenazi jews. *N. Engl. J. Med.* 354, 424–425. doi: 10.1056/NEJMc055509

Paillusson, S., Gomez-Suaga, P., Stoica, R., Little, D., Gissen, P., Devine, M. J., et al. (2017). α-Synuclein binds to the ER-mitochondria tethering protein VAPB to disrupt $Ca^{2+}$ homeostasis and mitochondrial ATP production. *Acta Neuropathol.* 134, 129–149. doi: 10.1007/s00401-017-1704-z

Paillusson, S., Stoica, R., Gomez-Suaga, P., Lau, D. H. W., Mueller, S., Miller, T., et al. (2016). There's something wrong with my MAM; the ER-mitochondria axis and neurodegenerative diseases. *Trends Neurosci.* 39, 146–157. doi: 10.1016/j.tins.2016.01.008

Paisán-Ruíz, C., Jain, S., Evans, E. W., Gilks, W. P., Simón, J., van der Brug, M., et al. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44, 595–600. doi: 10.1016/j.neuron.2004.10.023

Paisàn-Ruìz, C., Sàenz, A., de Munain, A. L., Martì, I., Martìnez Gil, A., Martì-Massò, J. F., et al. (2005). Familial Parkinson's disease: clinical and genetic analysis of four Basque families. *Ann. Neurol.* 57, 365–372. doi: 10.1002/ana.20391

Pan, P.-Y., Li, X., Wang, J., Powell, J., Wang, Q., Zhang, Y., et al. (2017). Parkinson's disease-associated LRRK2 hyperactive kinase mutant disrupts synaptic vesicle trafficking in ventral midbrain neurons. *J. Neurosci.* 37, 11366–11376. doi: 10.1523/JNEUROSCI.0964-17.2017

Parisiadou, L., Xie, C., Cho, H. J., Lin, X., Gu, X.-L., Long, C.-X., et al. (2009). Phosphorylation of Ezrin/Radixin/Moesin proteins by LRRK2 promotes the rearrangement of actin cytoskeleton in neuronal morphogenesis. *J. Neurosci.* 29, 13971–13980. doi: 10.1523/jneurosci.3799-09.2009

Parisiadou, L., Yu, J., Sgobio, C., Xie, C., Liu, G., Sun, L., et al. (2014). LRRK2 regulates synaptogenesis and dopamine receptor activation through modulation of PKA activity. *Nat. Neurosci.* 17, 367–376. doi: 10.1038/nn.3636

Perez Carrion, M., Pischedda, F., Biosa, A., Russo, I., Straniero, L., Civiero, L., et al. (2018). The LRRK2 variant E193K prevents mitochondrial fission upon MPP+ treatment by altering LRRK2 binding to DRP1. *Front. Mol. Neurosci.* 11:64. doi: 10.3389/fnmol.2018.00064

Perry, G., Zhu, X., Babar, A. K., Siedlak, S. L., Yang, Q., Ito, G., et al. (2008). Leucine-rich repeat kinase 2 colocalizes with α-synuclein in Parkinson's disease, but not tau-containing deposits in tauopathies. *Neurodegener. Dis.* 5, 222–224. doi: 10.1159/000113708

Piccoli, G., Condliffe, S. B., Bauer, M., Giesert, F., Boldt, K., De Astis, S., et al. (2011). LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. *J. Neurosci.* 31, 2225–2237. doi: 10.1523/jneurosci.3730-10.2011

Piccoli, G., Onofri, F., Cirnaru, M. D., Kaiser, C. J. O., Jagtap, P., Kastenmuller, A., et al. (2014). Leucine-rich repeat kinase 2 binds to neuronal vesicles through protein interactions mediated by its C-terminal WD40 domain. *Mol. Cell. Biol.* 34, 2147–2161. doi: 10.1128/mcb.00914-13

Plowey, E. D., Cherra, S. J., Liu, Y.-J., and Chu, C. T. (2008). Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. *J. Neurochem.* 105, 1048–1056. doi: 10.1111/j.1471-4159.2008.05217.x

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., et al. (2017). Parkinson disease. *Nat. Rev. Dis. Prim.* 3:17013. doi: 10.1038/nrdp.2017.13

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., et al. (1997). Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276, 2045–2047. doi: 10.1126/science.276.5321.2045

Poulopoulos, M., Levy, O. A., and Alcalay, R. N. (2012). The neuropathology of genetic Parkinson's disease. *Mov. Disord.* 27, 831–842. doi: 10.1002/mds.24962

Prabu, S. K., Anandatheerthavarada, H. K., Raza, H., Srinivasan, S., Spear, J. F., and Avadhani, N. G. (2006). Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. *J. Biol. Chem.* 281, 2061–2070. doi: 10.1074/jbc.m507741200

Price, A., Manzoni, C., Cookson, M. R., and Lewis, P. A. (2018). The LRRK2 signalling system. *Cell Tissue Res.* 373, 39–50. doi: 10.1007/s00441-017-2759-9

Przedborski, S. (2017). The two-century journey of Parkinson disease research. *Nat. Rev. Neurosci.* 18, 251–259. doi: 10.1038/nrn.2017.25

Puschmann, A. (2013). Monogenic Parkinson's disease and parkinsonism: Clinical phenotypes and frequencies of known mutations. *Parkinsonism Relat. Disord.* 19, 407–415. doi: 10.1016/j.parkreldis.2013.01.020

Qing, H., Wong, W., McGeer, E. G., and McGeer, P. L. (2009). Lrrk2 phosphorylates α synuclein at serine 129: Parkinson disease implications. *Biochem. Biophys. Res. Commun.* 387, 149–152. doi: 10.1016/j.bbrc.2009.06.142

Ramonet, D., Daher, J. P. L., Lin, B. M., Stafa, K., Kim, J., Banerjee, R., et al. (2011). Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. *PLoS One* 6:e18568. doi: 10.1371/journal.pone.0018568

Rassu, M., Del Giudice, M. G., Sanna, S., Taymans, J. M., Morari, M., Brugnoli, A., et al. (2017). Role of LRRK2 in the regulation of dopamine receptor trafficking. *PLoS One* 12:e0179082. doi: 10.1371/journal.pone.0179082

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 13, 566–578. doi: 10.1038/nrm3412

Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., et al. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial $Ca^{2+}$ responses. *Science* 280, 1763–1766. doi: 10.1126/science.280.5370.1763

Roosen, D. A., and Cookson, M. R. (2016). LRRK2 at the interface of autophagosomes, endosomes and lysosomes. *Mol. Neurodegener.* 11:73. doi: 10.1186/s13024-016-0140-1

Rowland, A. A., and Voeltz, G. K. (2012). Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat. Rev. Mol. Cell Biol.* 13, 607–615. doi: 10.1038/nrm3440

Rudenko, I. N., Kaganovich, A., Hauser, D. N., Beylina, A., Chia, R., Ding, J., et al. (2012). The G2385R variant of leucine-rich repeat kinase 2 associated with Parkinson's disease is a partial loss-of-function mutation. *Biochem. J.* 446, 99–111. doi: 10.1042/BJ20120637

Russo, I., Berti, G., Plotegher, N., Bernardo, G., Filograna, R., Bubacco, L., et al. (2015). Leucine-rich repeat kinase 2 positively regulates inflammation and down-regulates NF-κB p50 signaling in cultured microglia cells. *J. Neuroinflammation* 12:230. doi: 10.1186/s12974-015-0449-7

