NEUROTROPHINS BIODELIVERY TO CNS: INNOVATIVE APPROACHES FOR DISEASE-MODIFYING THERAPY

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PUBLISHED IN: Frontiers in Neuroscience and Frontiers in Pharmacology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-303-0
DOI 10.3389/978-2-88976-303-0

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NEUROTROPHINS BIODELIVERY TO CNS: INNOVATIVE APPROACHES FOR DISEASE-MODIFYING THERAPY

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Citation: Triaca, V., Imbimbo, B. P., Nistico, R., eds. (2022). Neurotrophins Biodelivery to CNS: Innovative Approaches for Disease-Modifying Therapy. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-303-0

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frontiers | Frontiers in Neuroscience

EDITORIAL
published: 10 May 2022
doi: 10.3389/fnins.2022.916563

Editorial: Neurotrophins Biodelivery to CNS: Innovative Approaches for Disease-Modifying Therapy

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Keywords: neurotrophins, neurodegenerative diseases, blood-brain barrier, focused ultrasound, intranasal route

Editorial on the Research Topic

Neurotrophins Biodelivery to CNS: Innovative Approaches for Disease-Modifying Therapy

Neurodegenerative diseases (NDs) are expected by 2050 to rise to 300% worldwide. Patients affected by Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington Disease (HD), and Amyotrophic Lateral Sclerosis (ALS) experience gradual and progressive neurons loss and neuronal function deterioration, which frequently result in cognitive dysfunctions and severe impairment of activities of daily living. Due to the unknown etiology of these neurological disorders and the difficulty of an early diagnosis, we are currently experiencing a lack of effective and disease-modifying treatments (Erkkinen et al., 2018).

Neurotrophins (NTs) belong to a class of growth factors which regulate survival, development and function of neurons through the Trk-p75 NTR receptors system (Chao and Hempstead, 1995). Secreted neurotrophic factors act by preventing the target neurons from initiating programmed cell death, thus allowing the neurons to survive (Patapoutian and Reichardt, 2001). NTs also induce differentiation of progenitor cells to form neurons. Although the vast majority of neurons in the mammalian brain are formed prenatally, parts of the adult brain (for example the dentate gyrus of the hippocampus and subventricular zone) retain the ability to grow new neurons from neural stem cells, a process known as adult neurogenesis. NTs are able to help to stimulate and control neurogenesis (Park and Poo, 2013).

The progressive deficient activity of neurotrophic factors like nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) in different neurodegenerative diseases has prompted intensive research to identify neurotrophic-based disease modifying therapies (Allen et al., 2013). BDNF has been reported to affect cognitive activity via its specific receptor tyrosin kinase B (TrkB) in a variety of neurological and psychiatric disorders (Lu et al., 2014), including PD (Stefani et al.) and chronic social stress (Cui et al.).

NTs and their pro-forms, namely the proneurotrophins (ProNTs), exert opposite effects on adult neurons, with the former generally being neuroprotective and the latter promoting a degenerative pattern (Patapoutian and Reichardt, 2001; Lu et al., 2005). In line, the inhibition of the BDNF precursor ProBDNF attenuates inflammation and encephalopathy in an animal paradigm of CNS sepsis (Jiang et al.).

NGF plays a major role in the maintenance of cholinergic baso-cortical and baso-hippocampal circuits involved in memory and higher cognition, and is considered a critical molecule for integrity and function of cholinergic neurons during development and adulthood. Numerous studies have also shown that NGF contributes to the survival and regeneration

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *09 April 2022*
Accepted: *12 April 2022*
Published: *10 May 2022*

Citation:
Triaca V, Imbimbo BP and Nisticò R (2022) Editorial: Neurotrophins Biodelivery to CNS: Innovative Approaches for Disease-Modifying Therapy. Front. Neurosci. 16:916563. doi: 10.3389/fnins.2022.916563

of neurons during aging and in age-related diseases such as AD (Cuello et al., 2019). Changes in neurotrophic signaling pathways are involved in the aging process and contribute to cholinergic and cognitive decline observed in AD (Volosin et al., 2006). Based on these biological properties, NGF has been proposed to restore cognition, halt neuroinflammation, and revert degeneration not only in AD, but also in Down's syndrome, neurotrauma, as well as in eye diseases, like macular degeneration (Alastra et al.; Capsoni and Cattaneo; Manni et al.; Amadoro et al.).

A mutated form of NGF has been proposed as a valuable neurotrophic substitute of the wild-type molecule without its pain-related side effects (Capsoni and Cattaneo).

Nonetheless, NGF- and BDNF-based clinical trials for AD and ALS, respectively, have failed probably due to poor brain penetration and insufficient target engagement. So far, the poor pharmacokinetic properties of neurotrophins render their use for the treatment of CNS disorders limited. To overcome this issue, in the last 15 years, the nasal route of administration for neurotrophins and peptidomimetics has been widely attempted in animal and human settings with promising preliminary results (Tessarollo and Yanpallewar; Alastra et al.; Manni et al.).

Several small neurotrophic agents that efficiently cross the blood-brain barrier (BBB) have been designed and tested in preclinical and clinical research. Recently, a European consortium, the first of its kind, has been intended to foster the study of neurotrophin mimetics in neuroinflammation and neurodegenerative diseases [EuroNeurotrophins, 2018-2022, H2020-MSCA-ITN-2017 (ETN), doi 10.3030/765704]. Among these molecules, the most promising are specific blockers of the p75NTR receptors. Additional compounds designed to activate Trk receptors signaling have been demonstrated to improve synaptic loss and memory deficits (Longo and Massa, 2013). Also, the TrkB.T1 truncated adult form of TrkB has gained attention for the distinct BDNF actions in brain physiopathology, pinpointing both epigenetic and splicing-related regulatory control of the neurotrophic pathway even beyond the nervous system (Tessarollo and Yanpallewar).

In the last decade, neuroscientists resorted to the nose-to-brain route of drugs delivery as non-invasive approach to bypass the BBB. Different forms of nanocarriers, including liposomes, nanopolymers, and emulsions have been attempted to transport drugs directly into the brain. However, and despite the potential of nasal delivery, optimal pharmacokinetics and target engagement are still waiting for significant optimization (Barbato et al.).

The transcranial high intensity focused ultrasound (FUS) is a ground-breaking technology for ablative surgery. FUS technology has been approved by FDA for the treatment of essential tremors and Parkinson's disease-related tremors. At lower intensity, FUS can stimulate or inhibit neural activity with potential applications in clinical practice for epileptic seizures and psychiatric disorders, as well as for chronic pain management by temporarily blocking nerves. Recently, preclinical studies demonstrated how low intensity FUS can transiently and finely regulate the BBB opening, thereby facilitating the brain penetrance of drugs especially when combined with intranasal drug delivery (Barbato et al.; Ji et al., 2019). FUS technology could therefore represent a game changer in the long-running battle against several neurodegenerative diseases with an emphasis on treating AD and PD.

CONCLUSIONS

Compelling evidence accumulated over the last two decades strongly pinpoint neurotrophins as neuroprotective and possibly disease-modifying treatments for the treatment of neurodegenerative diseases. Despite this growing hope, neurotrophins-based therapies are still suffering from major biological limitations including poor pharmacokinetic properties and very low penetration rates through the BBB. The exploitation of a new and adaptable technology like FUS, which allows non-invasive, efficient, and focused drug delivery to the brain, would possibly open a new era in the treatment of developmental and age-related deadly illnesses of the CNS.

There is promise for brain therapy. It will be up to the scientific community and drug developers to take a chance on it.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the editorial conceptualization and writing, approved the submitted version.

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Conflict of Interest: BI is an employee at Chiesi Farmaceutici. He is listed among the inventors of a number of Chiesi Farmaceutici's patents of anti-Alzheimer drugs.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Neuroscience

REVIEW
published: 15 July 2021
doi: 10.3389/fnins.2021.695592

Nerve Growth Factor Biodelivery: A Limiting Step in Moving Toward Extensive Clinical Application?

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *15 April 2021*
Accepted: *21 June 2021*
Published: *15 July 2021*

Citation:
Alastra G, Aloe L, Baldassarro VA, Calzà L, Cescatti M, Duskey JT, Focarete ML, Giacomini D, Giardino L, Giraldi V, Lorenzini L, Moretti M, Parmeggiani I, Sannia M and Tosi G (2021) Nerve Growth Factor Biodelivery: A Limiting Step in Moving Toward Extensive Clinical Application? Front. Neurosci. 15:695592. doi: 10.3389/fnins.2021.695592

Nerve growth factor (NGF) was the first-discovered member of the neurotrophin family, a class of bioactive molecules which exerts powerful biological effects on the CNS and other peripheral tissues, not only during development, but also during adulthood. While these molecules have long been regarded as potential drugs to combat acute and chronic neurodegenerative processes, as evidenced by the extensive data on their neuroprotective properties, their clinical application has been hindered by their unexpected side effects, as well as by difficulties in defining appropriate dosing and administration strategies. This paper reviews aspects related to the endogenous production of NGF in healthy and pathological conditions, along with conventional and biomaterial-assisted delivery strategies, in an attempt to clarify the impediments to the clinical application of this powerful molecule.

Keywords: nerve growth factor, nanomedicine, drug delivery, electrospinning, hydrogels

INTRODUCTION

Since its discovery in the 1950s (Levi-Montalcini, 1987) and the award of the Nobel Prize to Rita Levi-Montalcini and Stanley Cohen for their discoveries of growth factors in 1986, the number of basic science discoveries and preclinical studies supporting the use of nerve growth factor (NGF) for therapeutic purposes has constantly increased over the years, principally for neurodegenerative diseases (Alzheimer's disease, AD; and Parkinson's disease, PD) and brain injuries (perinatal hypoxia/ischemia, traumatic brain, and spinal cord injury), but also retinopathies, optic nerve degeneration, and peripheral neuropathies associated with diabetes and HIV. The potential applications have also extended from the nervous system as the primary target to an increasing number of tissues and organs, including epithelial tissue, parenchymal organs, and the osteoarticular system (Manni et al., 2013; Rocco et al., 2018), as well as inflammation (Skaper, 2017) and cancer (Griffin et al., 2018).

The road toward the clinical translation of NGF, however, has encountered numerous obstacles. In spite of the success of Genentech in translating NGF production from male mouse salivary gland extract (Bocchini and Angeletti, 1969) to the recombinant technology of the human form (Altar et al., 1991; Rogers, 1996), results from early clinical trials led Genentech to terminate the rhNGF (recombinant human NGF) project. This was based on side effects observed in two sets of phase

II clinical trials, suggesting that despite the efficacy of rhNGF administration at ameliorating the symptoms associated with both diabetic polyneuropathy and HIV-related neuropathy, side effects were dose limiting for NGF (Apfel, 2002). Moreover, a large-scale phase III clinical trial of 1019 patients randomized to receive either rhNGF or placebo for 48 weeks failed to confirm these earlier indications of efficacy (Apfel, 2002). Intravenous (IV), subcutaneous (SC), and intradermal (ID) NGF injection induces myalgia, mechanic and thermal hyperalgesia, emerging rapidly after injection and lasting for weeks (Mizumura and Murase, 2015). As concerns human studies, SC or intracerebroventricular (ICV) administration of rhNGF to healthy subjects or patients with diabetic polyneuropathy, HIV-associated peripheral neuropathy (SC, 0.03–1 μg/kg), AD (ICV, 75 μg/day for 3 months), PD (ICV, 3.3 mg infused over 23 days) or hypoxic-ischemic perinatal brain injury (0.1 mg/day for 10 days) (reviewed by Tria et al., 1994; Mizumura and Murase, 2015) always produced hyperalgesia at the injection site, and in some cases also mild to moderate-severe transient muscle pain (Petty et al., 1994; Rogers, 1996) (clinical trial NCT00000842).

Actually, the current evidence regarding the painful side effects of NGF administration is taking pharmacological research in two new directions: development of humanized anti-NGF monoclonal antibodies (anti-NGF mAbs) for conditions as osteoarthritis, lower back pain, and interstitial cystitis (Wise et al., 2021), and synthesis of TrkA ligands in an attempt to overcome this severe and limiting side effect (Carleton et al., 2018; Bagal et al., 2019). The rhNGF has finally received FDA approval as Cenegermin® eye drops by Dompé, first-in-class with the potential to completely heal rare neurotrophic keratitis (clinical trials NCT04293549, NCT03836859, NCT02101281, NCT03019627).

However, the major obstacles to clinical translation of NGF are also due to other factors, such as the biodistribution of this large molecule, including its crossing of blood-tissue barriers, and to issues of dosage, since NGF is produced by many different cell types (Gostynska et al., 2020), and because endogenous NGF production is altered in many of the pathologies included in a tentative list of potential targets for the NGF drug.

This review addresses some of these major issues affecting the development of innovative NGF delivery solutions, discussing possible reasons for their success, and in many cases their failures. Sections "Parenteral Administration" and "Topical Application" refer to both preclinical and clinical studies, these latter also indicated by the respective clinicaltrials.gov code; section "Biomaterial-Assisted Delivery" refers to *in vitro* and preclinical studies.

PARENTERAL ADMINISTRATION

NGF Endogenous Levels, Biodistribution and Metabolism

In the body, NGF is produced according to a delicate balance that varies from tissue to tissue also according to specific diseases and pathological states (Lorenzini et al., 2021), and that can be reflected by NGF blood levels

There is little data available on the biodistribution of exogenously administered NGF. In the initial human applications and clinical trials, mouse NGF or recombinant human NGF (rhNGF) was SC (Petty et al., 1994; Rogers, 1996; Apfel et al., 1998, 2000; McArthur et al., 2000; Schifitto et al., 2001) (clinical trial NCT00000842) or ICV administered (Olson et al., 1992; Jönhagen et al., 1998; Chiaretti et al., 2005, 2008), but basic information on absorption, distribution and excretion following parenteral administration derives from animal studies, in particular in adult rats and in cynomolgus monkeys. In this few biodistribution studies, mouse NGF was administrated intravenously (IV) and SC in rats as a single injection (35 μg/kg, single dose) or by continuous infusion via osmotic mini-pump (50–450 μg/pump) (Tria et al., 1994). In monkeys, rhNGF was administered SC (2 mg/kg), and pharmacokinetic analysis was conducted after single and multiple doses (for 15 days, every other day) (Nguyen et al., 2000). In both studies, the maximum plasma concentrations (C_{max}) confirmed that the drug is absorbed after SC administration. In rats, the maximum blood concentration of NGF after SC administration was 65-fold lower than after IV injection. The calculated time to reach maximum plasma concentrations (T_{max}) are very similar in two studies, despite the different doses employed. Multiple dosing in monkeys shifts the T_{max} from a mean value of 2.5 to 3.3 h (Nguyen et al., 2000; Tria et al., 1994).

Subcutaneous administration via osmotic mini-pump (450 μg/pump, corresponding to 37.5 μg/day) in rats resulted in detectable NGF plasma levels after 6 h, reaching peak values during day 1, confirming the T_{max} delay after multiple dosing (Tria et al., 1994). IV injection allows calculation of the half-life distribution phase ($t_{1/2\alpha}$), reached in 5–6 min, indicating a rapid disappearance from plasma, probably due to the binding of NGF to the α2-macroglobulin cleared from blood by hepatocytes (Tria et al., 1994). With regard to NGF metabolism, no degradation products were observed in plasma after immunoprecipitation and SDS-PAGE, suggesting a long-term stability of the protein (Tria et al., 1994; Nguyen et al., 2000). **Table 1** summarizes the basic pharmacokinetic parameters following comparable administration routes.

Data regarding tissue distribution were obtained following the administration of radiolabeled rhNGF (^{125}I-rhNGF) in primates (multiple dosing, for 15 days, every other day). The large central volume of distribution (V_2/F, 827 ml/kg) indicated distribution

TABLE 1 | Main PK parameters evaluated in animal studies, following comparable single subcutaneous injection (data from Tria et al., 1994; Nguyen et al., 2000).

	Monkeys	Rats
	rhNGF	mNGF
Dose	2 mg/kg	35 μg/kg
C_{max} (ng/ml)	1300 ± 120	3.57 ± 0.33
T_{max} (h)	2.5 ± 2.7	3.20 ± 0.49
$t_{1/2\beta}$ (h)	4.1 ± 1.0	4.47 ± 0.15

C_{max}, maximum plasma concentration; T_{max}, time to reach maximum plasma concentration; $t_{1/2\beta}$, elimination phase half-life.

in extravascular tissues. The organs were then collected at 8 and 24 h following dose 1 and dose 15. In non-neuronal tissues ^{125}I-rhNGF was detected at both time points and doses in all studied tissues, particularly in the thyroid, adrenals, kidneys, liver, spleen, peripheral and axillary tissues, and at the injection site. As expected, the radiolabeled drug was observed in the peripheral nervous system, whereas it was minimal in the spinal cord, and absent in the brain (Nguyen et al., 2000).

The plasma elimination half-life ($t_{1/2\beta}$) is very close both in rats and in monkeys (**Table 1**) and can be extended by varying the administration route, e.g., 4.47 h in SC versus 2.30 h in IV injection in rats. The administration schedule does not appear to affect this parameter, e.g., 4.1 h versus 4.8 h following single and multiple dosing. The clearance values (Cl) are not affected by administration route, e.g., 6.38 ml/min/kg in SC versus 6.93 ml/min/kg in IV injection in rats, but decrease following multiple administration, e.g., 140 ml/kg/hr versus 63 ml/kg/hr following single and multiple (Tria et al., 1994; Nguyen et al., 2000).

With regard to NGF metabolism, analysis of radiolabeled NGF in monkeys shows that urinary excretion represents the main route of elimination, although traces of radioactivity were found in the feces. No difference in elimination pattern was observed between 24 and 120 h following single and multiple dosing (Tria et al., 1994; Nguyen et al., 2000). There is also very little data available on specific NGF metabolic products, derived from immunoprecipitation and SDS-PAGE in monkey tissue lysates. In non-neuronal tissues, low molecular mass bands were observed in the kidney, liver and spleen, indicating that intensive metabolism occurs in these organs. SDS-PAGE from lysate of sympathetic ganglia and dorsal root ganglia also show intense NGF metabolism, while material present in the peripheral nerves (radial, sciatic, and tibial) was mostly negative (Tria et al., 1994; Nguyen et al., 2000).

NGF and the Blood Brain Barrier

Peripheral administration of NGF to target the CNS is limited by the poor ability of this molecule to cross the blood-brain barrier (BBB), and by peripheral enzymatic degradation. The unique properties of the BBB stem from CNS capillary histology, where endothelial cells are held together by tight junctions which limit the paracellular flux of solutes, and the presence of specific transporters which regulate the passage of molecules to the CNSs. Endothelial cells are covered by mural cells (pericytes and smooth muscle cells) which contribute to the dynamics of BBB control, and the microvascular tube is also surrounded by the inner vascular and the outer parenchymal basement membrane, providing an anchor for many signaling processes and an additional barrier for cells and molecules accessing the CNS. The blood vessels also interact with different immune cells, mainly perivascular macrophages, and microglial cells, representing the first line of innate immunity. Lastly, the direct bridge from the microvessel to the neurons is the astrocyte, a glial cell with extending processes which completely envelop the vascular tube, connecting the microvessels to the neurons (Daneman and Prat, 2015).