Russo, I., Bubacco, L., and Greggio, E. (2014). LRRK2 and neuroinflammation: partners in crime in Parkinson's disease? *J. Neuroinflammation* 11:52. doi: 10.1186/1742-2094-11-52

Saha, S., Guillily, M. D., Ferree, A., Lanceta, J., Chan, D., Ghosh, J., et al. (2009). LRRK2 modulates vulnerability to mitochondrial dysfunction in *Caenorhabditis elegans*. *J. Neurosci.* 29, 9210–9218. doi: 10.1523/jneurosci.2281-09.2009

Sanchez, G., Varaschin, R. K., Büeler, H., Marcogliese, P. C., Park, D. S., and Trudeau, L.-E. (2014). Unaltered striatal dopamine release levels in young parkin knockout, Pink1 knockout, DJ-1 knockout and LRRK2 R1441G

transgenic mice. *PLoS One* 9:e94826. doi: 10.1371/journal.pone.0094826

Sanders, L. H., Laganière, J., Cooper, O., Mak, S. K., Vu, B. J., Huang, Y. A., et al. (2014). LRRK2 mutations cause mitochondrial DNA damage in iPSC-derived neural cells from Parkinson's disease patients: reversal by gene correction. *Neurobiol. Dis.* 62, 381–386. doi: 10.1016/j.nbd.2013.10.013

Sanna, G., Del Giudice, M. G., Crosio, C., and Iaccarino, C. (2012). LRRK2 and vesicle trafficking. *Biochem. Soc. Trans.* 40, 1117–1122. doi: 10.1042/bst20120117

Schapansky, J., Khasnavis, S., DeAndrade, M. P., Nardozzi, J. D., Falkson, S. R., Boyd, J. D., et al. (2018). Familial knockin mutation of LRRK2 causes lysosomal dysfunction and accumulation of endogenous insoluble α-synuclein in neurons. *Neurobiol. Dis.* 111, 26–35. doi: 10.1016/j.nbd.2017.12.005

Schapansky, J., Nardozzi, J. D., Felizia, F., and LaVoie, M. J. (2014). Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy. *Hum. Mol. Genet.* 23, 4201–4214. doi: 10.1093/hmg/ddu138

Schapansky, J., Nardozzi, J. D., and LaVoie, M. J. (2015). The complex relationships between microglia, α-synuclein and LRRK2 in Parkinson's disease. *Neuroscience* 302, 74–88. doi: 10.1016/j.neuroscience.2014.09.049

Schapira, A. H. V. (2007). Mitochondrial dysfunction in Parkinson's disease. *Cell Death Differ.* 14, 1261–1266. doi: 10.1038/sj.cdd.4402160

Schapira, A. H. V., Chaudhuri, K. R., and Jenner, P. (2017). Non-motor features of Parkinson disease. *Nat. Rev. Neurosci.* 18, 435–450. doi: 10.1038/nrn.2017.62

Schirinzi, T., Madeo, G., Martella, G., Maltese, M., Picconi, B., Calabresi, P., et al. (2016). Early synaptic dysfunction in Parkinson's disease: insights from animal models. *Mov. Disord.* 31, 802–813. doi: 10.1002/mds.26620

Schreij, A. M., Chaineau, M., Ruan, W., Lin, S., Barker, P. A., Fon, E. A., et al. (2015). LRRK 2 localizes to endosomes and interacts with clathrin-light chains to limit Rac1 activation. *EMBO Rep.* 16, 79–86. doi: 10.15252/embr.201438714

Schwab, A. J., and Ebert, A. D. (2015). Neurite aggregation and calcium dysfunction in iPSC-derived sensory neurons with Parkinson's disease-related LRRK2 G2019S mutation. *Stem Cell Rep.* 5, 1039–1052. doi: 10.1016/j.stemcr.2015.11.004

Sepulveda, B., Mesias, R., Li, X., Yue, Z., and Benson, D. L. (2013). Short- and long-term effects of LRRK2 on axon and dendrite growth. *PLoS One* 8:e61986. doi: 10.1371/journal.pone.0061986

Shin, N., Jeong, H., Kwon, J., Heo, H. Y., Kwon, J. J., Yun, H. J., et al. (2008). LRRK2 regulates synaptic vesicle endocytosis. *Exp. Cell Res.* 314, 2055–2065. doi: 10.1016/j.yexcr.2008.02.015

Simmen, T., Lynes, E. M., Gesson, K., and Thomas, G. (2010). Oxidative protein folding in the endoplasmic reticulum: tight links to the mitochondria-associated membrane (MAM). *Biochim. Biophys. Acta* 1798, 1465–1473. doi: 10.1016/j.bbamem.2010.04.009

Singh, A., Zhi, L., and Zhang, H. (2019). LRRK2 and mitochondria: recent advances and current views. *Brain Res.* 1702, 96–104. doi: 10.1016/j.brainres.2018.06.010

Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., et al. (2003). α-Synuclein locus triplication causes Parkinson's disease. *Science* 302:841. doi: 10.1126/science.1090278

Sloan, M., Alegre-Abarrategui, J., Potgieter, D., Kaufmann, A.-K., Exley, R., Deltheil, T., et al. (2016). LRRK2 BAC transgenic rats develop progressive, L-DOPA-responsive motor impairment and deficits in dopamine circuit function. *Hum. Mol. Genet.* 25, 951–963. doi: 10.1093/hmg/ddv628

Steger, M., Tonelli, F., Ito, G., Davies, P., Trost, M., Vetter, M., et al. (2016). Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases. *eLife* 5:e12813. doi: 10.7554/eLife.12813

Strickland, D., and Bertoni, J. M. (2004). Parkinson's prevalence estimated by a state registry. *Mov. Disord.* 19, 318–323. doi: 10.1002/mds.10619

Su, Y.-C., and Qi, X. (2013). Inhibition of excessive mitochondrial fission reduced aberrant autophagy and neuronal damage caused by LRRK2 G2019S mutation. *Hum. Mol. Genet.* 22, 4545–4561. doi: 10.1093/hmg/ddt301

Su, B., Wang, X., Zheng, L., Perry, G., Smith, M. A., and Zhu, X. (2010). Abnormal mitochondrial dynamics and neurodegenerative diseases. *Biochim. Biophys. Acta* 1802, 135–142. doi: 10.1016/j.bbadis.2009.09.013

Surmeier, D. J., Obeso, J. A., and Halliday, G. M. (2017). Selective neuronal vulnerability in Parkinson disease. *Nat. Rev. Neurosci.* 18, 101–113. doi: 10.1038/nrn.2016.178

Sweet, E. S., Saunier-Rebori, B., Yue, Z., and Blitzer, R. D. (2015). The Parkinson's disease-associated mutation LRRK2–G2019S impairs synaptic plasticity in mouse hippocampus. *J. Neurosci.* 35, 11190–11195. doi: 10.1523/JNEUROSCI.0040-15.2015

Taymans, J.-M., and Greggio, E. (2016). LRRK2 kinase inhibition as a therapeutic strategy for Parkinson's disease, where do we stand? *Curr. Neuropharmacol.* 14, 214–225. doi: 10.2174/1570159x13666151030102847