While the restrictive nature of the BBB allows for proper neuronal function and protection of the neural tissue, and maintains CNS homeostasis, it also constitutes an obstacle for drug delivery. Whether NGF can penetrate the BBB and be absorbed by the brain tissue, and under what conditions, is still controversial, but the poor permeability of NGF through the BBB under physiological conditions has been widely described (Pan et al., 1998). However, the BBB is not a fixed structure, undergoing pathophysiological adaptations which are not yet fully understood. Although BBB formation starts during the embryonic stage, soon after vessel formation in the developing CNS (E11, in rats), the system continues to mature following birth, increasing the strength of the paracellular barrier and expression of the efflux transporter (Blanchette and Daneman, 2015), while pathological conditions, particularly inflammation, are known to modify its structure and dynamics. In general, different diseases (e.g., stroke, multiple sclerosis, epilepsy) are characterized by the internalization and down-regulation of tight junctions, increased rates of transcytosis, increased expression of adhesion molecules for leukocytes leading to increased leukocyte extravasation, degradation of the basal membrane, and reduced microvessel coverage by pericytes and astrocytes (Profaci et al., 2020). Specific pathologies may therefore offer time window opportunities for exploiting altered BBB permeability and increasing NGF transportation to the CNS.

Strategies to overcome the BBB for the CNS delivery of large molecules as NGF represent a major goal, driving alternative routes of administration [see section "Intranasal (IN)" and "Eye Drops"] and pharmaceutical technologies as nanocarrier (see section "Nanomedicines for NGF Delivery").

TOPICAL APPLICATION

Intranasal (IN)

Due to its large surface area, the high degree of vascularization, and the "nose-to-brain" pathways, the nasal cavity is an interesting portal for systemic delivery and to by-pass the BBB. The advantages of nose-to-brain drug delivery include safety and avoidance of the hepatic first pass metabolism, as well as its non-invasive nature and high patient compliance (Colombo et al., 2011). Limitations include the possible dosing volume through the nasal cavity and the consequent total amount of drug delivered systemically or into the brain (Dong, 2018), active mucociliary clearance of the mucosa, short retention time for drug absorption, low permeability for hydrophilic drugs, and low central nervous system (CNS) delivery for proteins (Erdõ et al., 2018). For these reasons, many strategies are currently being tested to enhance drug transport and distribution through the "nose-to-brain" pathways.

To give a brief anatomical overview, the nasal cavity consists of three anatomical areas, the nasal vestibule, respiratory region, and olfactory region, each characterized by different mucosal epithelia. **Figure 1** shows the cell composition of the olfactory part of nasal cavity, mucosa, olfactory epithelium and lamina propria, including the detail of the NGF, TrkA, and p75^{NTR} expression on different cell types.

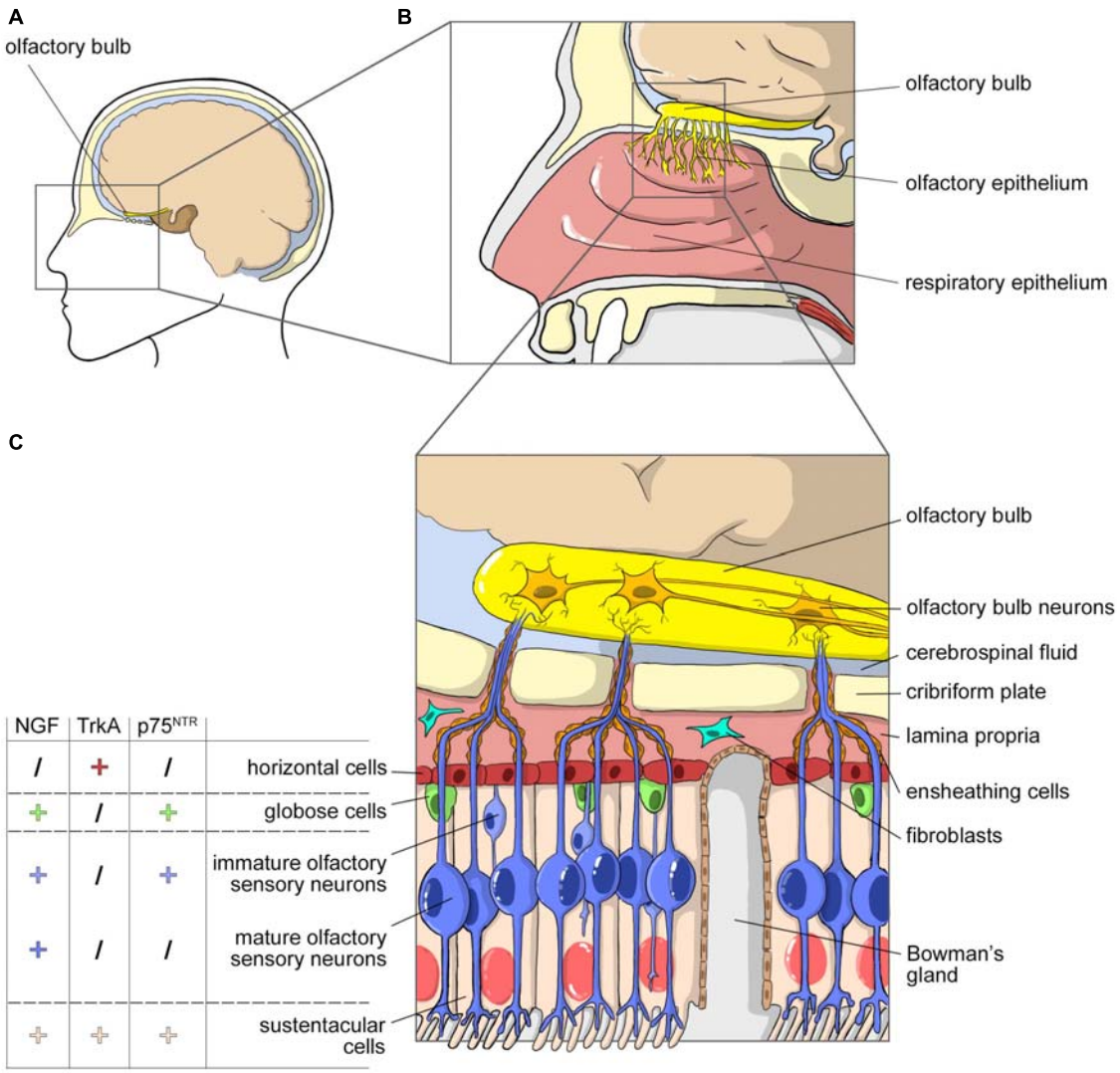

FIGURE 1 | NGF and olfactory system. **(A)** Sagittal view of the head with highlighted the area occupied by the nasal cavity. **(B)** Magnification of the nasal cavity with, in the square, the area occupied by the olfactory epithelium. **(C)** Cell composition of the olfactory part of the nasal cavity, mucosa, olfactory epithelium, and lamina propria. The olfactory epithelium is surrounded by layer globose and horizontal basal cells. It consists in many cellular types: sustentacular cells, Bowman's glands producing mucus, immature olfactory neurons and mature olfactory sensory neurons that project their axons toward the olfactory bulb through the cribriform plate. Axons are enclosed by olfactory ensheating cells and olfactory nerve fibroblasts. The picture includes data on the expression of NGF, TrkA and $p75^{NTR}$ in the different cellular populations of the olfactory epithelium (from Feron et al., 2008).

The respiratory epithelium is a ciliated pseudostratified columnar epithelium composed by four main cell types – ciliated and non-ciliated columnar cells, basal or horizontal cells and goblet cells – with high vascularization, supplied by the arterial branch of the maxillary artery. A mucus gel layer covers the epithelium, that, together with ciliary tip movements, constitutes the first protective barrier against inhaled particulates and irritants. The respiratory mucus layer is renewed every 10–20 min (Pardeshi and Belgamwar, 2013).

While the respiratory region is mainly involved in systemic drug absorption, the olfactory area is important not only for the ability of its neurons to provide the sense of smell, but also for the "nose-to-brain path," which delivers drugs directly into the brain. The olfactory mucosa consists of a ciliated chemosensory pseudostratified columnar epithelium that contains three types of cells – olfactory sensory neurons, immature olfactory sensory neurons and supporting (or sustentacular) cells – all connected by tight junctions (**Figures 1A,B**). The cilia are non-motile, and the overlying mucus gel has a very slow turnover (several days).

The olfactory mucosa presents the main inter-species anatomical differences, an aspect which must be considered when translating animal data to humans. In humans, this mucosa covers 10% of the total surface area, while in rodents, the most widely used species for intranasal administration studies, it can constitute up to 50% of the total area. This is an important aspect, because results obtained from animal models do not always

correlate with those of humans, a discrepancy which probably comes from an insufficient consideration of the anatomical and physiological differences between the respective nasal cavities (Cho et al., 2010). Rodents are more widely used for preliminary nose-to-brain drug absorption studies, while rabbits and dogs are used for pharmacokinetic studies.

Drugs transferred from the olfactory mucosa to the CNS bypassing the BBB follow two pathways, olfactory and trigeminal, with molecular transfer taking place outside or within the nerve axon. The olfactory path includes the neuronal cells of the olfactory epithelium, and the lamina propria and the olfactory bulb in the CNS. The olfactory bulb then projects to the cortex, amygdala and hypothalamus, providing an anatomical link between nasal administration and the brain structures (Khan et al., 2017). The trigeminal path consists of the trigeminal nerve with its three major branches, ophthalmic, maxillary, and mandibular, thus promoting the entrance of drugs to the caudal and rostral parts of the brain. The olfactory path delivers drugs to the rostral areas of the brain only, whereas the trigeminal pathway delivers to both the rostral and caudal areas.

Following drug administration into the nasal cavity, the first step of absorption is the passage through the mucus layer and ciliary movement. After crossing this barrier, several mechanisms are involved in the transmucosal transfer, such as the paracellular pathway (the passive transport of molecules between cells), or the transcellular pathway (active transport of the drug across the cells). Carrier-mediated transport, transcytosis, and transport through the intercellular tight junctions are other possible pathways.

The entry of a wide range of molecules into olfactory sensory neurons via an intracellular mechanism such as pinocytosis or receptor-mediated endocytosis was first demonstrated for BDNF (Deckner et al., 1993), and more recently for other drugs such as ribavirin, an antiviral drug potentially useful for the treatment of viral infections in both humans and animals (Colombo et al., 2011; Giuliani et al., 2018). This is the mechanism used by many viruses such as poliovirus or herpesvirus, as well as by the latest example, the SARS-CoV-2 virus. Following internalization in olfactory neurons, the molecules (or viruses) run down the soma via retrograde axonal transport. Neuronal transport is considered a slow process. For example, intranasal delivery of 70 μg radiolabeled BDNF, CNTF, NT-4, or erythropoietin (EPO) resulted in 0.1–1.0 nM neurotrophin concentrations within 25 min in brain parenchyma (Alcalá-Barraza et al., 2010). Intranasal studies using labeled IGF-1 suggest that the rapid distribution toward the CNS (∼30 min) is due to extracellular convection or intracellular transport rather than to diffusion (Thorne et al., 2004). Other studies report 45 min for the axonal transport phase (Crowe et al., 2018).

Despite the presence of tight junctions (TJs), the use of intercellular spaces has been hypothesized. These spaces are generated by channels through which proteins, peptides (such as insulin, IGF-1, albumin) and even stem cells can reach the CNS, as demonstrated in the nasal mucosa, and by a transient loosening of the BBB by decreasing expression of TJ proteins such as claudin-1, occludin, and tricellulin (Jackson et al., 2017).

Although interest in this delivery route for preclinical and clinical studies is increasing, very few studies of NGF pharmacokinetics or biodistribution are available. In a study on the Sprague Dawley rat hippocampus, for example, the bioavailability of intranasally administered NGF with or without chitosan was ∼14 fold greater than the group treated with NGF without chitosan (Vaka et al., 2009). In a preclinical model of AD, polymeric nanoparticles appear to be promising carriers for the nose-to-brain delivery of drugs (Rabiee et al., 2021). Following IN administration, rhNGF reached the brain within an hour, achieving a concentration of 3400 pM in the olfactory bulb, 660–2200 pM in other brain regions and, 240 and 180 pM in the hippocampus and the amygdala, respectively, while, little or no rhNGF was found in the brain following IV administration (Chen et al., 1998). The therapeutic efficacy of IN NGF administration has also been evaluated in many other brain diseases, and in clinical trials of traumatic brain injury, acute ischemic stroke and frontotemporal dementia (Eftimiadi et al., 2021). Notably, no systemic or local side-effects have been described in clinical trials using IN NGF administration in both adult (10 μl of NGF at 200 μg/ml concentration, daily, for a 1-year period) (de Bellis et al., 2018) and pediatric patients (0.1 mg/kg, three times daily for 7 consecutive days) (Chiaretti et al., 2020).

Eye Drops

The eye is regarded as one of the main therapeutic targets for NGF topical treatments. Local application of NGF exerts a healing action on corneal and cutaneous ulcers associated with pathological conditions such as inflammation, diabetes and rheumatoid arthritis (Aloe et al., 2008), and the use of NGF as a drug in ophthalmology is the best characterized and developed clinical use of this neurotrophin (Eftimiadi et al., 2021). Since the initial discovery that goldfish retinal cells are receptive to NGF action (Turner and Delaney, 1979), many studies have shown the potential therapeutic use of NGF to treat ophthalmic diseases (Aloe et al., 2012), leading to a number of pre-clinical research studies and clinical trials on different eye-related pathologies (Aloe et al., 2012; Manni et al., 2013).

The most recent research into NGF treatments has focused on neurotrophic keratitis, dry eye disease, optic neuropathy and optic pathway glioma (Eftimiadi et al., 2021), and the treatment of corneal ulcers of different etiologies, treated by topical NGF application in more than 200 patients, is of major interest (Lambiase et al., 2012). However, it was only in 2018, following 30 years of clinical trials (Bonini et al., 2018), that research finally led to the approval of a rhNGF produced in bacteria, named Cenegermin (Oxervate^{TM}; Dompè Farmaceutici SpA, Milan, Italy) for the treatment of neurotrophic keratitis. The topical administration of NGF leads to complete corneal healing (Bonini et al., 2018; Deeks and Lamb, 2020; Pflugfelder et al., 2020), without inducing the development of pain and circulating anti-NGF antibodies (Lambiase et al., 2007a).

But the retinal cells in the eye are part of the CNS and constitutes the visual system together with the brain areas receiving retinal input. The retina, which is part of the posterior segment, is composed of different layers of nerve cell bodies organized in nuclear and synaptic layers, transforming light into

nerve signals. From the retina, the retinal ganglion cell (RGCs) axons form the nerve fibers which converge in the optic disk and form the optic nerve.

Thanks to this neural connection to the brain, the topical application of NGF on eye is also regarded as a delivery route to the brain. In fact, and in addition to innervating primary visual areas, RGCs also extend their projections to the hypothalamus and direct/indirect projections to different limbic structures including the hippocampus and the septum (Tirassa et al., 2018; Murcia-Belmonte and Erskine, 2019; Eftimiadi et al., 2021). **Figure 2** shows the structures of the visual system and in particular of the eye (**Figures 2A,B**), including the detail of the cell composition of the retina and the expression of NGF, TrkA and $p75^{NTR}$ in the different cell populations (**Figure 2C**).

Although the eye has a number of anatomical and physiological barriers which limit the absorption and transport of molecules, topical application is nevertheless highly appealing. Although bioavailability and efficacy after this route are lower than a number of injection routes in different eye compartments (intravitreal, subconjunctival, and retrobulbar), different drugs are still capable of reaching the posterior segment of the eye. Topical application also reduces the chance of systemic side effects, and the drug can even be self-administered as eye drops.

The administration of NGF to the target areas of the brain via the ophthalmic route is theoretically hindered by the molecular weight of the active form of NGF (14.5 KDa) which does not permit its passage through the cornea. However, NGF is unexpectedly absorbed, albeit at a low concentration, reaching the retina, optic nerve and finally the brain (Lambiase et al., 2005; Di Fausto et al., 2007; Lambiase et al., 2007b) via different paths. From the optical surface, several routes direct the transport of the molecule to the posterior segment (Maurice, 2002; Koevary, 2005). NGF receptors are highly expressed throughout the visual system (Wang et al., 2014), and its voyage starts by binding the high affinity receptor TrkA in the anterior part of the eye (Roberti et al., 2014).

Nerve growth factor appears to be transported mainly by the *trans*-conjunctive/*trans*-sclera pathways, although systemic absorption and passage through the retrobulbar space have also been hypothesized (Maurice, 2002; Koevary et al., 2003).

Following the passage from the anterior to the posterior part of the eye, the cells in the retina and the RGCs transport NGF along their axons via anterograde or retrograde mechanisms (Carmignoto et al., 1991), indeed anterograde transport and systemic absorption may explain the increased levels of the molecule in the contralateral eye. NGF eye drops also induce c-Fos in the neurons of the primary visual areas of the CNS, supraoptic and paraventricular nuclei, hippocampus, frontal cortex and amygdala, indicating that all the retinal pathways are activated and that NGF also acts through post-synaptic modulation of cells localized in different brain areas which receive the retinal signals, either directly or indirectly (Tirassa et al., 2018).

An animal study on rats using radiolabeled NGF demonstrated that following eye drop administration, the molecule is present in the conjunctiva, sclera, choroid, retina and optic nerve. In the retina and the optic nerve, NGF was detected as early as 2 h after administration, reaching maximum level at 6 h and disappearing from the eye tissues after 48 h (Lambiase et al., 2005).

Ocular and intranasal application, with their ease of delivery, offer attractive alternatives to the systemic delivery of NGF, bypassing the BBB (Frey et al., 1997; Thorne and Frey, 2001; Aloe et al., 2014). A drawback is the low delivery efficiency. Moreover, the specificity of the treatment is uncertain and highly variable, with unpredictable, albeit minimal systemic effects.

Skin

Topical NGF applications also include the skin, where it acts locally and is highly effective in wound healing promotion. The cellular actors involved in epithelial tissue repair (keratinocytes, dermal fibroblasts, and myofibroblasts) are cells which produce or respond to NGF, expressing the TrkA high-affinity receptor (Palazzo et al., 2012; Matsumura et al., 2015; Samarasena et al., 2015). In this context, NGF also exerts an angiogenic action on endothelial cells (Calzà et al., 2001; Nico et al., 2008), a direct action on inflammatory and immune cells (Minnone et al., 2017), and a direct effect on the thinly myelinated Aδ- or unmyelinated C-fibers that innervate the dermis and epidermis (Indo, 2010). This is also demonstrated by the role of endogenous NGF in skin and mucosal wound healing in various animal models and human pathologies (Levi-Montalcini, 1987; Chéret et al., 2013).

Taking this evidence as a starting point, several reports have described the positive effect of NGF in epithelial wound healing, including chronic non-healing cutaneous ulcers in diabetic rodent models, where a defect of endogenous NGF is supposed (Tiaka et al., 2011). Our group demonstrated *in vitro* that NGF action is directed at the main cell types involved in wound healing (keratinocytes, fibroblasts, and endothelial cells), as well as at hyperglycemic conditions which mimic the pathological microenvironment of diabetes (Gostynska et al., 2019). We also tested the efficacy of a non-algogenic NGF derivative (hNGFP61S/R100E), named CHF6467 (Chiesi Farmaceutici). This molecule is a rhNGF containing an amino acid substitution, which removed the NGF-related hyperalgesic effect, while maintaining its ability to induce wound healing. CHF6467 treatments of pressure ulcers in diabetic mice accelerated skin repair, increasing re-epithelization, re-innervation, and re-vascularization (Giuliani et al., 2020). Our results confirmed other studies (Muangman et al., 2004), with the remarkable difference that we used a non-algogenic rhNGF, thus potentially overcoming the main limitation to the clinical application of NGF (Giuliani et al., 2020).

Besides its role in angiogenesis (Calzà et al., 2001; Ahluwalia et al., 2017; Li X. et al., 2018) and its action on skin cells (Gostynska et al., 2020), NGF may act by improving local re-innervation, fundamental to the wound healing process (Kiya and Kubo, 2019). Our transcriptomic study on the CHF6467 molecule also points to the modulation of Akt/mTOR signaling as the main driver of NGF action (Giuliani et al., 2020). This pathway is in fact in involved in the wound healing process (Huang et al., 2015; Jere et al., 2019) and is regarded as a therapeutic target (Squarize et al., 2010).

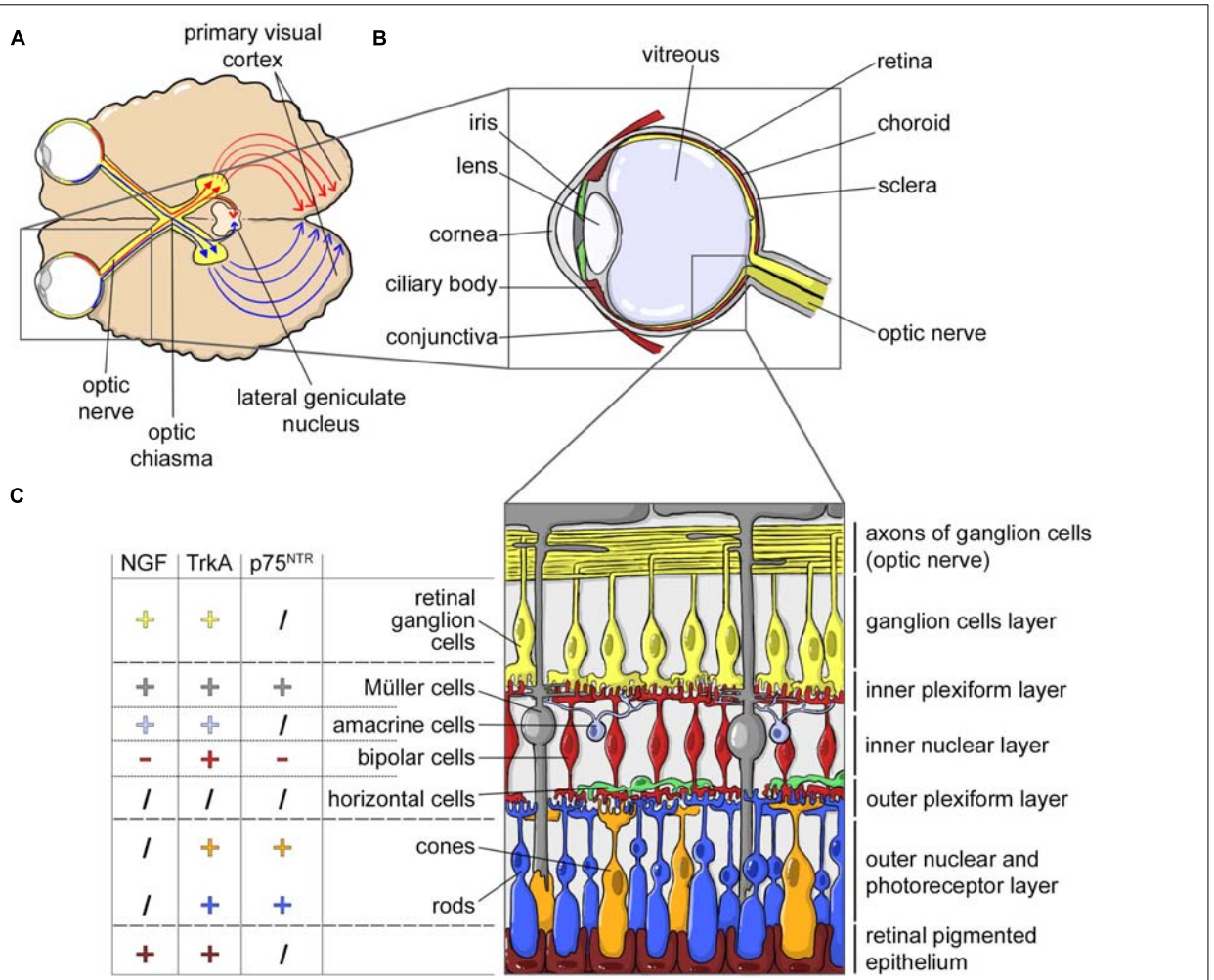

FIGURE 2 | NGF and the visual system. **(A)** Horizontal cross section of the brain, showing the optic nerves originating from retina and crossing at the optic chiasm. Each optic tract travels to its corresponding cerebral hemisphere to reach the lateral geniculate nucleus in the thalamus and to the contralateral hemisphere to reach the primary visual cortex. **(B)** Horizontal cross section of the eye showing the anterior (cornea, conjunctiva, iris, ciliary body, and lens) and a posterior (sclera, choroid, retina, and optic nerve) ocular segment, filled with the vitreous fluid. From the retina, the retinal ganglion cells axons form the nerve fibers converging in the optic disk and forming the optic nerve. **(C)** NGF, TrkA, and $p75^{NTR}$ expression in the different cellular populations of the retina (data from Garcia et al., 2017).

BIOMATERIAL-ASSISTED DELIVERY

Biomaterial-based systems (nanomedicine, hydrogels and scaffolds) are a common strategy to ameliorate drug delivery, and enormous advances have been made over the last decades to assist tissue repair and regeneration by biomaterial loading different growth factors (GF) (Lee et al., 2011). The use of biomaterials has been proposed to support macromolecule topical application and to facilitate the body's barriers crossing. For example, nanotherapeutics and nanomaterials improve the biodistribution of drugs throughout the brain for more effective treatments, not only via convection-enhanced delivery, but also via IN delivery (Keller et al., 2021).

But in spite of this progresses at the material side in controlling hydrophobicity/hydrophilicity, micro/nano-architectures, porosity, stiffness, and degradation rate, translation of materials to clinical applications is still limited due to difficulties such as scaling up reproducible manufacturing processes, the low stability of encapsulated proteins and their rapid inactivation by enzymes under physiological conditions.

Hydrogels for NGF Delivery

Hydrogels are polymers with a 3D network and a hydrophilic structure with the potential to absorb up to thousands of times their dry weight in water (Hoffman, 2002). Their unique properties, including gelation time and gelation temperature, mechanical strength, degradability together with their good affinity and compatibility with biological tissues, make hydrogels versatile materials for drug delivery and scaffolding for tissue engineering applications (Naahidi et al., 2017). The use of hydrogels as carrier materials for NGF is an important strategy to protect this protein from inactivation, ensure its sustained delivery over time, and improve its regenerative effects. Conventional hydrogels may be unsuited to wrapping NGF

due to a poor affinity to NGF, or to the lack of particular requirements such as a certain mechanical strength or shape at normal body temperature, and thermo-sensitive hydrogels may offer a valid alternative. These polymers are liquid at room temperature, changing into a 3D-network structure at normal body temperature, thus rapidly transforming from a solution to a viscoelastic gel making them particularly suitable for *in vivo* application.

Polysaccharide-Based-Hydrogels

Heparin poloxamer (HP) is a thermo-sensitive hydrogel with good affinity to NGF (Zhao et al., 2016). NGF-HP hydrogel maintains its thermosensitive nature and has a porous sponge-like structure which is ideal for carrying NGF and controlling its release. In an *in vivo* study on spinal cord injury (SCI) rat model, the NGF-HP hydrogel by *in situ* injection reduced the formation of a glial scar by inhibiting the generation of reactive astrocytes following SCI, promoting axon regeneration and inhibiting the formation of proteoglycans and collagen fibers, as well as promoting the formation of the new blood capillaries required for regeneration process. Moreover, and improvement in the locomotion performance was also observed.

Controlled delivery of multiple GFs to lesion areas is becoming an attractive strategy to achieve successful axonal regrowth following SCI. The HP hydrogel was therefore used for the delivery of both NGF and fibroblast growth factors (bFGF) (Hu et al., 2020). The release of these GFs from the hydrogel exhibited an initial rapid phase during the first week, and a slow sustained release. The GF-HP hydrogel was also used in a diabetic rat model with sciatic nerve crush injury to enhance the peripheral nerve regeneration with a single injection of GF-HP hydrogel. After 30 days, the GFs attenuated gastrocnemius muscle atrophy, and promoted the formation of myelinated axons, the proliferation of Schwann cells, and motor function recovery. However, the study lacks electrophysiology data and control experiments by single growth factor administration (Li R. et al., 2018).

Hydrogels carrying bioactive molecules can be used as cavity fillers in nerve conduits (NCs) for nerve reconstruction, in order to provide an ideal microenvironment for axonal regeneration. To promote the regeneration of a 5 mm gap in a rat facial nerve, an autologous vein was filled *in situ* with a thermosensitive Chitosan/β-glycerophosphate hydrogel loading NGF. While good functional recovery was achieved, the performance of the hydrogel was inferior to autologous nerve grafting (Cao et al., 2012). Alternatively, an electrospun conduit composed of aligned poly-L-Lactide-co-caprolactone (PCLC) nanofibers was filled with an NGF-loaded collagen/hyaluronan hydrogel (Jin et al., 2013). This NGF/PCLC/Hydrogel system enhanced neurite outgrowth from cultured dorsal root ganglia explants, compared to the plain PCLC/hydrogel. This result was not replicated *in vivo* to repair 10 mm gap in rat sciatic nerve, where no statistical difference in motor functional recovery and histomorphology were observed.

The combination of NGF with scaffolds presenting an ordered microstructure has also been employed (Singh et al., 2018). For example, an aligned open pore structure was generated inside a 3D printed conduit by directional cryogelation of a chitosan and gelatin solution, followed by physical absorption of NGF on the dried scaffold. When used in grafting a 15 mm gap in a rat sciatic nerve, these NCs showed significantly better results compared to the random scaffold, and even matched the performance of the autograft.

To treat chronically compressed nerves, a chitosan and sericin (CS-SS) scaffold cross-linked with genipin was developed for NGF delivery (Zhang et al., 2017). The round flake-like scaffolds were folded and adhered to the injured nerve after decompression in the *in vivo* rat model. The number and thickness of myelinated nerve fibers and axons increased, and atrophy and function impairment of the gastrocnemius muscle was suppressed.

Another scaffold-based strategy using hydrogels is aimed to obtain NGF concentration gradients, thus supporting axonal regeneration by adapting NGF release to the stage of the repair process. The use of such gradients *in vivo* to repair a challenging 20 mm gap in rat sciatic nerve was recently reported (Dodla and Bellamkonda, 2008). A polysulphone nerve guidance channel was filled with agarose hydrogel containing gradients of NGF and/or laminin, and nerve regeneration was evaluated in comparison with an autograft implant and an isotropic scaffold, containing a homogenous distribution of NGF and laminin. The anisotropic hydrogel with a concentration gradient in both NGF and laminin was the only one leading to an improved axonal regeneration, suggesting a synergistic effect, although the nerve autograft gave again the best results.

The ability of NGF to trigger the survival and neuronal differentiation of human adipose-derived stem cells (hADSCs) was exploited in the treatment of erectile disfunction in a rat model caused by an injury of the cavernous nerve. A biocompatible and biodegradable hydrogel composed of hyaluronic acid and polyethylene oxide was used as a delivery vehicle for both NGF and hADSCs by a single injection at the injury site. The hydrogel guaranteed a continuous release of NGF *in vitro* and led to an improved regeneration of the cavernous nerve, leading to a recovery of erectile function (Kim et al., 2013).

Other approaches have been used to exploit the biological effect of NGF without using the isolated protein itself, such as the use of NGF-overexpressing genetically modified hADSCs, which has been for example incorporated into a thermosensitive chitosan β-glycerophosphate/hydroxyethyl cellulose hydrogel to treat a spinal cord contusion in rats (Alizadeh et al., 2020).

Protein- and Peptide-Based-Hydrogels

The thermo-responsive hydrogel consisting of methoxy-poly (ethylene glycol)-*b*-poly(γ-ethyl-L-glutamate) (mPEG-PELG) was also successfully used to load NGF and obtain a controlled release (Liu et al., 2019). In a rat model, a 10 mm segment of sciatic nerve was dissected and removed, and the gap bridged using a chitosan conduit with the lumen filled of NGF/mPEG-PELG. The morphological, electrophysiological and functional analyses revealed that the chitosan scaffold with NGF/mPEG-PELG achieved superior regenerative outcomes compared to plain scaffolds or to a daily intramuscular injection of NGF.

Microporous hydrogels are another useful material. GelMA is a photo-crosslinking hydrogel composed of modified collagen components which retains cell adhesive peptide (arginyl-glycyl

aspartic acid, RGD) as well as matrix metalloprotease peptides (MMP). The GelMA hydrogel was used to create an adaptable microporous hydrogel (AMH), facilitating the formation of a stable 3D porous scaffold (Hsu et al., 2019). The adaptable microporous scaffold has cell-penetrable pore sizes and was integrated with a propagating gradient of NGF in a NC. The GelMA hydrogel loaded with NGF (NGF-G-AMH@) was implanted into the 5 mm transected sciatic nerve in SD mice. NGF-G-AMH@ directed axon outgrowth of up to 4.7 mm in 4 days *in vivo*, with well aligned axons and functional recovery within 30 days post-surgery. A gel material composed of collagen, nanohydroxyapatite and carrageenan (Col/nHA/Carr) closely mimics natural bone composition and microstructure, and provides a sustained release of human NGF-A upon loading (Wang et al., 2009). In a rabbit model of mandible distraction osteogenesis (DO), a single injection of NGF-A in a Col/nHA/Carr gel at the end of a distraction period enhanced histological and morphometric nerve parameters. A more rapid recovery from the inferior alveolar nerve injury was observed due to a sustained release of NGF from the gel, which continued to exert its biological activity for a prolonged period. However, neurophysiological and behavioral studies are needed to test the effects of the locally applied NGF/Col/nHA/Carr gel on neurosensory functions (Wang et al., 2010).

The Col/nHA/NGF construct also accelerated bone formation in the same model. Although *in vitro* release studies were not conducted, the authors hypothesized that the hydrogel system prevents biodegradation of the NGF and guarantees a sustained release *in vivo*, which, combined with the intrinsic osteoconductive action of COL/nHA, led to an improvement in bone regeneration (Chao et al., 2016).

Nerve growth factor concentration gradients have been recently achieved using a modified 3D printer apparatus to get a continuous NGF concentration gradient in a silk fibroin/collagen hydrogel then subjected to directional freezing to finally obtain a 3D scaffold displaying both biochemical gradient and longitudinally oriented microchannels. It was demonstrated that both the NGF gradient and the oriented structure synergistically promoted nerve regeneration on a 15 mm gap in rat sciatic nerve *in vivo*, accelerating functional recovery, but these results were not compared to an autograft nerve repair (Huang et al., 2020).

Amphiphilic diblock co-polypeptide hydrogels (DCH) using poly-leucine and poly-glutamate or poly-lysine can be deformed and thinnered by stress, thus injected through small-bore cannula, after which they self-assemble into rigid gel networks that degrades in about 56 days. NGF could be loaded in DCH which mediate its sustained release *in vivo* inside the BBB of the CNS (Song et al., 2012). When injected in the basal forebrain, depots of DCH-NGF provided a more prolonged delivery of NGF compared with NGF injected in buffer, which induced and maintained the hypertrophy of local forebrain cholinergic neurons for at least 28 days. This hypertrophic reaction of neurons seems to follow a gradient effect from the depot, and being more evident close and attenuate far from the depot.

Nerve growth factor loaded in a gelatin-polyethylene glycol-tyramine hydrogel together with bFGF loaded in heparin-pluronic nanogels and PCL beads as a passive bulking agent was tested to treat stress urinal incontinence (SUI). The combined action of NGF and bFGF, which were released at different rates, led to a significant improvement in regeneration and reinnervation of the damaged smooth muscle around the urethra in a rat model of SUI (Oh et al., 2015).

Finally, NGF and BDNF with mimicking peptides were used to functionalize RADA16-1, a self-assembling peptide capable of forming nanofibrous hydrogels under certain conditions (Lu et al., 2018). The hydrogel was used to fill a chitosan NC to graft a 10 mm gap in rat sciatic nerve.

Nanofibrous Electrospun Scaffolds for NGF Delivery

Among the more useful processing strategies to fabricate nanofibers, electrospinning is one of the best known methods (Greiner and Wendorff, 2007). Electrospun nanofibers with a defined micro/nanoarchitecture in terms of fiber size (fiber diameters range from a few hundreds of nanometers to tens of micrometers) and fiber orientation, have been used as a scaffold for a wide range of tissue engineering applications including neural, cardiovascular, bone and skin tissue engineering. Nanofibrous electrospun scaffolds offer a promising alternative to autologous grafting in peripheral nerve injuries, and have been extensively studied for neural tissue repair and regeneration (Ghane et al., 2021), due to their ability to act both as matrices for cells and as a delivery vehicle for various biomolecules such as NGF and glial cell line-derived neurotrophic factor (GDNF) (Liu et al., 2018; Bighinati et al., 2020). There are several reasons for the great interest in electrospun constructs in neural tissue engineering: ease of manufacture, production using a variety of natural and synthetic polymers, structural similarity with the extracellular matrix, and tunable morphology and mechanical properties. Of their various advantages, the ease of nanofiber functionalization is perhaps the most relevant, since biomolecules and drugs can easily be incorporated into electrospun scaffolds by means of several methods, including physical adsorption, blend electrospinning, coaxial electrospinning, and covalent immobilization. The nanometer scale of the fibers provides an extremely high surface-to-volume ratio, and contributes to improving biological functionality and biomolecule delivery (Ji et al., 2011). To tackle the problems related to the possible destabilization and denaturation of biomolecules such as growth factors when exposed to organic solvents in a traditional electrospinning process, variations in the technique, such as coaxial or emulsion electrospinning, have been employed to preserve the bioactivity of the incorporated biomolecules, thus enhancing the efficiency of incorporation, while controlling the release kinetics of the biomolecules at the same time.

A variety of natural and synthetic materials have been used to manufacture aligned structures for nerve regeneration, however only a few studies report significant results on the biomaterial-assisted delivery of NGF for *in vivo* applications.

In a detailed study recently published by Zhu et al. (2020) highly aligned poly(ε-caprolactone) (PCL) fibers with NGF gradients were developed for peripheral nerve regeneration. NGF was incorporated into the conduit following its manufacture,

preventing the biomolecule from being negatively affected by the organic solvents used during the electrospinning process. *In vitro* studies demonstrated that the conduits enhanced and attracted the longitudinal neurite growth of the dorsal root ganglion (DRG) neurons toward their high-concentration gradient side. *In vivo*, the conduits directed a stronger longitudinal attraction of axons and migration of Schwann cells in 15 mm rat sciatic nerve defects. At 12 weeks, rats transplanted with the conduits showed satisfactory morphological and functional improvements in g-ratio and total number and area of myelinated nerve fibers, as well as sciatic function index, compound muscle action potentials, and muscle wet weight ratio, as compared to aligned conduits with uniform NGF distribution. mRNA-seq and RT-PCR results also revealed that Rap1, MAPK, and cell adhesion molecule signaling pathways were closely associated with axon chemotactic response and attraction. The performance of the NGF-gradient aligned conduits was similar to that of autografts, demonstrating the great potential of the proposed scaffolds in repairing peripheral nerve defects.