Taymans, J.-M., Nkiliza, A., and Chartier-Harlin, M.-C. (2015). Deregulation of protein translation control, a potential game-changing hypothesis for Parkinson's disease pathogenesis. *Trends Mol. Med.* 21, 466–472. doi: 10.1016/j.molmed.2015.05.004

Taymans, J.-M., Van den Haute, C., and Baekelandt, V. (2006). Distribution of PINK1 and LRRK2 in rat and mouse brain. *J. Neurochem.* 98, 951–961. doi: 10.1111/j.1471-4159.2006.03919.x

Terheyden, S., Ho, F. Y., Gilsbach, B. K., Wittinghofer, A., and Kortholt, A. (2015). Revisiting the Roco G-protein cycle. *Biochem. J.* 465, 139–147. doi: 10.1042/bj20141095

Thirstrup, K., Dächsel, J. C., Oppermann, F. S., Williamson, D. S., Smith, G. P., Fog, K., et al. (2017). Selective LRRK2 kinase inhibition reduces phosphorylation of endogenous Rab10 and Rab12 in human peripheral mononuclear blood cells. *Sci. Rep.* 7:10300. doi: 10.1038/s41598-017-10501-z

Tong, Y., Yamaguchi, H., Giaime, E., Boyle, S., Kopan, R., Kelleher, R. J., et al. (2010). Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of -synuclein and apoptotic cell death in aged mice. *Proc. Natl. Acad. Sci. U S A* 107, 9879–9884. doi: 10.1073/pnas.1004676107

Toyofuku, T., Okamoto, Y., Ishikawa, T., Sasawatari, S., and Kumanogoh, A. (2020). LRRK2 regulates endoplasmic reticulum-mitochondrial tethering through the PERK-mediated ubiquitination pathway. *EMBO J.* 39:e100875. doi: 10.15252/embj.2018100875

Tozzi, A., de Iure, A., Bagetta, V., Tantucci, M., Durante, V., Quiroga-Varela, A., et al. (2016). α-synuclein produces early behavioral alterations *via* striatal cholinergic synaptic dysfunction by interacting with GluN2D N -methyl-D-aspartate receptor subunit. *Biol. Psychiatry* 79, 402–414. doi: 10.1016/j.biopsych.2015.08.013

Tozzi, A., Durante, V., Bastioli, G., Mazzocchetti, P., Novello, S., Mechelli, A., et al. (2018a). Dopamine D2 receptor activation potently inhibits striatal glutamatergic transmission in a G2019S LRRK2 genetic model of Parkinson's disease. *Neurobiol. Dis.* 118, 1–8. doi: 10.1016/j.nbd.2018.06.008

Tozzi, A., Tantucci, M., Marchi, S., Mazzocchetti, P., Morari, M., Pinton, P., et al. (2018b). Dopamine D2 receptor-mediated neuroprotection in a G2019S Lrrk2 genetic model of Parkinson's disease. *Cell Death Dis.* 9:204. doi: 10.1038/s41419-017-0221-2

Tsika, E., Kannan, M., Foo, C. S.-Y., Dikeman, D., Glauser, L., Gellhaar, S., et al. (2014). Conditional expression of Parkinson's disease-related R1441C LRRK2 in midbrain dopaminergic neurons of mice causes nuclear abnormalities without neurodegeneration. *Neurobiol. Dis.* 71, 345–358. doi: 10.1016/j.nbd.2014.08.027

Valsecchi, F., Ramos-Espiritu, L. S., Buck, J., Levin, L. R., and Manfredi, G. (2013). cAMP and Mitochondria. *Physiology* 28, 199–209. doi: 10.1152/physiol.00004.2013

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., and Bloch, D. A. (2003). Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. *Am. J. Epidemiol.* 157, 1015–1022. doi: 10.1093/aje/kwg068

Vancraenenbroeck, R., Lobbestael, E., De Maeyer, M., Baekelandt, V., and Taymans, J.-M. (2011). Kinases as targets for Parkinson's disease: from genetics to therapy. *CNS Neurol. Disord. Drug Targets* 10, 724–740. doi: 10.2174/187152711797247858

Vekrellis, K., Xilouri, M., Emmanouilidou, E., Rideout, H. J., and Stefanis, L. (2011). Pathological roles of α-synuclein in neurological disorders. *Lancet Neurol.* 10, 1015–1025. doi: 10.1016/S1474-4422(11)70213-7

Verstraeten, A., Theuns, J., and Van Broeckhoven, C. (2015). Progress in unraveling the genetic etiology of Parkinson disease in a genomic era. *Trends Genet.* 31, 140–149. doi: 10.1016/j.tig.2015.01.004

Vitte, J., Traver, S., Maués De Paula, A., Lesage, S., Rovelli, G., Corti, O., et al. (2010). Leucine-rich repeat kinase 2 is associated with the endoplasmic reticulum in dopaminergic neurons and accumulates in the core of lewy bodies in Parkinson disease. *J. Neuropathol. Exp. Neurol.* 69, 959–972. doi: 10.1097/nen.0b013e3181efc01c

Vogiatzi, T., Xilouri, M., Vekrellis, K., and Stefanis, L. (2008). Wild type α-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J. Biol. Chem.* 283, 23542–23556. doi: 10.1074/jbc.M801992200

Volpicelli-Daley, L. A., Abdelmotilib, H., Liu, Z., Stoyka, L., Daher, J. P. L., Milnerwood, A. J., et al. (2016). G2019S-LRRK2 expression augments -synuclein sequestration into inclusions in neurons. *J. Neurosci.* 36, 7415–7427. doi: 10.1523/JNEUROSCI.3642-15.2016

Volta, M., Beccano-Kelly, D. A., Paschall, S. A., Cataldi, S., MacIsaac, S. E., Kuhlmann, N., et al. (2017). Initial elevations in glutamate and dopamine neurotransmission decline with age, as does exploratory behavior, in LRRK2 G2019S knock-in mice. *eLife* 6:e28377. doi: 10.7554/eLife.28377

Volta, M., Cataldi, S., Beccano-Kelly, D., Munsie, L., Tatarnikov, I., Chou, P., et al. (2015). Chronic and acute LRRK2 silencing has no long-term behavioral effects, whereas wild-type and mutant LRRK2 overexpression induce motor and cognitive deficits and altered regulation of dopamine release. *Parkinsonism Relat. Disord.* 21, 1156–1163. doi: 10.1016/j.parkreldis.2015.07.025

Volta, M., and Melrose, H. (2017). LRRK2 mouse models: dissecting the behavior, striatal neurochemistry and neurophysiology of PD pathogenesis. *Biochem. Soc. Trans.* 45, 113–122. doi: 10.1042/bst20160238

von Campenhausen, S., Bornschein, B., Wick, R., Bötzel, K., Sampaio, C., Poewe, W., et al. (2005). Prevalence and incidence of Parkinson's disease in Europe. *Eur. Neuropsychopharmacol.* 15, 473–490. doi: 10.1016/j.euroneuro.2005.04.007

Wales, P., Pinho, R., Lázaro, D. F., and Outeiro, T. F. (2013). Limelight on α-synuclein: pathological and mechanistic implications in neurodegeneration. *J. Parkinsons Dis.* 3, 415–459. doi: 10.3233/jpd-130216