More commonly, NGF is incorporated homogeneously into the nanofibers by means of coaxial or emulsion electrospinning. In the study by Kuihua et al. (2014), an artificial nerve guidance conduit for nerve gap regeneration was designed and manufactured via coaxial electrospinning. Aligned core-shell nanofibers were obtained, with the shell made of a silk fibroin/poly(lactic-acid-co-caprolactone) blend [SF/P(LLACL)], and the core consisting of SF encapsulating NGF. This approach permitted stabilization of the NGF during the electrospinning process, and contributed to a controlled sustained release of NGF. A sustained release of biologically active NGF was observed, using ELISA and a PC12 cell-based bioassay, over a 60-day time period, although the number of neurons was lower than the positive control. The core-shell fibrous conduits were then used as a bridge implanted across a 15-mm defect in the sciatic nerve of rats. The outcome in terms of regenerated nerve at 12 weeks was evaluated by a combination of electrophysiological assessment, histochemistry, and electron microscopy, and the results, taken together, demonstrated that the NGF-aligned fibers promoted peripheral nerve regeneration significantly better than the same conduit without NGF, suggesting that the released NGF may effectively promote the regeneration of peripheral nerves. In an analogous study, very similar random core-shell nanofibers were prepared by coaxial electrospinning, consisting of a shell of P(LLA-CL) and a core of BSA/NGF (Liu et al., 2011), and the conduits used for sciatic nerve regeneration in rats. The functional and histological analyses revealed that the parameters related to the number and arrangement of regenerated nerve fibers, myelination, and nerve function reconstruction for the P(LLA-CL)/NGF group were similar to those obtained for the group where the autograph nerve was implanted, and were significantly better than for the group in which plain P(LLA-CL) electrospun fibers were implanted, even in the presence of an injection of NGF solution.

In the study by Zhang et al. (2015), a composite micro/nano-fibrous scaffold with core–shell structure was manufactured by coaxial electrospinning, combining synthetic polymers (polypyrrole, PPy) as a conductive polymer and poly(L-lactic acid, PLLA) with natural polymer and biomolecules (spider silk protein, Lysine and NGF). *In vitro* tests revealed that the scaffold was able maintain a stable structure for at least 4 months in buffered solution, with a degradation rate comparable to the nerve growth rate. Good biocompatibility and good cell adhesion with PC 12 cells were demonstrated. *In vivo* evaluation also showed that the composite fibrous conduit was effective at bridging a 20 mm sciatic nerve gap in adult rats within 10 months, and electrical stimulation through the conduit promoted Schwann cell migration and axonal regrowth.

In addition to coaxial electrospinning, emulsion electrospinning can be also used to incorporate biomolecules while preserving their bioactivity, a method used to load recombinant human NGF into the core of emulsion electrospun PLLA nanofibers (Xia and Lv, 2018). The resulting nanofibrous scaffold was then additionally loaded with recombinant human vascular endothelial growth factor (VEGF) on the surface to achieve a controlled dual-delivery of the biomolecules. *In vitro* studies showed a sequential release pattern of VEGF and NGF, with most of the VEFG released in the first few days, whereas the NGF loaded in the fiber core was continuously released for more than 1 month. After demonstrating that the scaffold enhanced neural differentiation of iPSC-NCSC cells *in vitro*, it was implanted into a critical-size defect in a rat sciatic nerve model. Footprint analysis, electrophysiological tests, and histological analysis revealed a significant improvement in neovascularization and nerve healing 3 months after surgery.

The potential of electrospinning to prepare an aligned fiber matrix able to influence the directionality and growth of axons in the CNS was investigated in the study by Colello et al. (2016). A composite material was prepared by electrospinning polydioxanone (PDO) in the presence of alginate beads incorporating NGF and chondroitinase ABC (ChABC). Upon implantation in a completely transected rat spinal cord, the composite matrices supplemented with NGF and (ChABC) promoted significant functional recovery. Examination of the conduits post-implantation revealed that electrospun aligned fibers induced a more robust cellular infiltration than random fibers. A vascular network was also generated in these matrices, since electrospun fibers acted as a growth substrate for endothelial cells. The presence of axons within the implanted electrospun matrix demonstrated that the aligned composite fibers containing NGF are able to provide trophic support and directional guidance cues to regenerating axons following spinal cord injury.

In a very recent and exhaustive study, emulsion electrospinning was used to develop innovative microenvironment-responsive (pH-responsive) immunoregulatory electrospun fibers to promote nerve function (Xi et al., 2020). PLLA-based scaffolds were manufactured, containing Rat-β-NGF microsol particles wrapped in the core of the fiber during the electrospinning process from a homogeneous and stable water-in-oil emulsion. IL-4 plasmid-loaded liposomes (pDNA) were then grafted onto the surface of the electrospun fiber scaffolds. The resulting biomimetic scaffold responded directly to the acidic microenvironment at focal areas, followed by triggered release of the IL-4 plasmid-loaded liposomes within

a few hours to suppress the release of inflammatory cytokines and promote the neural differentiation of mesenchymal stem cells *in vitro*. A Sprague Dawley (SD) rat spinal hemisection model was used to investigate the *in vivo* performance on inflammation suppression, nerve regeneration and functional recovery. Once implanted into the rats with acute spinal cord injury, the scaffold showed sustained NGF release, achieved by the core-shell structure, and brought a significantly shifted immune subtype to down-regulate the acute inflammation response, reduce scar tissue formation, promote angiogenesis and neural differentiation at the injury site, and enhance functional recovery *in vivo*.

Overall, electrospinning-based technologies allow an extraordinary range of manufacturing opportunities for finely tuned design suitable for topical application. Moreover, several studies have also demonstrated that NGF bioactivity is not compromised by the electrospinning processing, making this technology suitable for applications in dermatology, but also neurosurgery and orthopedics.

Nanomedicines for NGF Delivery

While biomacromolecules offer promising and possibly fundamental pharmaceutical treatments for controlling and tacking diseases, their action is hampered by severe limitations in delivery. This is due to chemical and physical instabilities, as well as difficulties in crossing physiological barriers, and to being accumulated and released over time at the correct site of action (Duskey et al., 2017; Tosi et al., 2019).

Conventional drug delivery strategies cannot address these limitations leading to the increase in the number of polymeric or lipidic nanomedicine (NMed) applications which have incredible potential for the medical field (Germain et al., 2020) to: (i) stabilization of the biomacromolecules by encapsulation within a polymeric or lipidic matrix, therefore assuring the required level of protection of biological activity, and (ii) a controlled release of pharmacologically relevant amounts of therapeutics at the site of action.

Depending on the material used, NMeds can be tuned in terms of size, shape, charge, binding capacity and hydrophobicity/hydrophilicity, and are easily scaled-up in view of future production on an industrial scale. This allows for a quality by design approach of an NMed with tunable characteristics to be compatible with (i) the drug characteristics; (ii) the required drug release profiles, and (iii) the characteristic or biological/pathological environment in order to control the pharmacokinetic half-life, biodistribution, stability, and overall therapeutic activity of the loaded macromolecule to be managed and regulated *ad hoc*.

One example, NGF is the most potent growth stimulating factor for cholinergic neurons and has been shown to prevent the degeneration of dopaminergic neurons, making it a promising candidate for the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Kurakhmaeva et al., 2008, 2009). Regarding NGF delivery by means of nanomedicines, several attempts have been made to improve loading and delivery across the BBB by engineering various polymers with different BBB targeting ligands. One example was the use of a poly(alkyl-cyanoacrylate) polymer coated with polysorbate 80 to promote BBB crossing (Kurakhmaeva et al., 2008, 2009). This coating promoted the adsorption of apolipoproteins onto the nanoparticle (NPs) surface, and the contact of the NPs with the brain capillary endothelial cells which promoted endocytosis and the intracellular release of the drug.

In the same study, NGF was adsorbed on the surface of polybutylcyanoacrylate (PBCA) NPs coated with polysorbate-80 (PS-80) surfactant for antiparkinsonian effects (Kurakhmaeva et al., 2008). Pre-treatment of the mice with NGF-loaded NPs coated with PS-80 15 min before MPTP (used to provoke parkinsonian syndrome) showed a considerable decrease in parkinsonian symptoms such as a 37% decrease in latero- and retropulsion and a 34% decrease in catalepsy as early as day 1 of observation when compared to control groups.

It is noteworthy that the total index of vertical and horizontal motor activity in the group receiving NGF-loaded NPs coated with PS-80 after MPTP was 1.78-fold higher compare to control, while treatment before MPTP induction was 2.86 times higher suggesting a potential protective effect. The effects of NGF-loaded NPs persisted for 7 and 21 days following a single injection of the neurotoxin proving to be one of the most promising NMed carriers by preventing the scavenging of the NGF by the cells of the reticuloendothelial system, prolonging circulation of these particles in the blood and increasing their concentration in cerebral vessels.

Similar experiments were conducted to explore the effect of NGF adsorbed on PBCA NPs coated with polysorbate-80 in Alzheimer's disease. Acute amnesia in mice was induced by subcutaneous injection of scopolamine before training in the step-through passive avoidance reflex (PAR) test to determine effects on memory (Kurakhmaeva et al., 2009). The NGF-loaded PBCA NP formulation produced significantly increased latent periods in the passive-avoidance reflex (PAR) test, compared to the control animals who only received scopolamine. In contrast, systemic administration of the NGF in solution did not induce any significant changes in the mental or cognitive activity of the animals after induction of these changes by scopolamine pretreatment.

Nerve growth factor was also encapsulated into a chemically crosslinked albumin nanocarrier matrix (HSA) with ultrasmall particles of iron oxide surface-modified with apolipoprotein E to facilitate active transport into the brain and allow it to be used as a theranostic agent (Feczkó et al., 2019). The HSA NPs exhibited a size of 212 $\pm$ 1 nm, a polydispersity index (PDI) of 0.075 $\pm$ 0.022 and a zeta potential of -48.3 mV. The biocompatibility of these nanocarriers and the bioactivity of NGF were confirmed in rat pheochromocytoma (PC12) cells. Following modification of the particle surface with Apo E, the particles were able to cross the BBB and remained bioactive in terms of neurite outgrowth regulation.

In addition to Apo E, Apolipoprotein A-I was used to coat NGF lipoprotein (HDL)-mimicking NPs (Zhu and Dong, 2017). High-density lipoprotein (HDL)-mimicking NPs is a natural NP consisting of a lipid core coated with apolipoproteins, and a phospholipid monolayer which plays a critical role in the transport of lipids, proteins, and nucleic acids via its

interaction with target receptors. The HDL-mimicking NPs successfully encapsulated NGF, resulting in a long half-life, prolonged release (10% over 72 h), *in vivo* stability, and increased physiological effects.

In another approach (Song et al., 2017), non-viral poly(lactic-co-glycolic acid) (PLGA) nanobubble (NBs) vectors, possessing unique advantages such as targeting, slow release and penetration, were used as gene carriers to deliver NGF. PLGA is one of the most successful polymers used in the development of drug delivery systems, offering excellent biocompatibility and biodegradability of NPs (Tan et al., 2013; Ruozi et al., 2015).

The NGF/PLGA NBs formed by double emulsion was 215.3 ± 55.29 nm, the PDI was 0.027 and the zeta potential was −11.3 ± 5.65 mV. It underwent Ultrasound (US)-mediated destruction to deliver NGF, resulting in diminished histological injury, neuron loss and neuronal apoptosis, and increased BBB scores in a rat model of spinal cord injury.

Chitosan, another widely used biodegradable and biocompatible polymer was used by Razavi et al. (2019) to encapsulate NGF in chitosan nanoparticles (NGF-CNPs). NGF-CNPs were characterized by photon-correlation spectroscopy analysis, which showed a mean NGF-CSNP diameter of 147.04 ± 8.09 nm, and a good stability of the nanoparticle surface charge (36.47 ± 1.88 mV). The encapsulation efficiency of NGF in chitosan nanoparticles is 83.93 ± 2.45%. These NMeds were evaluated for their differentiation potential of human adipose-derived stem cells (h-ADSCs) to Schwann-like cells as a source for treating various diseases such as peripheral nerve regeneration multiple sclerosis and diabetic neuropathy (Razavi et al., 2019). NGF-CNPs demonstrated no cytotoxicity and offered a sustained release of NGF reaching 74.63 ± 2.07% over 7 days without any initial burst release leading to an increased differentiation of h-ADSCs into Schwann-like cells and myelinating capacity *in vitro*.

Similarly, NGF was encapsulated into NPs [n(NGF)] of methacryloyloxyethyl phosphorylcholine (MPC), analogous to choline and acetylcholine, and polylactic acid (PLA) diacrylate to provide proof of CNS targeting in healthy mice following intravenous injection (Xu et al., 2019). The MPC-PLA exhibited an average diameter of 30.3 ± 3.6 nm under TEM, and a zeta potential of 24 mV. PC12 cells were treated with native NGF and NGF NPs to assess the activity of the NGF released from the nanocapsules. When the NGF was released from the NPs, it induced the differentiation and neurite outgrowth of these cells through intracellular pathways. The therapeutic benefit of n(NGF) for CNS repair following injury was evaluated in a mouse model of compression-induced acute spinal cord injury. After 21 days, extensive ankle movements and occasional plantar stepping was observed, representing a significant functional recovery in locomotion.

Besides these results, widely reviewed in the past literature (Ruozi et al., 2012; Srikanth and Kessler, 2012; Angelova et al., 2013; Géral et al., 2013), we would like to highlight some key factors which may significantly improve the chances of success for NMed in the field of biomacromolecule delivery.

Regarding the choice of NMed, its design and production, a major concern, still hotly debated, regards two main aspects of nanoproduction. The first is the absolute conviction that it is possible to develop one single nanomedicine for every drug (or macromolecule), the so-called "magic bullet" (Strebhardt and Ullrich, 2008; Flühmann et al., 2019), is neither more a reality nor the future. This erroneous view of the *magic bullet* led to many years of research without any real or concrete advances in the translatability of NMeds to a clinical setting. Therefore, it is pivotal, when approaching a NMed design to consider the future NMed as a single product together with the embedded drug.

The second aspect, especially when considering biomacromolecules such as NGF, proteins or enzymes, relates to the stability of the biological drugs throughout the preparation procedure and during storage. The greatest drawback concerns the requirements of nanoproduction (such as stirring, heating, sonication, organic solvents, etc.), which severely impact the stability and maintenance of the biological drug's pharmacological activity (Duskey et al., 2020). Some of these requirements relate to the polymer/lipid used in the formulation, and should be carefully designed and always adapted to the "stability features" of the embedded drug. Failure to take these aspects into consideration when designing the NMed risks rendering the loaded drug ineffective, thus defeating its purpose.

GENE THERAPY AND CELL-ASSISTED BIODELIVERY

Nerve growth factor cell and gene therapy for the CNS, in particular to target cholinergic degeneration in AD, has been investigated in preclinical models and also tested in human studies (Hosseini et al., 2018; Mitra et al., 2019), particularly in the United States (Rafii et al., 2018) and Sweden (Eyjolfsdottir et al., 2016).

Results from the US study on gene therapy in AD patients have recently been reviewed to include CNS analysis following autopsy. Intraparenchymal adeno-associated virus serotype 2 (AAV2)-NGF delivery was safe but did not improve cognition. Neuropathological analysis then aimed to establish whether (AAV2)-NGF engaged the target cholinergic neurons of the basal forebrain. Patients with clinically diagnosed early- to middle-stage AD received a total dose of 2 × 1011 vector genomes of AAV2-NGF by stereotactic injection of the nucleus basalis of Meynert. Following a mean survival of 4.0 years, AAV2-NGF targeting, spread, and expression indicated that NGF gene expression persisted for at least 7 years at the sites of AAV2-NGF injection. However, the mean distance of AAV2-NGF spread was only 0.96 ± 0.34 mm, indicating that NGF did not directly reach the cholinergic neurons at any of the 15 injection sites. Given that AAV2-NGF did not directly engage the target cholinergic neurons, the authors cannot conclude that growth factor gene therapy is effective for AD (Castle et al., 2020).

In the Swedish study, biodelivery of NGF (NGF-ECB) by encapsulated cell was used in AD patients in a first-in-human study. Results were gathered from a third dose cohort of patients with mild to moderate AD, receiving second-generation NGF-ECB implants with improved NGF secretion, in

an open-label, phase Ib dose escalation study with a 6-month duration. Each patient underwent stereotactic implant surgery with four NGF-ECB implants generated using the Sleeping Beauty transposon gene expression technology targeted at the cholinergic basal forebrain, resulting in production of about 10 ng NGF/device/day. The data derived from this patient cohort demonstrate the safety and tolerability of sustained NGF release by a second-generation NGF-ECB implant to the basal forebrain. Moreover, the patients' responses to the NGF-treatment indicated that approximately half of the patients responded to the ECB-NGF-treatment with increased cholinergic markers (e.g., ChAT activity) in the CSF, correlating to improved cognition and brain glucose metabolism (Karami et al., 2015), less brain atrophy (Ferreira et al., 2015), and normalization of the EEG-pattern (unpublished data).

DISCUSSION

The discovery of endogenous GF production of GFs during adulthood as well as during development opened new perspectives for mature CNS biology, moving away from the dogma of prominent histologist Ramon y Cajal: "Once development ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree." We are now in the science of the future, knowing that endogenous regeneration may occur in the mature CNS, albeit to a limited extent. NGF can now be produced using human recombinant technologies, and molecules which can limit adverse side-effects are available either as modified full-length proteins or as TrkA short peptides analogs. However, we still need to better understand that NGF-based therapies should be considered as "hormonal" therapies rather than conventional pharmacological therapies, in view of the endogenous production of NGF. We also need to protect the molecule from protein degradation, and promote the crossing of blood-tissue barriers, in order to bring the appropriate molecule concentration to the appropriate place for the appropriate time. The use of modern biomaterial technologies is an essential strategy for rapidly achieving this goal. Scaffolds obtained by different fabrication procedures, such as hydrogels and composite materials are providing significant indications about efficacy of NGF delivery in peripheral nerve, but also in other tissues repairs, as bone, while nanoparticle conjugation is regarded as a promising strategy also to overcome physiological barriers.

However, in view of clinical translation, we also need to move forward in preclinical research, which, at the present, provides a puzzling and incomplete picture of biomaterial potentiality. In particular, we need well-designed proof-of-concept studies for both safety and efficacy, thus including appropriate control groups and defined functional end-points in the efficacy studies, and the Good Laboratory Practice standard for safety studies. Because of this, a more stringent interdisciplinary collaboration would be desirable, such as a more stringent editorial policy in both biomaterial and biomedical journals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Emilia-Romagna POR-FESR 2014-20, project Mat2Rep.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fnins.2021.665757

Injection of Anti-proBDNF Attenuates Hippocampal-Dependent Learning and Memory Dysfunction in Mice With Sepsis-Associated Encephalopathy

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OPEN ACCESS

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: 08 February 2021
Accepted: 26 May 2021
Published: 20 July 2021

Citation:
Cui Y-H, Zhou S-F, Liu Y, Wang S, Li F, Dai R-P, Hu Z-L and Li C-Q (2021) Injection of Anti-proBDNF Attenuates Hippocampal-Dependent Learning and Memory Dysfunction in Mice With Sepsis-Associated Encephalopathy. Front. Neurosci. 15:665757. doi: 10.3389/fnins.2021.665757

Sepsis-associated encephalopathy (SAE) is a risk factor for cognitive and memory dysfunction; however, the mechanism remains unclear. Brain-derived neurotrophic factor (BDNF) was reported to have a positive effect on cognition and emotion regulation, but the study of its precursor, proBDNF, has been limited. This study aimed to elucidate the effects and associated mechanisms of hippocampal proBDNF in a lipopolysaccharide (LPS)-induced SAE mouse model. In this study, we found that the mice exhibited cognitive dysfunction on day 7 after LPS injection. The expression of proBDNF and its receptor, $p75^{NTR}$, was also increased in the hippocampus, while the levels of BDNF and its receptor, TrkB, were decreased. A co-localization study showed that proBDNF and $p75^{NTR}$ were mainly co-localized with neurons. Furthermore, LPS treatment reduced the expression of NeuN, Nissl bodies, GluR4, NR1, NR2A, and NR2B in the hippocampus of SAE mice. Furthermore, an intrahippocampal or intraperitoneal injection of anti-proBDNF antibody was able to ameliorate LPS-induced cognitive dysfunction and restore the expression of NeuN, Nissl bodies, GluR4, NR1, NR2A, NR2B, and PSD95. These results indicated that treatment with brain delivery by an intrahippocampal and systemic injection of mAb-proBDNF may represent a potential therapeutic strategy for treating patients with SAE.

Keywords: sepsis associated encephalopathy, proBDNF, $p75^{NTR}$, hippocampus, cognition and memory dysfunction

INTRODUCTION

Sepsis-associated encephalopathy (SAE) is defined as a response of the central nervous system (CNS) to a systemic inflammatory response syndrome and diffuse brain dysfunction (Iwashyna et al., 2010). SAE is associated with mortality in patients with sepsis in the form of delirium, epileptic seizure, and shock (Cohen et al., 2015). A recent study indicated that SAE might

induce sustained brain lesions (Semmler et al., 2013). About 40% of patients displayed long-term and irreversible sequela, including memory impairment, depression, anxiety, and cognitive disturbances (Widmann and Heneka, 2014). Thus, the prevention and treatment of SAE, especially the cognitive impairment, is crucial for patients with sepsis.

A clinical study shows that the CNS is indirectly infected during the development of SAE (Gofton and Young, 2012) and that the pathogenesis of SAE involves multiple brain regions (Widmann and Heneka, 2014). The hippocampus, as the primary region of the brain responsible for learning and memory, is sensitive to ischemia, anoxia, and inflammation (Semmler et al., 2005; Hagena et al., 2016). Sepsis survivors were reported to have significant hippocampus atrophy (Semmler et al., 2013). SAE animal models are impaired in hippocampal-dependent learning and memory tasks (Abramova et al., 2013; Chugh et al., 2013). SAE-induced cognitive impairment is related to the dysfunction of neurons and synaptic plasticity of the hippocampus (Christian et al., 2014; Valero et al., 2014). The basis of learning and memory is the modulation of synaptic proteins which influence synaptogenesis and dendritic spine formation that regulate synaptic plasticity (Kariolis et al., 2020). Thus, understanding the mechanisms leading to synaptic and neuronal dysfunction will help to develop the tailored neuronal synapse-targeted therapies for cognitive impairment in SAE.

A reduction in the levels of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), has been reported in connection with neurogenic defects (Shirayama et al., 2002; Angelucci et al., 2005). BDNF is one of the most abundant and widely distributed neurotrophic factors, which is a major protector of the CNS. BDNF supports neuronal survival, development, and differentiation to regulate learning and memory (Chao, 2003; Reichardt, 2006; Hempstead, 2015). Additionally, BDNF is a critical regulator of synaptic plasticity in the hippocampus (Thoenen, 2000). Previous studies have reported that BDNF protects the CNS by inhibiting apoptosis and impairing neuronal excitation (McAllister, 2002; Counts and Mufson, 2010). Reduced BDNF expression maintains the dysfunction of the CNS in patients with severe sepsis (Ritter et al., 2012).

The precursor form of BDNF, proBDNF, is released extracellularly and can bind its receptor to play an opposing role to mature BDNF (mBDNF) (Koshimizu et al., 2010). proBDNF plays a key role in regulating the development of the CNS and the neurogenesis of the hippocampus *via* binding its high-affinity receptor, $p75^{NTR}$, or sortilin (Teng et al., 2005). Studies found that the proBDNF/$p75^{NTR}$/sortilin complex stimulated neuronal apoptosis, amyloid deposition, depression, and learning and memory dysfunction in neurodegenerative disease models (Sun et al., 2012; Chen et al., 2016) and that proBDNF levels increased in the brain of patients with Alzheimer's disease (Chen et al., 2017). Additionally, proBDNF negatively regulates the migration of cerebellar granule cells, and this effect is mediated by $p75^{NTR}$ during development and pathological conditions (Xu et al., 2011). Similarly, proBDNF has been reported to inhibit neuronal proliferation and neurogenesis (Li et al., 2017), which may be due, in part, to the activation of RhoA through the $p75^{NTR}$ signaling pathway that damages neurite outgrowth and filapodial growth cones *in vitro* (Sun et al., 2012). Furthermore, in a study of the mechanism of pain, proBDNF and $p75^{NTR}$ were upregulated in the inflammatory cells of local tissues with inflammatory pain, which was alleviated upon injection of an anti-proBDNF antibody (Luo et al., 2016). Our previous study established that an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) (20 mg/kg) induced the upregulation of proBDNF in T cells of the mesenteric lymph node (Wang et al., 2019). Furthermore, our recent study demonstrated that the upregulated proBDNF in the immune system promoted the pathogenesis of SAE through downregulating the circulating levels of $CD4^+$ T cells, thus limiting its infiltration into the meninges and perturbing the meningeal pro-/anti-inflammatory homeostasis (Luo et al., 2020). Hence, it is plausible that proBDNF modulates the functions of the hippocampus and is involved in regulating the cognitive impairment of SAE.

Here we report that proBDNF and its receptor $p75^{NTR}$ were upregulated in hippocampal neurons after the induction of SAE. The systemic administration or intrahippocampal microinjection of anti-proBDNF antibodies (mAb-proB) attenuated cognitive impairment, which may be due to the expansion of neuronal function and the synaptic transmission-associated protein level of the hippocampus in SAE progression. Thus, mAb-proB is a promising therapeutic with the potential to alleviate SAE by regulating the hippocampal neuron function.

Abbreviations: SAE, sepsis-associated encephalopathy; BDNF, brain-derived neurotrophic factor; LPS, lipopolysaccharide; TrkB, tropomyosin receptor kinase B; CNS, central nervous system; mBDNF, mature brain-derived neurotrophic factor; OFT, open field test; NOR, novel object recognition test; PVDF, polyvinylidene fluoride; TBST, Tris-buffered saline Tween-20.

MATERIALS AND METHODS

Animals

Male 7- to 8-week-old C57BL/6 mice (18–23 g) were purchased from the Laboratory Animal Co., Ltd., of Slack King (Longping High-Tech Park, Changsha, China) SCXK (Hunan) (2013-0004). The animals were housed at four to five individuals per cage, with a 12-h light/dark cycle at a constant temperature (22°C) and in a humidity-controlled (50 ± 5%) animal facility, with food and water *ad libitum*. All the animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996, and were approved by the Animal Ethics Committee of the Third Xiangya Hospital (Changsha, China). All efforts were made to minimize suffering and the number of mice used.

SAE Mice Model

Lipopolysaccharide derived from *Escherichia coli* serotype 055:B5 (catalog no. L2880, Sigma-Aldrich, United States) was dissolved in 0.9% normal saline and administrated to the mice *via* intraperitoneal injection (i.p. 10 mg/kg) to induce SAE (Zhu et al., 2019; Luo et al., 2020). The control animals were injected with an equivalent volume of saline. The animals were randomly divided into a control group and an LPS group.

Systemic Administration of Anti-proBDNF Monoclonal Antibody

The neutralizing mAb-proB was developed by Shanghai Yile Biotechnology Company, and its biological function has been well characterized by our previous studies (Luo et al., 2016; Wang et al., 2019; Luo et al., 2020). To investigate the role of mAb-proB, the mice were treated with 100 μg mAb-proB in 0.3 ml normal saline *via* i.p. injection 12 h after the induction of SAE. In the control group, an equal volume of normal mouse IgG isotype (catalog no. AT1596, CMCTAG, United States) was administrated to the mice *via* i.p. injection.

Intrahippocampal Microinjection of Anti-proBDNF Monoclonal Antibody

MAb-proB was dissolved in saline to a concentration of 1.00 μg/μL. After anesthetization by sevoflurane inhalation and pentobarbital sodium (50 mg/kg), the mice were placed in a stereotaxic apparatus and administered with mAb-proB or normal mouse control IgG (1.00 μg) *via* intrahippocampal injection (AP, 2.06 mm; ML, ±2.30 mm; SV, 2.25 mm relative to the bregma) as previously described (Luo et al., 2020). Bilateral hippocampus infusion was administered *via* 4.20 μl Nanoliter Microinjection with a glass micropipette. Approximately 1.00 μl of mAb-proB or IgG was slowly infused at a rate of 100 nL/s. After an additional 10 min to ensure adequate diffusion, the glass micropipette was slowly retracted from the mouse. At 2 h after the intrahippocampal injection, the mice were administrated with LPS or saline *via* i.p. injection, and their behavior was assessed at 5–9 days after LPS treatment.

Behavioral and Cognitive Tests

All behavioral procedures were implemented from 9 a.m. to 5 p.m. in a sound-isolated room. Tests were operated and recorded by the same experimenter who was blinded to the grouping of the mice.

Open Field Test

The open field is composed of a white polyester resin chamber ($50 \times 50 \times 50$ cm^3). Each mouse was placed in the center of the arena and was free to explore for 5 min. The total distance traveled and the time spent in the central square were recorded and analyzed by the ViewPoint Video Tracking Software (ViewPoint Behavior Technology, Lyon, France).

Novel Object Recognition Test

The novel object recognition (NOR) test was carried out in an open field box ($50 \times 50 \times 50$ cm^3). Before the test, the mice were habituated to the box for 5 min without any objects. Then, each mouse was placed in the center of the box and exposed to two identical objects for 5 min (familiarization session) and then returned to their cage. A 30-min interval between the familiarization and test session was set for short-term memory tasks, and a 24-h interval was set for long-term memory tasks. Each mouse was then permitted to explore both the familiar object and a completely different object (novel object) for 5 min (test session). The time spent exploring the familiar object (TA) and the novel object (TB) were recorded and analyzed by the ViewPoint Video Tracking Software (ViewPoint Behavior Technology, Lyon, France). A recognition index, defined as the amount of time spent in exploring the novel object divided by the total time spent in exploring both objects and multiplied by 100 {[TB/(TA + TB)] × 100}, was used to measure recognition memory (Lin et al., 2018).

Y Maze Test

The Y maze apparatus was made up of three divided gray polyvinylidene passages, with a 120° angle between each arm ($30 \times 7 \times 16$ cm^3). The apparatus was placed in a soundproof and isolated room. The three arms include the start arm, in which the mouse explored first (always open), the novel arm, which is blocked at the training time but opened at the test time, and the other arm (always open). The Y maze test included two trials–training and testing–separated by an interval time. In the training, each mouse was permitted to explore the start arm and the other arm randomly to avoid spatial memory errors. After a 30-min interval time, each mouse was free to explore all three arms. After a 24-h inter-trial interval, the tags attached to two of these arms were altered, and the mice were allowed to explore the three arms again. The time spent in and the number of entries into each arm were recorded and analyzed *via* the ViewPoint Video Tracking Software (ViewPoint Behavior Technology, Lyon, France).

Western Blot Analysis

Total protein was extracted from mouse hippocampal tissue using the Minute^{TM} total protein extraction kit (catalog no. SD-001, Invent Biotechnologies Inc., United States) according to the manufacturer's instructions. Proteins (40 μg) were separated on a 10% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (catalog no. IPVH15150, Millipore, Billerica, MA, United States). After blocking with 1% gelatin in Tris-buffered saline plus Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: BDNF antibody, 1:1,000, catalog no. ab108319, Abcam; p75 antibody, 1:1,000, catalog no. ab8874, Abcam; sortilin antibody, 1:2,000, catalog no. ab16640, Abcam; tropomyosin receptor kinase B (TrkB) antibody, 1:1,000, catalog no. 13129-1-AP, Proteintech; β-actin antibody, 1:5,000, catalog no. 66009-1-Ig, Proteintech; GluR1 antibody, 1:1,000, catalog no. ab183797, Abcam; GluR4 antibody, 1:1,000, catalog no. SAB4501296, Sigma; NR1 antibody, 1:1,000, catalog no. ab174309, Abcam; NR2A antibody, 1:1,000, catalog no. 19953-1-AP, Proteintech; NR2B antibody, 1:1,000, catalog no. ab254356, Abcam; and PSD95 antibody, 1:1,000, catalog no. 2507, Cell Signaling Technology. The membranes were rinsed completely and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:2,000, catalog nos. ab6721 and ab6789, Abcam) for 2 h at room temperature. Lastly, the membranes were rinsed and exposed to photographic film with Immobilon western chemiluminescent HRP substrate (catalog no. WBKLS0500, Millipore, United States). The signals were quantified by NIH Image J 7.0 software and standardized to β -actin.

Immunofluorescence Staining

Paraffin-embedded brain tissues were serially cut into 4-μm sections. The slides were regularly deparaffinized and hydrated. The sections were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 20 min and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline for 60 min at 37°C and incubated with the following primary antibodies overnight at 4°C: anti-proBDNF antibody, catalog no. ANT006, 1:500, Alomone lab; anti-NeuN antibody, 1:1,000, catalog no. ab104224, Abcam; and anti-p75NTR antibody, 1:500; catalog no. ab25958, Abcam. The sections were incubated with the following secondary antibodies for 1 h at 37°C [goat polyclonal secondary antibody to rabbit IgG (Goat Anti-Rabbit IgG H&L; Alexa Fluor® 488), 1:1,000 for proBDNF and p75NTR, ab150077, Abcam; goat anti-mouse IgG H&L (Alexa Fluor® 594), 1:1,000 for NeuN]. The coverslips were stained with DAPI (1:2,000, SC-3598, Santa Cruz Biotechnology, Inc., Dallas, TX, United States) for 2 min at room temperature. Immunofluorescence images were acquired using a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corporation, Tokyo, Japan).

Nissl Staining

The sections were transferred onto gelatin-coated slides for airing. The sections were immersed into Nissl dye (catalog no. G1430, Solarbio, China) for 10 min at 56°C and washed mildly with water. Then, the sections were differentiated in 80 and 95% ethyl alcohol and in absolute ethyl alcohol for 5 min separately and cleared in xylene for 10 min. Finally, the sections were examined *via* a microscope.

Immunohistochemistry Staining

The immunohistochemistry staining protocol is identical to the immunofluorescence staining protocol before secondary antibody incubation and differs with respect to immersing the sections in H_2O_2. The sections were rinsed in PBS buffer and then incubated in 3% H_2O_2 solution for 20 min to remove endogenous peroxidase. Next, the slides were blocked with 5% BSA in 0.01% Triton X-100 in PBS for 1 h at 37°C and then incubated with the following antibodies overnight at 4°C: proBDNF antibody (catalog no. ANT006, 1:1,000, Alomone lab) and NeuN antibody (1:1,000, catalog no. ab104224, Abcam). To test the efficiency of humanized mAb-proB, the same protocol was performed as mentioned above except without the primary antibody. The sections were incubated with biotinylated goat anti-mouse immunoglobulin secondary antibody (1:1,000, ab6788, Abcam) or sheep anti-human IgG H&L (1:1,000, ab6869, Abcam) for 1 h at 37°C. The slides were then washed and incubated in an ABC universal plus kit (catalog no. PK-8200, Vector Laboratories, United States). Finally, 3,3′-diaminobenzidine staining of the sections was performed according to the ABC universal plus kit protocol. Images of the sections were obtained using an optical microscope (Nikon, Japan).

Reverse Transcription and Quantitative Real-Time PCR

Total RNA extraction from the hippocampus was performed as previously described (Wang et al., 2019; Yu et al., 2020). Briefly, cDNA was obtained using a reverse transcription kit (catalog no. 4368814, Thermo Fisher Scientific, United States). RT-qPCR was carried out with SYBR Green (Bio-Rad) on a CFX96 Touch™ Deep Well Real-Time PCR Detection system (Bio-Rad, Hercules, CA, United States). The primer sequences were as follows: 5′-GGGTGTGAACCACGAGAAAT-3′ and 5′-ACAGTCTTCTGGGTGGCAGT-3′ (*GAPDH*); 5′-AGTGGAGAGTGCTGCAAAGC-3′ and 5′-GTCAGAGAA CGTAACACTGTCCA-3′ ($p75^{NTR}$); 5′-ATTAGGGAGTGGGT CACAGC-3′ and 5′-GATTGGGTAGTTCGGCATTG-3′ (*BDNF*) ; 5′-GAAAATGGCCTGTGGGTGTC-3′ and 5′-ACCAAGATC AGCTTTGCAGG-3′ (*sortilin*); 5′-CGCAAACGGCAGGAGA AAGA-3′ and 5′-TGCGCACCTCAGGGCTATTT-3′ (*Trkb*).

Statistical Analysis

All the experiments were independently performed in triplicate. Data are expressed as mean ± SEM. An unpaired two-tailed Student's *t*-test, one-way analysis of variance, or two-way analysis of variance was used for statistical analysis. A p-value < 0.05 was considered to be statistically significant. The statistical analysis was performed using GraphPad Prism 7.0 (San Diego, CA, United States).

RESULTS

Intraperitoneal Injection of LPS Impaired Learning and Memory in Mice

The behavior routines are shown in **Figure 1A** for the analysis of the SAE mice model. First, we measured the locomotor activity and emotional behavior using the open field test (OFT) experiment. In the OFT, compared with the control group, there was no significant difference in the locomotor performance of the LPS group on the 5th day after LPS injection (unpaired *t*-test; $t(16) = 2.012$, $p = 0.0614$, **Figure 1B**) and in the time spent in the center square on the 6th day after LPS injection (unpaired *t*-test; $t(16) = 1.884$, $p = 0.0779$, **Figure 1C**).

To further assess the influence of LPS on learning and memory in mice, NOR and Y maze tests were conducted (Lin et al., 2018; van der Kooij et al., 2018). Using the NOR and Y maze tests, we found that the mice displayed decreased short-term memory (inter-trial interval: 30 min) (unpaired *t*-test; $t(16) = 3.035$, $p = 0.0079$, **Figure 1D**; $t(16) = 4.793$, $p = 0.0002$, **Figure 1F**) and long-term memory (inter-trail interval: 1 day; unpaired *t*-test; $t(16) = 3.008$, $p = 0.0083$, **Figure 1E**; $t(16) = 3.059$, $p = 0.0075$, **Figure 1G**) after LPS injection. These data together suggest that i.p. LPS treatment damages the learning and memory functions of mice.