Walker, M. D., Volta, M., Cataldi, S., Dinelle, K., Beccano-Kelly, D., Munsie, L., et al. (2014). Behavioral deficits and striatal DA signaling in LRRK2 p.G2019S transgenic rats: a multimodal investigation including PET neuroimaging. *J. Parkinsons Dis.* 4, 483–498. doi: 10.3233/jpd-140344

Wallings, R., Connor-Robson, N., and Wade-Martins, R. (2019). LRRK2 interacts with the vacuolar-type H$^+$-ATPase pump a1 subunit to regulate lysosomal function. *Hum. Mol. Genet.* 28, 2696–2710. doi: 10.1093/hmg/ddz088

Wallings, R., Manzoni, C., and Bandopadhyay, R. (2015). Cellular processes associated with LRRK2 function and dysfunction. *FEBS J.* 282, 2806–2826. doi: 10.1111/febs.13305

Wang, X., Yan, M. H., Fujioka, H., Liu, J., Wilson-Delfosse, A., Chen, S. G., et al. (2012). LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. *Hum. Mol. Genet.* 21, 1931–1944. doi: 10.1093/hmg/dds003

Weng, Y.-H., Chen, C.-Y., Lin, K.-J., Chen, Y.-L., Yeh, T.-H., Hsiao, I.-T., et al. (2016). (R1441C) LRRK2 induces the degeneration of SN dopaminergic neurons and alters the expression of genes regulating neuronal survival in a transgenic mouse model. *Exp. Neurol.* 275, 104–115. doi: 10.1016/j.expneurol.2015.09.001

West, A. B. (2017). Achieving neuroprotection with LRRK2 kinase inhibitors in Parkinson disease. *Exp. Neurol.* 298, 236–245. doi: 10.1016/j.expneurol.2017.07.019

West, A. B., Cowell, R. M., Daher, J. P. L., Moehle, M. S., Hinkle, K. M., Melrose, H. L., et al. (2014). Differential LRRK2 expression in the cortex, striatum and substantia nigra in transgenic and nontransgenic rodents. *J. Comp. Neurol.* 522, 2465–2480. doi: 10.1002/cne.23583

West, A. B., Moore, D. J., Biskup, S., Bugayenko, A., Smith, W. W., Ross, C. A., et al. (2005). Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc. Natl. Acad. Sci. U S A* 102, 16842–16847. doi: 10.1073/pnas.0507360102

Westerlund, M., Belin, A. C., Anvret, A., Bickford, P., Olson, L., and Galter, D. (2008). Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain and other rodent and human organs: implications for

Parkinson's disease. *Neuroscience* 152, 429–436. doi: 10.1016/j.neuroscience. 2007.10.062

Wood, A. R., Perry, J. R. B., Tanaka, T., Hernandez, D. G., Zheng, H.-F., Melzer, D., et al. (2013). Imputation of variants from the 1000 genomes project modestly improves known associations and can identify low-frequency variant—phenotype associations undetected by hapmap based imputation. *PLoS One* 8:e64343. doi: 10.1371/journal.pone.0064343

Xiong, Y., Neifert, S., Karuppagounder, S. S., Liu, Q., Stankowski, J. N., Lee, B. D., et al. (2018). Robust kinase- and age-dependent dopaminergic and norepinephrine neurodegeneration in LRRK2 G2019S transgenic mice. *Proc. Natl. Acad. Sci. U S A* 115, 1635–1640. doi: 10.1073/pnas.17126 48115

Xiong, Y., Neifert, S., Karuppagounder, S. S., Stankowski, J. N., Lee, B. D., Grima, J. C., et al. (2017). Overexpression of Parkinson's disease-associated mutation LRRK2 G2019S in mouse forebrain induces behavioral deficits and α-synuclein pathology. *eNeuro* 4:ENEURO.0004–17.2017. doi: 10.1523/ENEURO.0004-17.2017

Young, K. W., Bampton, E. T. W., Pinòn, L., Bano, D., and Nicotera, P. (2008). Mitochondrial $Ca^{2+}$ signalling in hippocampal neurons. *Cell Calcium* 43, 296–306. doi: 10.1016/j.ceca.2007.06.007

Yue, M., Hinkle, K. M., Davies, P., Trushina, E., Fiesel, F. C., Christenson, T. A., et al. (2015). Progressive dopaminergic alterations and mitochondrial abnormalities in LRRK2 G2019S knock-in mice. *Neurobiol. Dis.* 78, 172–195. doi: 10.1016/j.nbd.2015.02.031

Zhao, Y., and Dzamko, N. (2019). Recent developments in LRRK2-targeted therapy for Parkinson's disease. *Drugs* 79, 1037–1051. doi: 10.1007/s40265-019-01139-4

Zhou, H., Huang, C., Tong, J., Hong, W. C., Liu, Y.-J., and Xia, X.-G. (2011). Temporal expression of mutant LRRK2 in adult rats impairs dopamine reuptake. *Int. J. Biol. Sci.* 7, 753–761. doi: 10.7150/ijbs.7.753

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., et al. (2004a). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 44, 601–607. doi: 10.1016/j.neuron.2004. 11.005

Zimprich, A., Müller-Myhsok, B., Farrer, M., Leitner, P., Sharma, M., Hulihan, M., et al. (2004b). The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval. *Am. J. Hum. Genet.* 74, 11–19. doi: 10.1086/380647

# The Future of GDNF in Parkinson's Disease

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## INTRODUCTION

At the 2019 annual meeting for American Society of Neural Therapy and Repair (ASNTR) a special panel assembled to discuss the future of neurotrophic factor delivery in Parkinson's disease (PD), particularly those factors belonging to the Glial cell line-derived neurotrophic factor family of ligands (GFLs; GDNF and Neurturin). The panel consisted of representatives from academia, industry, and non-profit organizations with primary backgrounds in neurology or neurosurgery and the impetus for the assembly was data from the a recent GDNF clinical trial (Whone A. et al., 2019; Whone A. L. et al., 2019) that utilized an enhanced method of protein infusion to facilitate improved spread of GDNF. Despite preclinical success, this trial, as all previously published trials, failed to demonstrate clinical efficacy (Whone A. L. et al., 2019), leaving the field wondering if there is a future for these neurotrophic factors in PD (Kirkeby and Barker, 2019). This opinion piece will summarize the discussion and the overarching recommendations from the meeting.

## RECENT RESULTS FROM GDNF TRIALS

Over the last few decades there have been numerous clinical trials utilizing central delivery of GDNF or neurturin via direct protein infusion or overexpression using viral vector-based gene therapy (Merola et al., 2010) with the latest trial reporting dosing of the first patient in September 2020 [Brain Neurotherapy Bio; adeno-associated virus (AAV)-GDNF]. Despite preliminary reports of efficacy in the open-label phase of trials, placebo-controlled studies have failed to replicate any favorable outcomes (e.g., Marks et al., 2008, 2010). Although the reasons behind these apparent failures are unknown, one of the issues may be lack of sufficient target engagement—either via poor diffusion in protein delivery trials (Salvatore et al., 2006) or poor transduction in viral vector trials (Bartus et al., 2011). To that end, the most recent trial rationalized that improving delivery with convection enhanced delivery (CED) might overcome the limitation of insufficient putamenal and nigral drug coverage and achieve improvements in motor function (Whone A. et al., 2019). Regrettably, the results from this trial closely resembled those seen in previous trials—improved $^{18}$F-DOPA uptake in the absence of clinical improvements. Here, we discuss what additional potential inadequacies have confounded various clinical trials and whether any rational hope remains in regard to utilizing this family of growth factors in the treatment of PD.