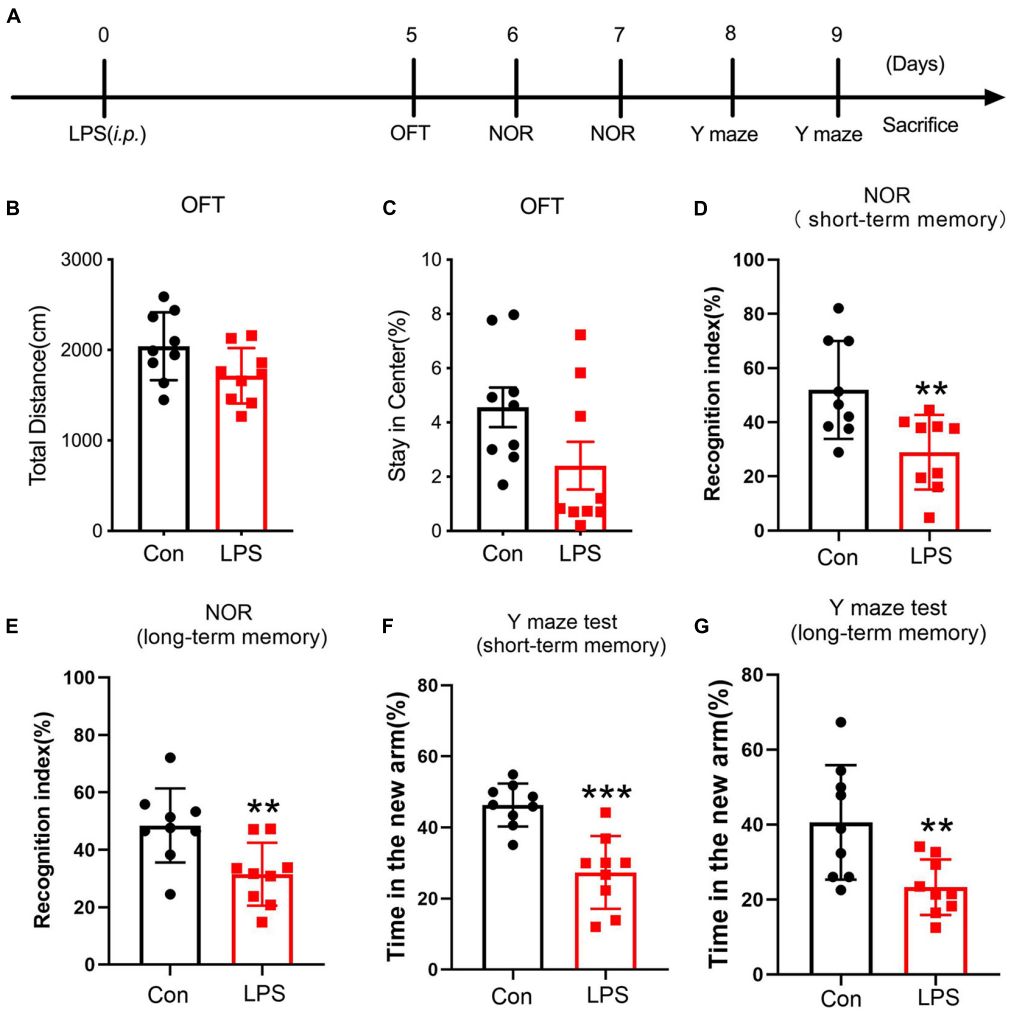

FIGURE 1 | Behavioral tests after the establishment of the sepsis-associated encephalopathy mouse model. **(A)** Timeline of behavioral tests after administration of lipopolysaccharide. **(B,C)** The total distance and the time spent in the center field of the open field test (n = 9/group). **(D,E)** The recognition index of short-term or long-term memory tasks in the novel object recognition test (n = 9/group). **(F,G)** The percentage of time mice stay in the novel arm in the short-term or long-term memory task in the Y maze test (n = 9/group). Data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

LPS Injection Altered the Expression of proBDNF, BDNF, and Its Receptors in Mice

We first examined the expression level of *BDNF*,*TrkB*, *sortilin*, and $p75^{NTR}$ in the hippocampus of mice on the first, third, seventh, and 14th day after LPS injection (**Figures 2A–D**). Compared with the control group, the $p75^{NTR}$ transcripts (**Figure 2D**) increased on the 7th day after LPS administration (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 6.093$, $p = 0.0048$), the *BDNF* transcripts (**Figure 2A**) decreased on the third day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 4.838$, $p = 0.0267$), seventh day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 4.901$, $p = 0.0245$), and 14th day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 6.087$, $p = 0.0048$) after LPS administration, and the *TrkB* transcripts (**Figure 2B**) decreased on the seventh day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 5.406$, $p = 0.0123$) and 14th day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 7.015$, $p = 0.0014$) after LPS administration, while the *sortilin* transcripts (**Figure 2C**) showed no significant change (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 2.580$, $p > 0.05$).

Next, we evaluated the protein levels of BDNF, TrkB, sortilin, and $p75^{NTR}$ in the hippocampus of LPS-treated mice (**Figures 2E–K**). The results showed that, compared with the control group, the protein levels of proBDNF and its high-affinity receptor $p75^{NTR}$ increased on day 3 (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 5.466$, $p = 0.0113$ for proBDNF), day 7 (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 6.569$, $p = 0.0025$ for proBDNF; $F(4, 15) = 6.624$, $p = 0.023$ for $p75^{NTR}$), and day 14 (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 5.529$, $p = 0.0104$

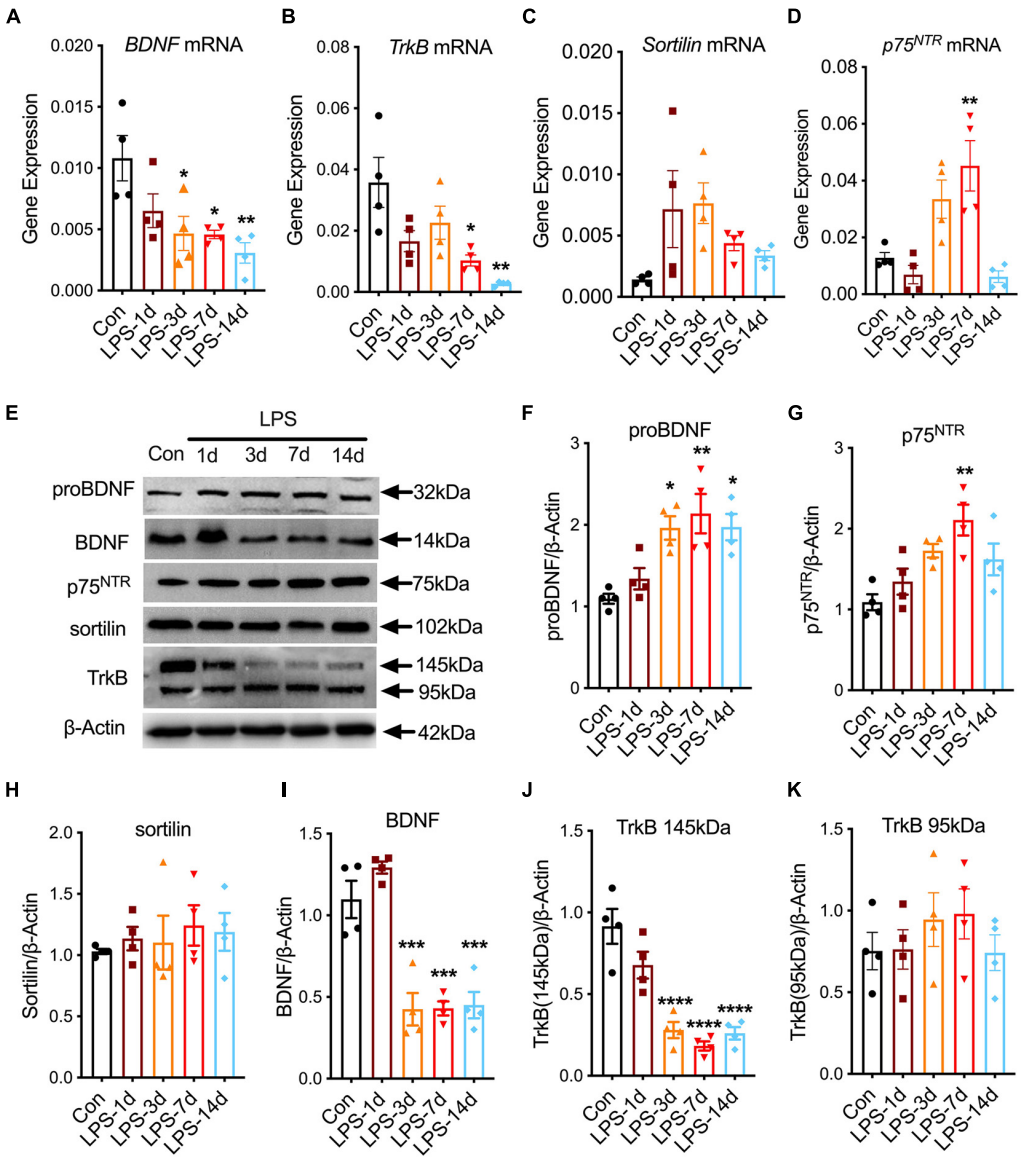

FIGURE 2 | The expression pattern of proBDNF, BDNF, and their receptors in sepsis-associated encephalopathy mice. **(A–D)** The expression level of *BDNF, TrkB, sortilin, and p75^{NTR}* at different time points after the lipopolysaccharide injection (n = 4/group). **(E–K)** Immunoblot analysis of different time points and the quantitative results of the expression of proBDNF, p75^{NTR}, sortilin, BDNF, and TrkB (n = 4/group). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

for proBDNF) after LPS administration, while sortilin showed no significant differences (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 0.3041$, $p > 0.05$; **Figures 2E–H**). In contrast, the protein levels of mBDNF and its receptor TrkB (145 kDa) decreased on the third day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 8.324$, $p = 0.0002$ for BDNF; $F(4, 15) = 9.392$, $p < 0.0001$ for TrkB), seventh day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 8.262$, $p = 0.0003$ for BDNF; $F(4, 15) = 10.83$, $p < 0.0001$ for TrkB), and 14th day (one-way ANOVA, Tukey's multiple-comparisons test; $F(4, 15) = 8.014$, $p = 0.0004$ for BDNF; $F(4, 15) = 9.688$, $p < 0.0001$ for TrkB) after LPS administration. However, the change in TrkB (95 kDa) levels in SAE mice was not statistically significant (**Figures 2E,K**). Thus, these results indicate that proBDNF and p75^{NTR} are upregulated and BDNF and TrkB (145 kDa) are downregulated following LPS administration.

proBDNF and Its Receptor p75^{NTR} Are Expressed Mainly in Hippocampal Neurons

Immunohistochemistry staining demonstrated that proBDNF was highly expressed (unpaired t-test; $t(6) = 3.197$, $p = 0.0187$) in the hippocampus on the seventh day after LPS injection

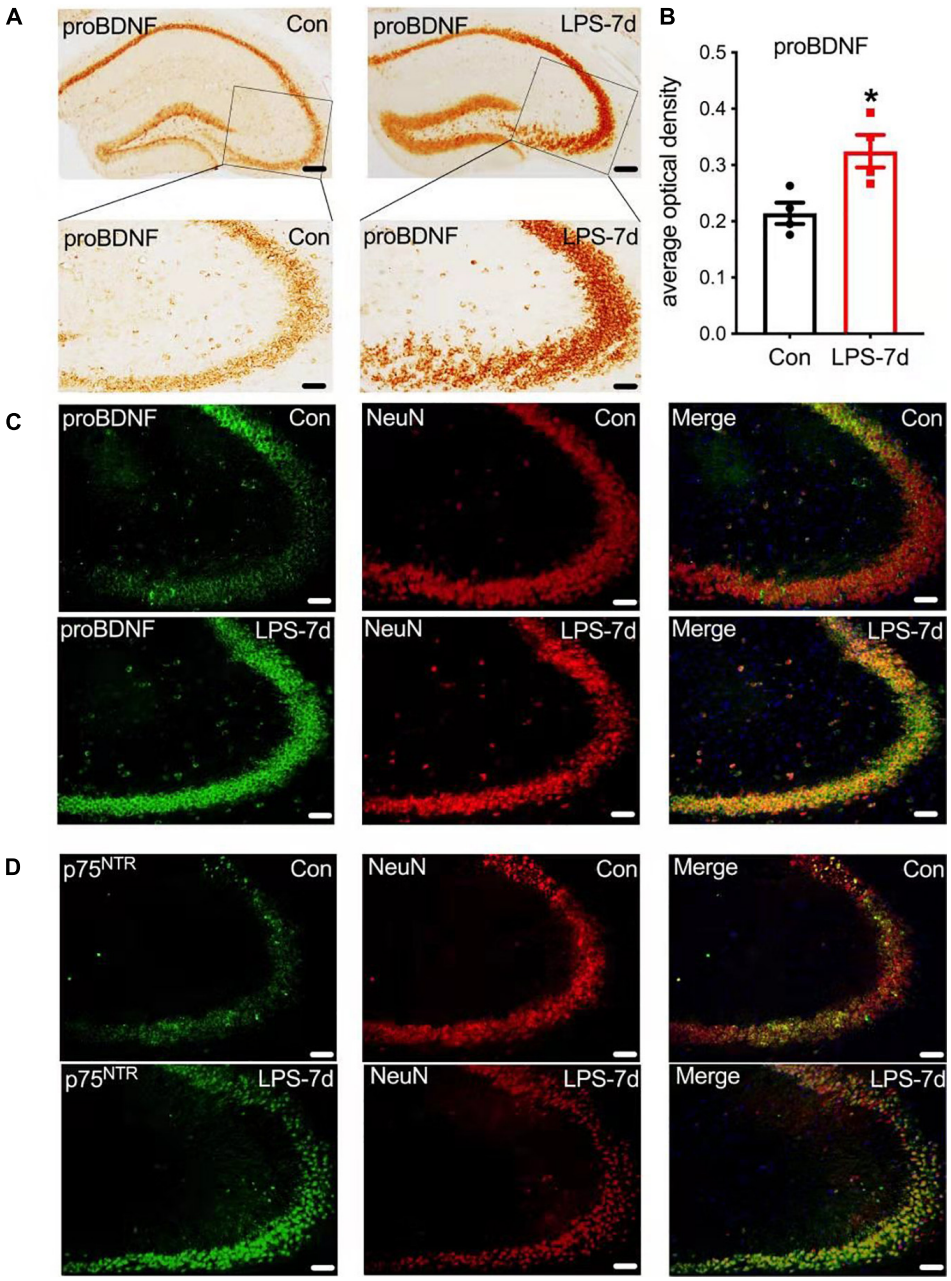

FIGURE 3 | proBDNF and its receptor $p75^{NTR}$ are mainly expressed in hippocampal neurons. **(A,B)** proBDNF accumulated in hippocampal neurons (upper scale bar = 100 μm, lower scale bar = 50 μm, n = 4/group). **(C,D)** proBDNF and its receptor $p75^{NTR}$ mainly co-existed in the neuron (scale bar = 50 μm, n = 4/group). Data are expressed as mean ± SEM. *$p < 0.05$.

(**Figures 3A,B**). To further confirm the distribution of proBDNF and $p75^{NTR}$ in hippocampal cells, we applied dual-color immunofluorescence staining of hippocampus tissue sections from SAE mice (**Figures 3C,D**). The representative tissue stains showed that both proBDNF and $p75^{NTR}$ are expressed mainly in neurons in the hippocampus CA3 region (**Figures 3C,D**). Taken together, the above-mentioned results indicate that upregulated proBDNF may bind $p75^{NTR}$ to affect learning and cognition by regulating the neuron function in SAE mice.

Intrahippocampal Microinjection of the Anti-proBDNF Neutralizing Antibody Improved SAE-Induced Cognitive Dysfunction

To confirm the role of proBDNF in the learning and memory behavior of LPS-induced SAE mice model, we administered the anti-proBDNF neutralizing antibody (mAb-proB) *via* intrahippocampal microinjection. At 2 h before the

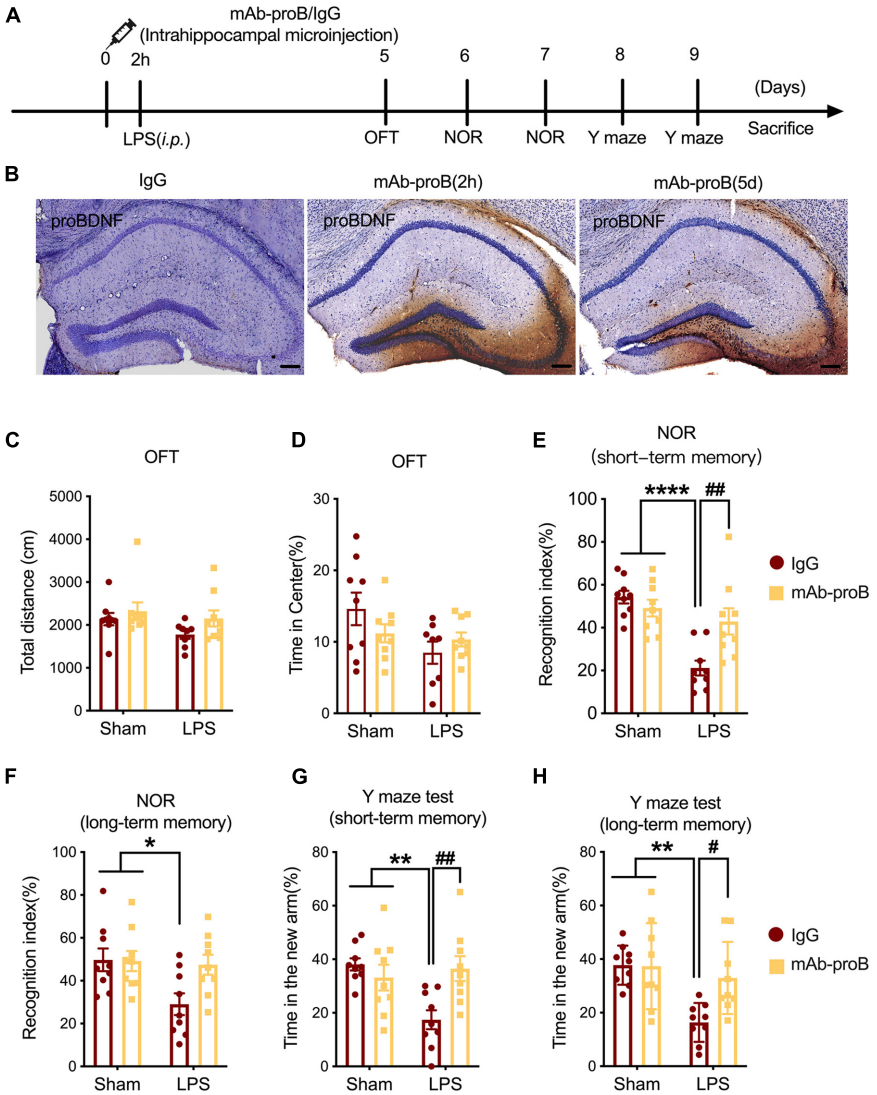

FIGURE 4 | Intrahippocampal microinjection of mAb-proB improved the sepsis-associated encephalopathy-induced cognitive impairment. **(A)** The behavioral intervention flowchart of the experiment. **(B)** The efficiency of neutralizing mAb-proB in hippocampus tissue. Scale bar = 100 μm. **(C,D)** The total traveled distance and percentage of time mice spent in the center of the field *via* open field test (n = 9/group). **(E,F)** The recognition index of short/long-term memory tasks of the novel object recognition test (n = 9/group). **(G,H)** The percentage of the time that mice stay in the novel arm of the short/long-term memory tasks in the Y maze test (n = 9/group). Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, LPS + IgG vs. LPS + mAb-proB.

i.p. injection of normal saline or LPS, the mice underwent intrahippocampal microinjection with 1 μg of mAb-proB or an equal volume of control IgG. The mice treated with normal saline plus IgG or mAb-proB were defined as the Sham + IgG group and Sham + mAb-proB group, respectively. Behavioral tests in the normal saline group were performed from day 5 to 9 after injection to evaluate the influence of mAb-proB on the cognitive behaviors of four different groups of mice (Sham + IgG, Sham + mAb-proB, LPS + IgG, and LPS + mAb-proB; **Figure 4A**).

We first measured the efficiency of mAb-proB by immunohistochemistry staining under the intrahippocampal microinjection. To complete this test, we added secondary antibody to test the neutralizing efficiency of mAb-proB, and IgG was used as a negative control. The representative image shows that mAb-proB was still present from 2 h to 5 days after stereotaxic injection, before we performed the behavioral evaluation (**Figure 4B**).