## LOST IN TRANSLATION

The most obvious question that remains is whether neurotrophic factors such as GDNF truly hold disease modifying potential for PD. A wealth of preclinical data supports the notion that GDNF may prevent or retard nigrostriatal degeneration. Moreover, preclinical and human clinical data clearly indicate that GFLs are, in fact, CNS dopaminergic trophic factors. Therefore, treatment with GFLs should promote survival of striatal dopamine (DA) innervation and thereby improve the motor symptoms of PD. So why have clinical trials utilizing this approach largely failed?

One key aspect of GDNF and similar factors is that the therapeutic mode of action is not fully defined, and that the degenerating PD brain may be resistant to the neuroprotective potential of these proteins. The lack of clarity on GDNF's mechanism of action may be causing issues in appropriate model selection for preclinical therapeutic testing. For instance, GDNF preclinical data is largely based on acute, toxin-induced models–such as 6-hydroxydopamine and MPTP. While administration of GDNF under these settings have provided both neuroprotection and neurorestoration, the same claims of GDNF efficacy have not been substantiated in other models of PD—such as in alpha-synuclein (α-syn) overexpression models (Decressac et al., 2011). In fact, work in the AAV α-syn overexpression model shows that GDNF exhibits no neuroprotective effect (Decressac et al., 2011).

There is some controversy as to the cause of this resistance to neuroprotection. On one hand, the lack of GDNF-induced beneficial effects in the α-syn model has been argued to be due to the downregulation of Nurr1 and its downstream product, the GDNF receptor component receptor tyrosine kinase (RET) (Decressac et al., 2012), although other groups have failed to reproduce the downregulation of Nurr1. Importantly, some key caveats to α-syn overexpression models lie in the finding that α-syn mRNA is in fact decreased in PD and few have reported changes in Nurr1 and RET in human disease (Chu et al., 2006; Su et al., 2017). Moreover, this and other α-syn models of PD overexpress α-syn by 4–10 times normally seen in human studies. Although animal models provide valuable insight into certain disease processes, it is clear that the PD field suffers from a lack of clinically-predictive animal models that faithfully recapitulate all key aspects of parkinsonian neurodegeneration and disease progression. Thus, until we have models more encompassing of the etiopathological features of PD, future reports of preclinical efficacy, or the lack thereof, must be interpreted with caution.

In regard to translation, it is also important to note that the chief risk-factor in PD is age (Collier et al., 2011), yet a majority of preclinical studies have largely neglected this crucial variable. However, it is clear that age alters the local environment, and confers impediments in delivery modalities such as viral vector transduction (Polinski et al., 2016), amongst others. In addition, many intracellular processes change with aging. Thus, performing the preclinical experimentation in models whose age corresponds to that of the average human patient is as important as choosing the model with the most appropriate pathological insult. Nonetheless, it is important to note that GDNF retains at least some function with advanced age as intraputamenal infusion into aged monkeys reduces age-related motoric deficits (Maswood et al., 2002).

Another equally important variable is disease duration. It is well-known that PD patients with longer duration of disease have more disease related complications, and such patients are the target for surgical experimental therapeutics for valid reasons of clinical morbidity. However, this variable introduces a critical barrier to translating animal studies to humans as animal studies do not recapitulate the longevity of disease duration as they are cost prohibitive. Also, in the open label studies as well as in the blinded placebo controlled studies, the average age of onset of disease was much younger than the average age (<50) of onset of PD (Gill et al., 2003; Slevin et al., 2005; Lang et al., 2006). This younger age of onset for PD represents a unique subpopulation as has been recognized by many contemporary researchers (Mehanna and Jankovic, 2019; Espay et al., 2020). This raises the important question if such younger onset patients are the best candidates and if they are indeed chosen based on preclinical age equivalency, then, the GDNF intervention must be performed much earlier in the course of their illness (the mean duration of illness was 10 years in these studies). This raises important ethical issues of risk vs. benefits in early onset PD subjects from invasive neurosurgical interventions. Early onset PD patients have a slower disease progression trajectory and so are treated effectively with pharmacotherapy during this "honeymoon" period that lasts well over 5 years. Yet, based on preclinical testing data, treating these patients earlier within 5 years of disease onset may be the best possible use of GDNF. The only possible ethical solution to this conundrum is to reduce the risks of the neurosurgical intervention. Developing less invasive and more safe methods for intracranial delivery of either GDNF protein or GDNF delivery vectors will allow testing such therapies in early disease in such younger patients with ethical equipoise.

## HAVE WE PERFORMED THE RIGHT CLINICAL TRIAL?

One question in the neurotrophic factor field has always been that of the timing of neurotrophic factor administration. Studies that utilized toxin-induced models clearly demonstrated that GDNF must be administered prior to, or during the insult, in order to achieve efficacy. Administration later may enhance the dopaminergic tone of nigral neurons, but does not provide neuroprotection (Mandel et al., 1997, 1999; Salvatore et al., 2004; Manfredsson et al., 2009a). There is clear evidence from human trials that GFLs can induce DA dendritic sprouting (Love et al., 2005; Kordower et al., 2013) or F-dopa uptake (Gill et al., 2003; Whone A. L. et al., 2019). Therefore, if the theorem that increasing striatal DA should confer therapeutic benefit is correct, then it may be that GFLs are biologically effective but have not reached a necessary threshold of striatal DA regeneration to achieve this benefit. Intervention at earlier stages of disease when more of the nigrostriatal DA pathway is intact or has not undergone plastic changes due to ongoing degeneration, should give GFLs a greater opportunity

to reach this threshold of striatal regeneration to provide clinical benefit.

Nevertheless, during the early days of neurotrophic factor delivery, questions regarding the integrity of the nigrostriatal system during disease progression remained. It was not until recently that histopathological characterization by Kordower et al. clearly delineated the significant degree of nigrostriatal denervation during the years immediately following diagnosis (Kordower et al., 2013). Thus, the GFL clinical trials to date targeting late stage patients in Hoehn & Yahr stage 3–4 (where reports have been available) clinical trials are seemingly at odds with the preclinical studies administering the intervention when the nigrostriatal system is mostly intact. To that fact, how many times have we heard variations on the statement "you cannot save what is no longer there"? Thus, the only way to reconcile the field is to test the neuroprotective potential of GDNF and neurturin in early stage patients. During our discussion, it was proposed that the quintessential clinical trial would be performed in patients with unilateral onset, prior to contralateral progression. This, of course, yet again brings up the question about patient safety and advocacy.