The locomotive activity and anxiety-like behaviors of the mice were evaluated on the fifth day after mAb-proB stereotaxic injection. The analysis found no significant differences among the four cohorts in the total distance and percentage of time spent in the center square in the OFT experiment (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 31) = 0.3238$, $p > 0.05$ for total distances **Figure 4C**; $F(1, 31) = 2.719$, $p > 0.05$ for percentage of time in center; **Figure 4D**). Compared with mice in the Sham group, LPS induced short/long-term

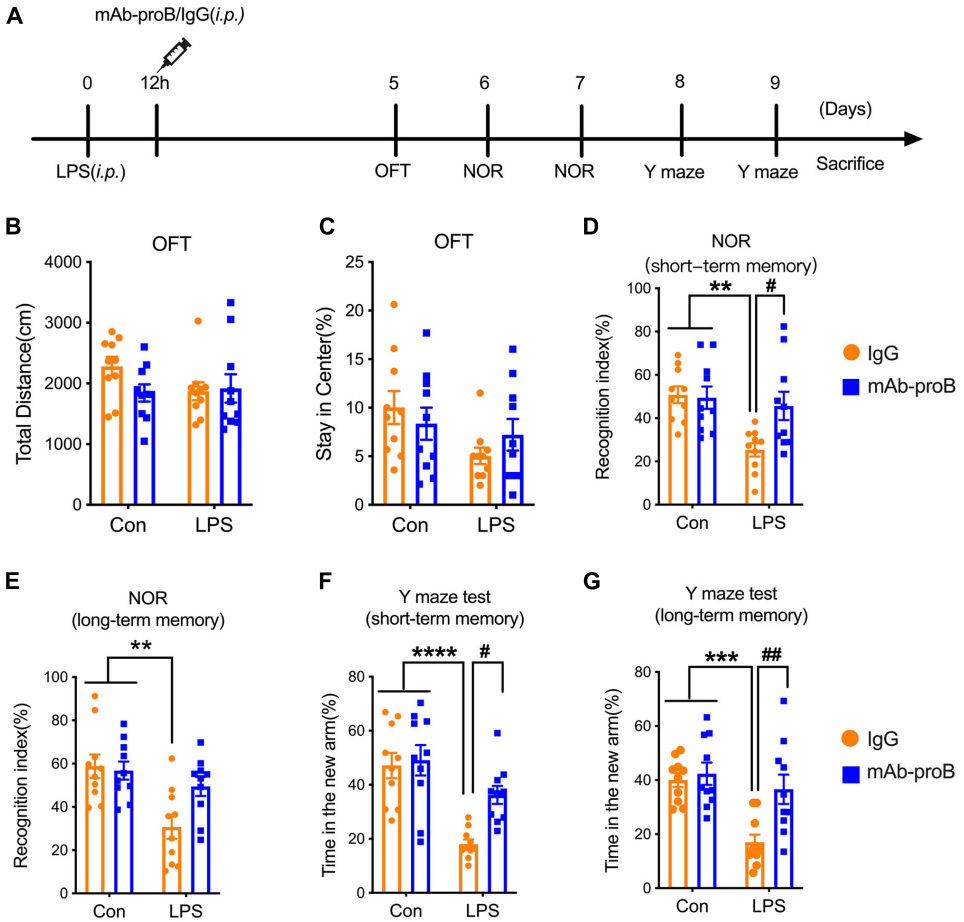

FIGURE 5 | Systemic delivery of neutralizing mAb-proB partially reversed the sepsis-associated encephalopathy-induced cognitive impairment. **(A)** The behavior schedule of the experiment. **(B,C)** The total distance traveled and percentage of time during which mice stay in the center of the field (n = 9–10/group). **(D,E)** The recognition index of the short/long-term memory tasks of the novel object recognition test (n = 10/group). **(F,G)** The percentage of time that mice stay in the novel arm of the short/long-term memory tasks in the Y maze test (n = 10/group). Data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, LPS + IgG vs. LPS + mAb-proB.

memory dysfunction in the LPS + IgG group mice (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 32) = 7.711$, $p < 0.0001$, for NOR short-term memory **Figure 4E**; $F(1, 32) = 6.996$, $p = 0.0283$, for NOR long-term memory; **Figure 4F**; $F(1, 32) = 5.562$, $p = 0.0041$, for Y maze test short-term memory; **Figure 4G**; two-way ANOVA, $F(1, 32) = 5.485$, $p = 0.0026$, for Y maze test long-term memory; **Figure 4H**). While the cognitive index of the NOR short-term memory tasks in the LPS + mAb-proB group mice was noticeably higher than that of the LPS + IgG group (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 32) = 5.063$, $p = 0.0059$, **Figure 4E**), there was no significant difference in the improvement of long-term memory between the LPS + IgG and LPS + mAb-proB groups (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 32) = 3.719$, $p > 0.05$, **Figure 4F**). Additionally, the percentage of time spent in the novel arm of the short/long-term memory tasks of the Y maze test (two-way ANOVA, Tukey's multiple comparisons test; $F(1, 32) = 4.856$, $p = 0.0086$, for short-term memory; **Figure 4G**; $F(1, 32) = 4.265$, $p = 0.0244$, for long-term memory; **Figure 4H**) was noticeably higher in the LPS group mice following mAb-proB treatment.

Systemic Delivery of mAb-proB Partially Reversed the SAE-Induced Cognitive Impairment

While we have demonstrated that an intracerebral injection of mAb-proB can improve SAE-induced cognitive impairment, intracerebral injection is an unlikely delivery method in a clinical setting. Thus, it is important to optimize the delivery of mAb-proB with respect to feasibility and efficiency in our experimental applications. To this effect, we delivered the mAb-proB (100 μg) or the same concentration of mouse IgG *via* i.p. injection 12 h after LPS administration and performed the behavioral tests from day 5 to 9 after LPS administration (**Figure 5A**).

The OFT results showed that the total distance traveled and the percentage of time spent in the center field was similar

between the four groups (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 1.956$, $p > 0.05$ for total distances; **Figure 5B**; $F(1, 36) = 1.656$, $p > 0.05$ for percentage of time in center; **Figure 5C**). From the NOR and Y maze tests, we found that LPS administration decreased the short/long-term memory in the SAE mice group (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 5.233$, $p = 0.0038$, for ORT short-term memory; **Figure 5D**; $F(1, 36) = 5.673$, $p = 0.0016$, for ORT long-term memory; **Figure 5E**; $F(1, 36) = 7.075$, $p < 0.0001$, for Y maze test short-term memory; **Figure 5F**; $F(1, 36) = 5.905$, $p = 0.0010$, for Y maze test long-term memory; **Figure 5G**). Compared with the LPS + IgG group, we found that mAb-proB treatment noticeably rescued the short-term memory impairment of SAE mice (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 4.173$, $p = 0.0272$, **Figure 5D**) but had no effect on the long-term memory function in the NOR experiment (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 3.791$, $p > 0.05$, **Figure 5E**). The results of the Y maze test showed that the mAb-proB treatment increased the time that the mice stayed in the novel arm not only with regards to short-term memory (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 4.426$, $p = 0.0174$, **Figure 5F**) but also with long-term memory (two-way ANOVA, Tukey's multiple-comparisons test; $F(1, 36) = 5.030$, $p = 0.0057$, **Figure 5G**).

MAb-proB Enhanced the Expression of NeuN and Synapse-Associated Proteins

To investigate the mechanism of systemic mAb-proB delivery on enhancing cognitive function in SAE mice, we assessed the levels of synapse-associated proteins, Nissl bodies, and NeuN-positive neuronal cells (**Figure 6**). Compared with the control group, the systemic administration of LPS resulted in the decreased expression of Nissl bodies and NeuN-positive neuronal cells (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 5.916$, $p = 0.0035$, for Nissl staining; **Figures 6A,B**; $F(3, 16) = 6.228$, $p = 0.0023$, for NeuN staining; **Figures 6A–C**), while mAb-proB treatment can improve the expression of Nissl bodies (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 4.385$, $p = 0.0314$, **Figure 6A**) and NeuN (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 4.725$, $p = 0.0195$, **Figures 6A–C**) in the hippocampus of SAE mice.

Next, we assessed the levels of synapse-associated proteins at 7 days after mAb-proB treatment in the SAE mice. The immunoblot analysis showed that the expression of GluR4 (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 4.761$, $p = 0.0185$), NR1 (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 4.089$, $p = 0.0472$), NR2A (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 4.966$, $p = 0.0138$), and NR2B (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 7.498$, $p = 0.0004$) decreased remarkably in the hippocampus after LPS injection (**Figures 6D,E**). Compared with the LPS + IgG group, the systemic delivery of mAb-proB resulted in an increase in the hippocampal protein levels of GluR4 (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 7.142$, $p = 0.0006$), NR1 (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 5.426$, $p = 0.0071$), NR2A (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 5.225$, $p = 0.0095$), NR2B (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 5.324$, $p = 0.0244$), and PSD95 (one-way ANOVA, Tukey's multiple-comparisons test; $F(3, 16) = 5.282$, $p = 0.0248$) (**Figures 6D,E**).

To understand the effects of mAb-proB treatment on the expression of BDNF, TrkB, and sortilin in the hippocampus, BDNF, TrkB, and sortilin expression was analyzed by RT-qPCR. Compared with the IgG group, the mAb-proB treatment of SAE mice had no effect on the gene expression of BDNF, TrkB, and sortilin (**Supplementary Figure 1**).

Taken together, these results suggest that mAb-proB may improve learning and memory dysfunction through enhancing the expression of NeuN and synapse-associated proteins in the hippocampus of SAE mice.

DISCUSSION

This study illustrated the role of proBDNF in the regulation of learning and memory dysfunction in SAE mice. We found that SAE induced cognitive impairment in mice and was associated with an increased expression of proBDNF and $p75^{NTR}$ and a decreased expression of BDNF and TrkB in the hippocampus. Furthermore, intrahippocampal microinjection or systemic delivery of neutralizing mAb-proB attenuated the cognitive impairment of SAE mice. The mechanism by which mAb-proB (i.p.) ameliorates SAE-induced cognitive impairment may be related to the enhanced expression of NeuN and synapse-associated proteins in the hippocampus.

Studies reported SAE as an early indication of sepsis; a critical clinical sign for SAE is cognitive dysfunction (Gofton and Young, 2012). As a major component of Gram-negative bacteria cell wall (Tang et al., 2007), LPS was reported to induce neuron toxicity (Zhu et al., 2015), brain dysfunction, and memory disorders (Choi et al., 2017; Han et al., 2018). LPS injection is a widely used and easily replicated experimental model of SAE. This model induces an overwhelming activation of the innate immune system, which has a number of similarities to SAE (Fink, 2014; Liao et al., 2020). Hence, we performed ORT and Y maze tests to evaluate the short- and long-term learning and memory behavior in this LPS-based mice model. In this study, after 5 days of LPS administration, serious short- and long-term cognitive impairment was observed in SAE mice in ORT and Y maze tests, while no anxiety-like behaviors was found *via* the OFT experiment (**Figure 1**). Thus, systemic LPS administration led to cognitive impairment in the SAE mouse model.

The hippocampus is the key region for learning and memory (Zeidman and Maguire, 2016; Fares et al., 2019; Zeidman and Maguire, 2016; Fares et al., 2019) which is frequently and easily affected by inflammation (Zong et al., 2019). However, the mechanism of SAE-induced cognitive impairment has not been fully elucidated. Studies indicated that the downregulation of neurotrophic factors, especially BDNF, plays an important role in cognitive impairment and neurodegeneration (Fleitas et al., 2018; Caffino et al., 2020). BDNF is one of the neurotrophic

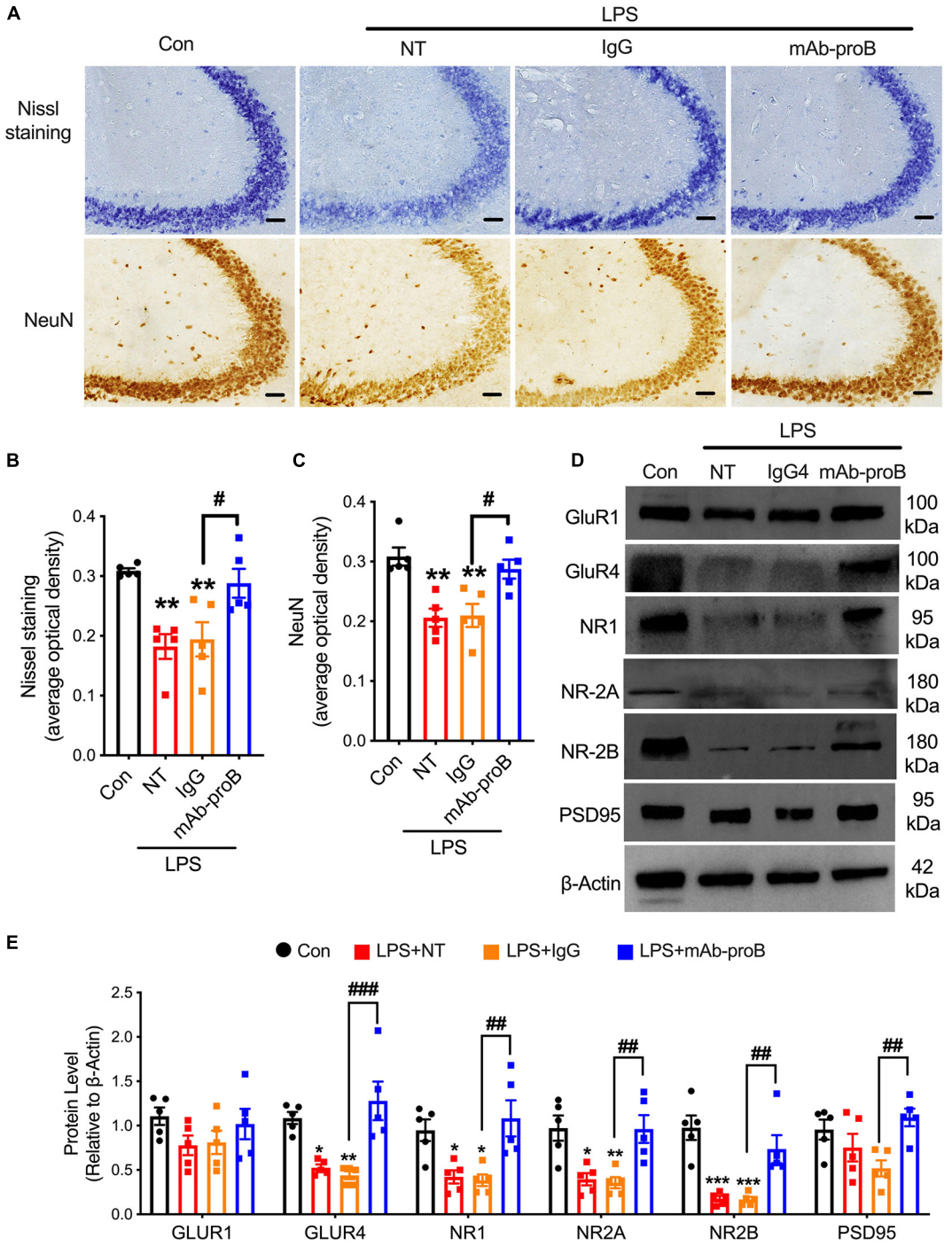

FIGURE 6 | MAb-proB upregulated the expression of neuronal cells and synapse-associated proteins in sepsis-associated encephalopathy mice. **(A–C)** Representative images and quantitation of Nissl bodies and NeuN-positive neuronal cells (scale bar = 50 μm, n = 4/group). **(D,E)** Immunoblot analysis of GluR1, GluR4, NR1, NR-2A, NR-2B, and PTSD95 (n = 5/group). Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, LPS + IgG vs. LPS + mAb-proB.

factors that are expressed widely in the brain, regulating neuronal survival, synaptic transmission, synaptic plasticity, and learning and memory (Song et al., 2017). Della Giustina et al. (2020) found that BDNF was remarkably decreased on day 10 after sepsis induction in rats, and those animals displayed a severe cognitive impairment. Pang et al. (2004) reported that BDNF supported neuronal survival, long-term potentiation, and learning and memory. As the precursor of BDNF, proBDNF, other than

producing BDNF, also binds to its receptors to perform multiple biological functions in the CNS (Luo et al., 2016). Upregulated proBDNF-induced apoptosis suppressed hippocampal neuron remodeling (Montroull et al., 2019), synaptic transmission (Yang et al., 2009), and synaptic plasticity (Yang et al., 2014). Increased proBDNF and its receptor $p75^{NTR}$ were detected in SAE mice (Thomas et al., 2016; Ji et al., 2018). In our study, we identified that proBDNF and $p75^{NTR}$ increased steadily upon LPS injection and reached a peak on day 7 after LPS administration. Concurrently, the expression of BDNF and its receptor TrkB decreased in the hippocampus of SAE mice (**Figure 2**). These results suggest that the imbalance between proBDNF and BDNF might be associated with cognitive impairment in the SAE model. Additionally, immunofluorescence staining revealed that proBDNF and $p75^{NTR}$ are mainly expressed in neurons (**Figure 3**). Thus, elevated proBDNF may bind to p75NTR and affect learning and cognition by regulating the neuron function in SAE mice.

Previous studies showed that mAb-proBDNF treatment leads to the growth of synapses and the improvement of emotional disorders in animal models (Sun et al., 2012; Zhong et al., 2019). An et al. (2018) reported that a hippocampus injection of mAb-proBDNF promotes the location learning strategy of rats. Additionally, an intraperitoneal injection of mAb-proBDNF remarkedly alleviated emotional dysfunction (Bai et al., 2016; Zhong et al., 2019). We also observed that an intraperitoneal injection of mAb-proBDNF highly alleviated the fear conditioning memory in SAE mice, which was accomplished by perturbing the peripheral proinflammatory response (Luo et al., 2020). In our latest study, we confirmed that mAb-proBDNF treatment can attenuate multiple sclerosis in a mouse model and inhibit the proinflammatory response in the spinal cord and spleen of diseased mice (Hu et al., 2021). In the current study, our results showed that both intrahippocampal microinjection (**Figure 4**) and the systemic delivery (**Figure 5**) of mAb-proBDNF improved the cognitive impairment in SAE mice, this may be due to the inhibition of the inflammatory response of the CNS and/or peripheral system.

Interestingly, our results also found that mAb-proBDNF is not helping to improve long-term memory impairment in the NOR test. The hippocampus was known to be involved in object memorization and long-term object recognition (Reger et al., 2009; Antunes and Biala, 2012). Studies have shown that the downregulation of BDNF impaired long-term, but not short-term, memory recognition (Alonso et al., 2002; Lee et al., 2004; Seoane et al., 2011). BDNF was shown to regulate object recognition memory reconsolidation through the induction of long-term potentiation (Radiske et al., 2017; Rossato et al., 2019). In our study, we found that mAb-proBDNF treatment did not influence the expression of BDNF, TrkB, and sortilin in the hippocampus (**Supplementary Figure 1**). This indicates that downregulated BDNF may impair the long-term memory of novel objects in SAE mice. Notably, it is well established that i.p. LPS (10 mg/kg) can disrupt the integrity of the blood–brain barrier (BBB) in mice (Liu et al., 2020). Taken together, we speculate that mAb-proBDNF may cross the BBB to regulate learning and memory dysfunction directly in the SAE mice.

To further explore the function of systemic mAb-proBDNF treatment in SAE mice, we assessed the expression of NeuN and Nissl bodies in the hippocampus. An i.p. LPS administration resulted in a marked decrease in the expression of Nissl bodies and NeuN, while a systemic mAb-proB treatment enhanced the expression of those proteins (**Figure 6**). NeuN, a neuron-specific nuclear protein located in the neuronal nucleus, is involved in the regulation of mRNA splicing and played a role in regulating neural cell differentiation and the development of the nervous system (Kim et al., 2009; Li et al., 2015; Duan et al., 2016). The Nissl body was composed of many rough endoplasmic reticula and scattered ribosomes which synthesize the proteins during organelle renewal (Kádár et al., 2009). These results suggested that mAb-proB may regulate the function of hippocampal neuronal cells in LPS-induced SAE mice.