## SAFETY OF GFLs

Following diagnosis, PD progresses slowly on average, and pharmacological restoration of the dopaminergic tone in the caudate/putamen (e.g., with Sinemet) provides a fairly lengthy "honeymoon period." Thus, performing a rather complex neurosurgical procedure at a time shortly following diagnosis is not to be taken lightly. This provides an ethical dilemma whereby a clinician is faced with a patient that will maintain acceptable quality of life for some time, yet the disease will progress relentlessly albeit asymmetrically. At this point, how can you justify the testing of an invasive therapeutic paradigm that remains unproven in PD? Neurosurgical experience and advances would support lowering intervention thresholds. Safety data from a wealth of procedures with an indwelling lead/cannula–such as deep brain stimulation (DBS) where a number of anatomical locations, including deep structures, have been targeted—support the lower thresholds as a relatively low rate of serious adverse events are now reported (Budman et al., 2018). In line with DBS safety, striatal intraparenchymal fetal mesencephalic transplantation has demonstrated that the neurosurgical procedure itself is very safe (Lindvall, 2015). Still, despite all the current improvements with stereotactic neurosurgical techniques, one can argue that the risks of a neurosurgical intervention do not match up with the risks associated with currently effective pharmacotherapy in early stages of PD. Therefore, the justification to perform such a procedure must provide disproportionately high benefit to the risks or the risks themselves must get mitigated via the use of less invasive methods of delivery.

Moreover, even if currently optimized surgical methods are used with the least possible adverse events, there are still open questions as to the safety of GDNF itself. Although all indications from preclinical research into neurotrophic factors belonging to this family of proteins suggest that GDNF is safe, perhaps the most compelling data comes from the long-term safety profile of Neurturin, GDNF and other gene therapy-based candidates in human clinical trials (Tenenbaum and Humbert-Claude, 2017; Chu et al., 2020). Nevertheless, there could be consequences of long-term activity with the possibility that secondary issues such as aberrant sprouting of neurons (Georgievska et al., 2002) may lead to a new set of symptoms. Certainly, the use of a clinical approach that allows for cessation of protein delivery (i.e., cannulation/pump infusion, or regulated vectors) would provide a safety mechanism whereby treatment could be halted in the case of an adverse event.

## DISEASE DIAGNOSIS AND TRACKING OF PROGRESSION

Most panelists agreed that GFL delivery could be clinically therapeutic if treatment were initiated earlier in disease progression for PD patients. However, even if all clinicians would agree that the delivery of GFLs was of a similar risk to pharmacological treatment (an agreement that is not in place at present), it is currently impossible to reliably detect very early PD (Rizzo et al., 2016). Despite being easily recognized in the public eye, PD is a rather complex disorder, and early diagnosis is not unequivocal (Berg et al., 2018). In fact, a diagnosis of early PD is extremely uncertain, especially when performed outside of a specialty movement disorders Center of Excellence. For instance, other neurodegenerative disorders such as multiple system atrophy (Krismer and Wenning, 2017) and progressive supranuclear palsy (Owolabi, 2013) can often times be misdiagnosed as PD early in the course of disease (Tolosa et al., 2006). This is obviously a complication that makes clinical trial design for early PD increasingly complex. Along the same lines, disease modifying clinical trials in PD, especially early in the disease, are hampered by the fact that there are no good metrics whereby one can measure progression without very large sample sizes or utilizing exceptionally long trial periods. Moreover, trials thus far have been powered to detect improvement in the Unified Parkinson Disease Rating Scale (UPDRS) when perhaps we should be looking for stabilization in decline. Finally, PD is also an extremely heterogenous disorder: Progression rates vary widely, there is heterogeneity in the predominant symptom (e.g., tremor-dominant vs. gait/balance-dominant) that may not respond the same to GDNF, or may not be homogeneous enough for current progression markers (like UPDRS), to detect changes, Thus, as crucial as future biomarkers are in PD for earlier definitive diagnosis (Parnetti et al., 2019) and to track progression, they will be equally important to enable definitive clinical trials in early disease. Such a shift in treatment paradigms would have the greatest impact for PD in the immediate future.

## ALTERNATIVES TO GFLs

Although GDNF and neurturin undoubtedly has undergone the highest scrutiny of all potential trophic factors in PD, there are alternatives worth mentioning. Damage to the

striatum results in increased astrocytic production of ciliary neurotrophic factor (CNTF), which belongs to the interleukin-6 family of neuropoietic cytokines. CNTF signaling occurs via a variety of heteroreceptors complexes following binding to CNTF receptor alpha (CNTFRα) (Schuster et al., 2003). Although the exact signaling mechanism is unknown, increased CNTF can protect DA neurons from toxicity, both via direct interaction with neurons as well as by reducing the inflammatory potential of microglia (Hagg and Varon, 1993; Nam et al., 2015; Baek et al., 2018). The closely related trophic factors mesencephalic astrocyte-derived neurotrophic factor (MANF) (Petrova et al., 2003) and cerebral/conserved dopamine neurotrophic factor (CDNF) (Lindholm et al., 2007) similarly provide neuroprotection in various animal models of PD (Airavaara et al., 2012). The exact mode of action of these proteins is unknown, although neuroprotection seems to be, at least in part, conferred via modulation of endoplasmic reticulum stress and autophagy (Zhang et al., 2018). A clinical trial is currently ongoing (ClinicalTrials.gov Identifier: NCT03775538) assessing the safety of putamenal delivery of CDNF (Huttunen and Saarma, 2019) and anecdotal reports suggests that the treatment has been well-tolerated. Finally, small molecule GDNF family receptor (GFR) agonists are being investigated as a potential alternative to the invasive neurosurgical approach otherwise required (Ivanova et al., 2018). However, GFR receptors are heavily expressed in organs throughout the body [The Human Protein Atlas (Uhlen et al., 2015)]. For example, an intracerebroventricularly delivered GDNF trial was halted due to side-effects (Nutt et al., 2003) which is likely due to GDNF's actions in hypothalamus (Manfredsson et al., 2009b). Thus, GDNF administration for PD likely requires site-specific putamenal delivery rendering this strategy the rare case where intraparenchymal delivery is more advantageous than a global small molecule paradigm. Nonetheless, regardless of the therapeutic modality one chooses, the same critical GFL safety factors discussed above apply.

## CONCLUSIONS

In summary, the panel participants, as well as the audience, expressed cautious optimism for the future of neurotrophic factors, maintaining that GDNF remains a highly promising target in the treatment of PD progression. The preclinical data remain strong, and we simply may not have unleashed the full potential of these proteins, because they have thus not been properly delivered and tested in the context of human disease at feasible points of intervention. Surely, recent improvements such as enhanced vector biodistribution (Kanaan et al., 2017; Davidsson et al., 2019) and less invasive delivery techniques such as focused ultrasound-assisted delivery (Noroozian et al., 2019), are moving us closer to the reinvention of clinical trials. Nevertheless, the path forward is not clear cut, and with current means at our disposal, the execution of early stage clinical trials may not be feasible. It is very possible that the repeated failure to find positive GFL-based clinical trial outcomes mar the field and effectively prohibit future trials from being proposed due lack of financial interests and/or negative public perception. What will the threshold be for investing in new and redesigned trials that are likely to be more expensive than those in the past? In essence, the future of GFL treatment to intervene in the progression of PD symptoms is dependent on significant improvements to preclinical models, improvements to clinical striatal delivery methods, discovery of alternate less invasive methods, improvements to very early PD diagnosis, and especially improvements to PD clinical trial design that would facilitate the prosecution of conclusive clinical trials.

## AUTHOR CONTRIBUTIONS

FM and RM wrote the first version of this manuscript. All authors contributed to the editing of subsequent drafts.

## REFERENCES

Airavaara, M., Harvey, B. K., Voutilainen, M. H., Shen, H., Chou, J., Lindholm, P., et al. (2012). CDNF protects the nigrostriatal dopamine system and promotes recovery after MPTP treatment in mice. *Cell Transpl.* 21, 1213–1223. doi: 10.3727/096368911X600948

Baek, J. Y., Jeong, J. Y., Kim, K. I., Won, S. Y., Chung, Y. C., Nam, J. H., et al. (2018). Inhibition of microglia-derived oxidative stress by ciliary neurotrophic factor protects dopamine neurons *in vivo* from MPP(+) neurotoxicity. *Int. J. Mol. Sci.* 19:3543. doi: 10.3390/ijms19113543

Bartus, R. T., Herzog, C. D., Chu, Y., Wilson, A., Brown, L., Siffert, J., et al. (2011). Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains. *Mov. Disord.* 26, 27–36. doi: 10.1002/mds.23442

Berg, D., Adler, C. H., Bloem, B. R., Chan, P., Gasser, T., Goetz, C. G., et al. (2018). Movement disorder society criteria for clinically established early Parkinson's disease. *Mov. Disord.* 33, 1643–1646. doi: 10.1002/mds.27431

Budman, E., Deeb, W., Martinez-Ramirez, D., Pilitsis, J. G., Peng-Chen, Z., Okun, M. S., et al. (2018). Potential indications for deep brain stimulation in neurological disorders: an evolving field. *Eur. J. Neurol.* 25, 434–e30. doi: 10.1111/ene.13548

Chu, Y., Bartus, R. T., Manfredsson, F., Olanow, C. W., and Kordower, J. H. (2020). Long-term post-mortem studies following neurturin gene therapy in patients with advanced Parkinson's disease. *Brain* 143, 960–975. doi: 10.1093/brain/awaa020

Chu, Y., Le, W., Kompoliti, K., Jankovic, J., Mufson, E. J., and Kordower, J. H. (2006). Nurr1 in Parkinson's disease and related disorders. *J. Comp. Neurol.* 494, 495–514. doi: 10.1002/cne.20828

Collier, T. J., Kanaan, N. M., and Kordower, J. H. (2011). Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates. *Nat. Rev. Neurosci.* 12, 359–366. doi: 10.1038/nrn3039

Davidsson, M., Wang, G., Aldrin-Kirk, P., Cardoso, T., Nolbrant, S., Hartnor, M., et al. (2019). A systematic capsid evolution approach performed *in vivo* for the design of AAV vectors with tailored properties and tropism. *Proc. Natl. Acad. Sci. U.S.A.* 116, 27053–27062. doi: 10.1073/pnas.1910061116

Decressac, M., Kadkhodaei, B., Mattsson, B., Laguna, A., Perlmann, T., and Bjorklund, A. (2012). Alpha-synuclein-induced down-regulation of Nurr1 disrupts GDNF signaling in nigral dopamine neurons. *Sci. Transl. Med.* 4:163ra156. doi: 10.1126/scitranslmed.3004676

Decressac, M., Ulusoy, A., Mattsson, B., Georgievska, B., Romero-Ramos, M., Kirik, D., et al. (2011). GDNF fails to exert neuroprotection in a rat alpha-synuclein model of Parkinson's disease. *Brain* 134, 2302–2311. doi: 10.1093/brain/awr149

Espay, A. J., Kalia, L. V., Gan-Or, Z., Williams-Gray, C. H., Bedard, L., Rowe, S. M., et al. (2020). Disease modification and biomarker development

in parkinson disease: revision or reconstruction? *Neurology* 94, 481–494. doi: 10.1212/WNL.0000000000009107

Georgievska, B., Kirik, D., and Bjorklund, A. (2002). Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp. Neurol.* 177, 461–474. doi: 10.1006/exnr.2002.8006

Gill, S. S., Patel, N. K., Hotton, G. R., O'Sullivan, K., McCarter, R., Bunnage, M., et al. (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat. Med.* 9, 589–595. doi: 10.1038/nm850

Hagg, T., and Varon, S. (1993). Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons *in vivo. Proc. Natl. Acad. Sci. U.S.A.* 90, 6315–6319. doi: 10.1073/pnas.90.13.6315

Huttunen, H. J., and Saarma, M. (2019). CDNF protein therapy in Parkinson's disease. *Cell Transpl.* 28, 349–366. doi: 10.1177/0963689719840290

Ivanova, L., Tammiku-Taul, J., Sidorova, Y., Saarma, M., and Karelson, M. (2018). Small-molecule ligands as potential GDNF family receptor agonists. *ACS Omega* 3, 1022–1030. doi: 10.1021/acsomega.7b01932

Kanaan, N. M., Sellnow, R. C., Boye, S. L., Coberly, B., Bennett, A., Agbandje-McKenna, M., et al. (2017). Rationally engineered AAV capsids improve transduction and volumetric spread in the CNS. *Mol. Ther. Nucleic Acids.* 8, 184–197. doi: 10.1016/j.omtn.2017.06.011

Kirkeby, A., and Barker, R. A. (2019). Parkinson disease and growth factors - is GDNF good enough? *Nat. Rev. Neurol.* 15, 312–314. doi: 10.1038/s41582-019-0180-6

Kordower, J. H., Olanow, C. W., Dodiya, H. B., Chu, Y., Beach, T. G., Adler, C. H., et al. (2013). Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain* 136, 2419–2431. doi: 10.1093/brain/awt192

Krismer, F., and Wenning, G. K. (2017). Multiple system atrophy: insights into a rare and debilitating movement disorder. *Nat. Rev. Neurol.* 13, 232–243. doi: 10.1038/nrneurol.2017.26

Lang, A. E., Gill, S., Patel, N. K., Lozano, A., Nutt, J. G., Penn, R., et al. (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann. Neurol.* 59, 459–466. doi: 10.1002/ana.20737

Lindholm, P., Voutilainen, M. H., Lauren, J., Peranen, J., Leppanen, V. M., Andressoo, J. O., et al. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons *in vivo. Nature* 448, 73–77. doi: 10.1038/nature05957

Lindvall, O. (2015). Treatment of Parkinson's disease using cell transplantation. *Philos. Trans. R Soc. Lond. B Biol. Sci.* 370:20140370. doi: 10.1098/rstb.2014.0370

Love, S., Plaha, P., Patel, N. K., Hotton, G. R., Brooks, D. J., and Gill, S. S. (2005). Glial cell line-derived neurotrophic factor induces neuronal sprouting in human brain. *Nat. Med.* 11, 703–704. doi: 10.1038/nm0705-703

Mandel, R. J., Snyder, R. O., and Leff, S. E. (1999). Recombinant adeno-associated viral vector-mediated glial cell line-derived neurotrophic factor gene transfer protects nigral dopamine neurons after onset of progressive degeneration in a rat model of Parkinson's disease. *Exp. Neurol.* 160, 205–214. doi: 10.1006/exnr.1999.7203

Mandel, R. J., Spratt, S. K., Snyder, R. O., and Leff, S. E. (1997). Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14083–14088. doi: 10.1073/pnas.94.25.14083

Manfredsson, F., Okun, M. S., and Mandel, R. J. (2009a). Gene therapy for neurological disorders: challenges and future prospects for the use of growth factors for the treatment of Parkinson's disease. *Curr. Genet. Ther.* 9, 375–388. doi: 10.2174/156652309789753400

Manfredsson, F., Tumer, N., Erdos, B., Landa, T., Broxson, C. S., Sullivan, L. F., et al. (2009b). Nigrostriatal rAAV-mediated GDNF overexpression induces robust weight loss in a rat model of age-related obesity. *Mol. Ther.* 17, 980–991. doi: 10.1038/mt.2009.45

Marks, W. J. Jr., Bartus, R. T., Siffert, J., Davis, C. S., Lozano, A., Boulis, N., et al. (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. *Lancet Neurol.* 9, 1164–1172. doi: 10.1016/S1474-4422(10)70254-4

Marks, W. J. Jr., Ostrem, J. L., Verhagen, L., Starr, A., Larson, S., Bakay, R. A., et al. (2008). Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *Lancet Neurol.* 7, 400–408. doi: 10.1016/S1474-4422(08)70065-6

Maswood, N., Grondin, R., Zhang, Z., Stanford, J. A., Surgener, S., Gash, D. M., et al. (2002). Effects of chronic intraputamenal infusion of glial cell line-derived neurotrophic factor (GDNF) in aged rhesus monkeys. *Neurobiol. Aging.* 23, 881–889. doi: 10.1016/S0197-4580(02)00022-2

Mehanna, R., and Jankovic, J. (2019). Young-onset Parkinson's disease: its unique features and their impact on quality of life. *Parkinson. Relat. Disord.* 65, 39–48. doi: 10.1016/j.parkreldis.2019.06.001

Merola, A., Van Laar, A., Lonser, R., and Bankiewicz, K. (2010). Gene therapy for Parkinson's disease: contemporary practice and emerging concepts. *Expert. Rev. Neurother.* 20, 577–590. doi: 10.1080/14737175.2020.1763794

Nam, J. H., Park, E. S., Won, S. Y., Lee, Y. A., Kim, K. I., Jeong, J. Y., et al. (2015). TRPV1 on astrocytes rescues nigral dopamine neurons in Parkinson's disease via CNTF. *Brain* 138, 3610–3622. doi: 10.1093/brain/awv297

Noroozian, Z., Xhima, K., Huang, Y., Kaspar, B. K., Kugler, S., Hynynen, K., et al. (2019). MRI-guided focused ultrasound for targeted delivery of rAAV to the brain. *Methods Mol. Biol.* 1950, 177–197. doi: 10.1007/978-1-4939-9139-6_10

Nutt, J. G., Burchiel, K. J., Comella, C. L., Jankovic, J., Lang, A. E., Laws, E. R. Jr., et al. (2003). Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology* 60, 69–73. doi: 10.1212/WNL.60.1.69

Owolabi, L. (2013). Progressive supranuclear palsy misdiagnosed as Parkinson's disease: a case report and review of literature. *Ann. Med. Health Sci. Res.* 3 (Suppl. 1):S44–47. doi: 10.4103/2141-9248.121221

Parnetti, L., Gaetani, L., Eusebi, P., Paciotti, S., Hansson, O., El-Agnaf, O., et al. (2019). CSF and blood biomarkers for Parkinson's disease. *Lancet Neurol.* 18, 573–586. doi: 10.1016/S1474-4422(19)30024-9

Petrova, P., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M. K., et al. (2003). MANF: a new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. *J. Mol. Neurosci.* 20, 173–188. doi: 10.1385/JMN:20:2:173

Polinski, N. K., Manfredsson, F., Benskey, M. J., Fischer, D. L., Kemp, C. J., Steece-Collier, K., et al. (2016). Impact of age and vector construct on striatal and nigral transgene expression. *Mol. Ther. Methods Clin. Dev.* 3:16082. doi: 10.1038/mtm.2016.82

Rizzo, G., Copetti, M., Arcuti, S., Martino, D., Fontana, A., and Logroscino, G. (2016). Accuracy of clinical diagnosis of Parkinson disease: a systematic review and meta-analysis. *Neurology* 86, 566–576. doi: 10.1212/WNL.0000000000002350

Salvatore, M. F., Ai, Y., Fischer, B., Zhang, A. M., Grondin, R. C., Zhang, Z., et al. (2006). Point source concentration of GDNF may explain failure of phase II clinical trial. *Exp. Neurol.* 202, 497–505. doi: 10.1016/j.expneurol.2006.07.015

Salvatore, M. F., Zhang, J. L., Large, D. M., Wilson, E., Gash, C. R., Thomas, T. C., et al. (2004). Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. *J. Neurochem.* 90, 245–254. doi: 10.1111/j.1471-4159.2004.02496.x

Schuster, B., Kovaleva, M., Sun, Y., Regenhard, P., Matthews, V., Grotzinger, J., et al. (2003). Signaling of human ciliary neurotrophic factor (CNTF) revisited. The interleukin-6 receptor can serve as an alpha-receptor for CTNF. *J. Biol. Chem.* 278, 9528–9535. doi: 10.1074/jbc.M210044200

Slevin, J. T., Gerhardt, G. A., Smith, C. D., Gash, D. M., Kryscio, R., and Young, B. (2005). Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. *J. Neurosurg.* 102, 216–222. doi: 10.3171/jns.2005.102.2.0216

Su, X., Fischer, D. L., Li, X., Bankiewicz, K., Sortwell, C. E., and Federoff, H. J. (2017). Alpha-synuclein mRNA is not increased in sporadic PD and alpha-synuclein accumulation does not block GDNF signaling in parkinson's disease and disease models. *Mol. Ther.* 25, 2231–2235. doi: 10.1016/j.ymthe.2017.04.018

Tenenbaum, L., and Humbert-Claude, M. (2017). Glial cell line-derived neurotrophic factor gene delivery in parkinson's disease: a delicate balance

between neuroprotection, trophic effects, and unwanted compensatory mechanisms. *Front. Neuroanat.* 11:29. doi: 10.3389/fnana.2017.00029

Tolosa, E., Wenning, G., and Poewe, W. (2006). The diagnosis of Parkinson's disease. *Lancet Neurol.* 5, 75–86. doi: 10.1016/S1474-4422(05)70285-4

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. *Science* 347:1260419. doi: 10.1126/science.1260419

Whone, A., Luz, M., Boca, M., Woolley, M., Mooney, L., Dharia, S., et al. (2019). Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson's disease. *Brain* 142, 512–525. doi: 10.1093/brain/awz023

Whone, A. L., Boca, M., Luz, M., Woolley, M., Mooney, L., Dharia, S., et al. (2019). Extended treatment with glial cell line-derived neurotrophic factor in Parkinson's Disease. *J. Parkinsons Dis.* 9, 301–313. doi: 10.3233/JPD-191576

Zhang, Z., Shen, Y., Luo, H., Zhang, F., Peng, D., Jing, L., et al. (2018). MANF protects dopamine neurons and locomotion defects from a human alpha-synuclein induced Parkinson's disease model in *C. elegans* by regulating ER stress and autophagy pathways. *Exp Neurol.* 308, 59–71. doi: 10.1016/j.expneurol.2018.06.016

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