Synapse-associated proteins, such as NMDA receptors and PSD95, play a key role in learning and memory. Previous studies suggested that synaptic protein damage was associated with cognitive impairment (Zhang et al., 2017; Merino-Serrais et al., 2019). The GluR2B levels in the frontal cortex decreased 28 h after LPS treatment (Savignac et al., 2016), and the selectivity of NMDA receptors was also reduced (Zhang et al., 2017). Consistent with previous studies (Liraz-Zaltsman et al., 2016; Zhang et al., 2017; Muhammad et al., 2019), we found that the GluR4, NR1, NR2A, and NR2B levels decreased in hippocampal tissue on day 7 after LPS administration in SAE mice. Interestingly, the expression of GluR4, NR1, NR2A, NR2B, and PSD95 can be rescued after mAb-proB treatment (**Figure 6**). In summary, these data indicate that the neutralizing proBDNF antibody may improve the learning and memory dysfunction *via* enhancing the function of neuronal cells and the expression of synapse-associated proteins in the hippocampus in a mouse model of sepsis.

The current therapies of SAE disease are, to a large extent, hampered by the inability of drugs to cross the BBB. Thus, targeting the BBB should be incorporated as part of a short- and long-term therapeutic strategy in sepsis patients. The proBDNF antibody acts as a macromolecular substance; under normal circumstances, it is difficult to penetrate the BBB. In our study, we speculated that the peripheral delivery of the proBDNF antibody may partially reach the brain and play a regulatory function under the LPS-induced SAE disease mice model. Nevertheless, the ability and efficiency of the proBDNF antibody to penetrate the BBB should be improved in our future study. Recent studies by Kariolis et al. (2020) and Ullman et al. (2020) provided a feasible method to modify the BBB penetration ability of proBDNF antibodies. Using the Fc fragment BBB transport vehicle platform to design the fusion protein combined with the proBDNF antibody will help to improve the penetration ability of the proBDNF antibody into the brain. This method will provide more possibilities for the proBDNF antibody for the treatment of human CNS diseases, such as SAE. In addition, $p75^{NTR}$ is the main high-affinity receptor of proBDNF. Therefore, the development of BBB-permeable small-molecule $p75^{NTR}$ signaling modulator also has the potential to treat pathogenetic diseases related to proBDNF.

CONCLUSION

In conclusion, the present study reports a regulatory function of proBDNF on hippocampal neuronal cells and its detrimental role in the pathogenesis of SAE. Treatment with mAb-proBDNF may effectively attenuate cognitive impairment and represent a potential therapeutic strategy for treating patients with SAE.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/ **Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Central South University.

AUTHOR CONTRIBUTIONS

Y-HC, S-FZ, Z-LH, and C-QL contributed to conception and design of the study. S-FZ organized the database. SW performed the statistical analysis. Y-HC wrote the first draft of the manuscript. YL, S-FZ, R-PD, and FL wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This research was supported by the National Natural Science Foundation of China (31371212 to C-QL, 81771354 and 81471106 to R-PD, 81471372 to FL, and 81901231 to Z-LH), the Natural Science Foundation of Hunan Province, China (2018JJ3635 to FL and 2019JJ40369 to C-QL), China Postdoctoral Science Foundation (no. 2020M672516 to Z-LH), and the Hunan Province Science Foundation for Young Scientists of China (2018JJ3864 to SW and 2020JJ5809 to Z-LH).

ACKNOWLEDGMENTS

We would like to thank Shanghai Yile Biotechnology Corp. for providing the monoclonal anti-proBDNF antibody and AiMi Academic Services (www.aimieditor.com) for English language editing and review services.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.665757/full#supplementary-material

Supplementary Figure 1 | mAb-proB has no impact on the expression of BDNF, TrkB, and sortilin in the hippocampus of sepsis-associated encephalopathy mice. The expression levels of BDNF **(A)**, TrkB **(B)**, and sortilin **(C)** in the hippocampus tissue were measured by RT-qPCR (n = 6/group). Data are expressed as mean ± SEM.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 09 September 2021
doi: 10.3389/fnins.2021.735928

Nerve Growth Factor-Based Therapy in Alzheimer's Disease and Age-Related Macular Degeneration

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OPEN ACCESS

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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: 03 July 2021
Accepted: 10 August 2021
Published: 09 September 2021

Citation:
Amadoro G, Latina V, Balzamino BO, Squitti R, Varano M, Calissano P and Micera A (2021) Nerve Growth Factor-Based Therapy in Alzheimer's Disease and Age-Related Macular Degeneration. Front. Neurosci. 15:735928. doi: 10.3389/fnins.2021.735928

Alzheimer's disease (AD) is an age-associated neurodegenerative disease which is the most common cause of dementia among the elderly. Imbalance in nerve growth factor (NGF) signaling, metabolism, and/or defect in NGF transport to the basal forebrain cholinergic neurons occurs in patients affected with AD. According to the cholinergic hypothesis, an early and progressive synaptic and neuronal loss in a vulnerable population of basal forebrain involved in memory and learning processes leads to degeneration of cortical and hippocampal projections followed by cognitive impairment with accumulation of misfolded/aggregated Aβ and tau protein. The neuroprotective and regenerative effects of NGF on cholinergic neurons have been largely demonstrated, both in animal models of AD and in living patients. However, the development of this neurotrophin as a disease-modifying therapy in humans is challenged by both delivery limitations (inability to cross the blood–brain barrier (BBB), poor pharmacokinetic profile) and unwanted side effects (pain and weight loss). Age-related macular degeneration (AMD) is a retinal disease which represents the major cause of blindness in developed countries and shares several clinical and pathological features with AD, including alterations in NGF transduction pathways. Interestingly, nerve fiber layer thinning, degeneration of retinal ganglion cells and changes of vascular parameters, aggregation of Aβ and tau protein, and apoptosis also occur in the retina of both AD and AMD. A protective effect of ocular administration of NGF on both photoreceptor and retinal ganglion cell degeneration has been recently described. Besides, the current knowledge about the detection of essential trace metals associated with AD and AMD and their changes depending on the severity of diseases, either systemic or locally detected, further pave the way for a promising diagnostic approach. This review is aimed at describing the employment of NGF as a common therapeutic approach to AMD and AD and the diagnostic power of detection of essential trace metals associated with both diseases. The multiple approaches employed to allow a sustained release/targeting of NGF to the brain and its neurosensorial ocular extensions will be also discussed, highlighting innovative technologies and future translational prospects.

Keywords: neuroprotection, brain degeneration, trace metals, Alzheimer's disease, age-related macular degeneration, nerve growth factor, retinal degeneration, biomarkers

INTRODUCTION

As an integral part/extension of the central nervous system (CNS), ocular structures display several cytological, anatomical, and developmental similarities with the brain so that the retina and cerebral areas, receiving directly (visual system) and indirectly (amygdala, hippocampus, and other hypothalamic nuclei) its inputs, constitute the so-called "eye–brain connection" (Erskine and Herrera, 2014; Tirassa et al., 2018). Because of the biological connection between the brain and eyes, analogous and corresponding pathological processes can lead to dysfunction of both of them. Oxidative injury, chronic activation of inflammatory pathway(s), mitochondrial impairment, vascular changes, neurotrophin(s) imbalance, protein quality control deficits and proteostasis, metal ion dyshomeostasis, genetics causes, or a combination of them are proposed to be involved in age-related disorders featured by brain and retinal degeneration, including Alzheimer's disease (AD) and age-related macular degeneration (AMD; Kaarniranta et al., 2011; Masuzzo et al., 2016; Xu et al., 2018; Acevedo et al., 2019; Micera et al., 2019; Mufson et al., 2019; Wang and Mao, 2021). AD is a complex and multifactorial neurodegenerative disorder representing the main cause of dementia in the elderly (Walsh and Selkoe, 2004; Querfurth and LaFerla, 2010). The extracellular neuritic plaques, composed of β-amyloid (Aβ), and the intracellular neurofibrillary tangles (NFTs), composed of hyperphosphorylated tau protein, are the two key neuropathological hallmarks associated with this disease. Both Aβ plaques and NFTs impair synaptic plasticity and neural circuit networks causing the clinical symptoms of progressive memory loss (Spires-Jones and Hyman, 2014; Masters et al., 2015). AMD is a late-onset retinal neurodegenerative disorder representing the major cause of blindness in Western countries (Fine et al., 2000; Gehrs et al., 2006; Jager et al., 2008; Çerman et al., 2015). Accumulation of insoluble extracellular aggregates called drusen in the retina, degeneration of retinal pigment epithelium (RPE) cells, and choroidal neovascularization (CNV) manifest in AMD-suffering subjects leading to irreversible loss of vision (Ambati and Fowler, 2012; van Lookeren Campagne et al., 2014).

Here, we discuss how the understanding of the common pathophysiological molecular mechanisms linking these two overlapping disorders will help in the development of novel diagnostic/prognostic biomarkers and effective therapeutic avenues. Furthermore, the power of the nerve growth factor (NGF) in maintaining the functional phenotype(s) of different neuronal populations and retinal cells along with the recent optimization of innovative strategies allowing both local and systemic delivery of this neurotrophin jointly prospects its utilization as a promising, neuroprotective option for the treatment of both AD and AMD.

ALZHEIMER'S DISEASE AND AMD PATHOPHYSIOLOGY: SIMILARITIES AND DIFFERENCES

Epidemiological, genetic, pathological, and clinical lines of evidence have documented a strong relationship between AD and AMD (Wang and Mao, 2021) which have prompted scientists to speculate that AMD could be a form of "Alzheimer's disease in the eye" (Kaarniranta et al., 2011; Ohno-Matsui, 2011) (**Figure 1**).

To better understand the burden on public health of these two age-related multifactorial neurodegenerative disorders, it is worth pointing out that the prevalence of AD is estimated to be 46 million of cases worldwide (Brookmeyer et al., 2007; Reitz and Mayeux, 2014), with the number of AD patients expected to triple in 2050 (Baumgart et al., 2015), reaching 131 million of cases (Prince et al., 2013). In a similar way, 15% (age 65–74), 25% (age 75–84), and 30% (age 85% or more) of subjects are affected with AMD (Klein et al., 2004; Wong et al., 2014). Indeed, investigations have demonstrated that, in addition to a strong genetic component, aging is the principal common risk factor for both AD and AMD (Coleman et al., 2008; Mayeux and Stern, 2012; Rudnicka et al., 2012). Genetic heritable components play a role in the development of AD, with rare autosomal mutations in the three genes *APP*, *PSEN1*, and *PSEN2* and the APOE epsilon-4 allele associated with familial early-onset and sporadic late-onset AD forms, respectively (Mayeux and Stern, 2012). On the other hand, CFH (encoding for a complement inhibitor factor) and ARMS2/HTRA1 (encoding for a serine protease) are linked with AMD (Logue et al., 2014; Woo et al., 2015; Tsiloulis et al., 2016). Interestingly, genome-wide association studies (GWAS) have shown that single nucleotide polymorphisms (SNPs) in *ABCA7* (encoding for ATP-binding cassette transporter subfamily A member 7), *HGS* (encoding for a member of the clathrin-mediated endocytosis signaling pathway), and *PILRA/ZCW1P1* genes (encoding for the paired-immunoglobulin-like type 2 receptor which binds with herpes simplex virus-1 and for

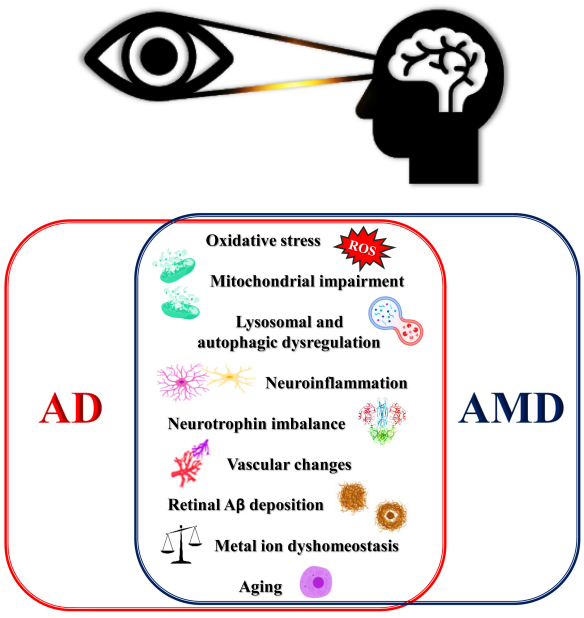


FIGURE 1 | Schematic for overlapping degenerative mechanisms shared by AMD and AD. These pathways have been associated with brain and retina degeneration, cognitive, deficits and loss of vision. AD, Alzheimer's disease; AMD, age-related macular degeneration; ROS, reactive oxygen species.

a zinc finger protein functioning as a histone modification, respectively) are also common in the pathogenesis of both AMD and AD (Ding et al., 2009; Kaarniranta et al., 2011). Besides, hypercholesterolemia, hypertension, atherosclerosis, obesity, diabetes, and environmental risk factors also strongly contribute to the development of both AD and AMD in mid-life (Kivipelto et al., 2001, 2002, 2005; Newman et al., 2005; Polidori et al., 2001; Seddon et al., 2005; Age-Related Eye Disease Study Research Group (AREDSRG), 2000; Kaarniranta et al., 2011; Ohno-Matsui, 2011). Relevantly, patients suffering AMD exhibit an increased risk of developing AD as compared with people without AMD (Frost et al., 2016; Wen et al., 2021), even though a direct association between these two disorders has not been confirmed (Williams et al., 2014). Besides, compelling studies have demonstrated that vision abnormalities featured by degeneration of the visual cortex and/or RGC loss or AMD-like retinal degeneration are prominent in AD patients and manifest even before clinical signs of cognitive decline (Ashok et al., 2020).

The phenotypic parallelism between AD and AMD is strongly highlighted by experimental evidence that the beta-amyloid (Aβ) peptide – the main biochemical component of extracellular senile plaques featuring the AD brain – is also found in ocular drusen deposits characterizing AMD (Anderson et al., 2004). In particular, these latter inclusions generally localized in the macula, the central region of the retina, are focal depositions of acellular debris accumulating between the basal lamina of postmitotic neuroepithelial cells called RPE – which take care of neural cells, rods, and cones – and the inner collagenous layer of the Bruch's membrane (Usui et al., 2018; Blasiak, 2020). The majority of patients manifest a dry form of AMD which is characterized by the presence of amorphic drusens classified as "hard" and "soft" drusen. "Hard" drusen is focal and thickening of the basement membrane of RPE, whereas "soft" drusen is hardening confined to the separation of the basement membrane from Bruch's membrane at the inner collagenous zone. On the other hand, pseudodrusen, which is associated with clinical progression to more severe stages of AMD, is a deposit located above the basement membrane of RPE (Miller, 2013). Advanced AMD manifests with progressive RPE deterioration and develops into the exudative AMD (eAMD) with CNV or non-exudative AMD (neAMD) with geographic atrophy (GA) in which drusen coalesces and causes damage to RPE and photoreceptors (Jindal, 2015; Hernandez-Zimbron et al., 2018). Interestingly, the amyloid deposits possessing a central core with non-radiating fibrils have been also detected in the inner layers of the retina of AD patients, in contrast to classical/neuritic plaques found in their brain (Koronyo-Hamaoui et al., 2011). Relevantly, just as in AD, the role of these deposits is controversial in AMD and other types of macular degeneration since it is still unclear whether they are causing the RPE dysfunction or are a mere consequence of the impairment of the RPE function (Biscetti et al., 2017). In addition to Aβ, several protein and lipid constituents of drusens – including clusterin, vitronectin, amyloid P, esterified cholesterol and phosphatidylcholine, apolipoprotein E, and inflammatory mediators, such as acute phase reactants and complement components (C5, C5b9, and C3 fragments) – are also present in insoluble cerebral aggregates of AD (Mullins et al., 2000; Crabb et al., 2002; Luibl et al., 2006; Isas et al., 2010; Wang et al., 2010). Likewise, metal elements such as Zn^{2+}, Fe^{3+}, Cu^{2+}, ubiquitin, and lipofuscin account for the biochemical composition of lesions from both AD and AMD (Atwood et al., 2002; Ryhänen et al., 2009; Ohno-Matsui, 2011). Although rodents have neither functional macula nor sharp vision in the retina, an important demonstration of commonalities occurring between AMD and AD is represented by the human APOE4-expressing transgenic mice, an animal model carrying the APOE4 allelic variant which is one of the major risk factors for AD onset/progression. Interestingly, old animals of this strain, when fed a high-fat diet, exhibit an ocular phenotype which resembles several crucial features of AMD. Besides, their retinal as well as memory dysfunction is strongly attenuated by the delivery of antibody Aβ-neutralizing (Ding et al., 2008, 2011). Additional parallel pathophysiological events occurring in both AMD and AD pathogenesis encompass increased oxidative stress and mitochondrial and lysosomal dysfunctions. Owing to the relative enrichment of unsaturated lipids, the abundance of redox-active transition metals, the modest antioxidant defense, the neurotransmitter auto-oxidation and RNA oxidation, and the Ca^{2+} signaling along with the high energetic demand, the brain is largely susceptible to oxidative stress especially at terminal synaptic ends (Cobley et al., 2018). In a corresponding manner, the retina is particularly prone to undergo injury linked to excessive generation of reactive oxygen species (ROS) due to its high oxygen consumption, exposure to continuous light, high intracellular levels of polyunsaturated fatty acids (PUFAs) in photoreceptor outer segments, presence of photosensitizers in the RPE and neurosensory retina, and daily phagocytosis of the retinal outer segment originating from rods and cones (Beatty et al., 2000; Fine et al., 2000; Klein et al., 2004; Buch, 2005; Gehrs et al., 2006; Katta et al., 2009). Thus, it is not surprising that proteins isolated from RPE cells are heavily modified by oxidative stress markers, including malondialdehyde, 4-hydroxynonenal, AGE, and RAGE modifications (Schutt et al., 2002, 2003). Degradation-resistant aggregates of lipofuscin consisting of cross-linked, oxidized proteins and lipids also accumulate in the brain and in the eye of AD and AMD in association with the occurrence of pro-oxidant environmental conditions, especially those caused by light stimulation (Kaarniranta et al., 2011). Damage of mtDNA, which is more vulnerable than nuclear DNA to damage from oxidation and blue light, is proven to accumulate during aging in the retina along with changes in morphology and function of respiration-competent mitochondria (Barreau et al., 1996; Ballinger et al., 1999; Barron et al., 2001; Liang and Godley, 2003; King et al., 2004; Godley et al., 2005; Feher et al., 2006). Correspondingly, mitochondrial dysfunction and oxidative injury marked by peroxidation, nitration, reactive carbonyls, and nucleic acid oxidation are early and prominent into selectively damaged hippocampal and cortical neuronal populations in AD brain (Nunomura et al., 2001; Ganguly et al., 2021; Ke et al., 2021). The autophagic, lysosomal, and proteasomal signal transduction pathways, the main protein quality control mechanisms endowed with the capacity of repairing oxidative stress-induced damages, are also

altered both in AMD eyes and AD brain (Kaarniranta et al., 2011). This imbalance in clearance systems likely causes an intracellular buildup in misfolded/damaged proteins, including Aβ and phospho-tau (ptau; Loeffler et al., 1993; Johnson et al., 2002; Ohno-Matsui, 2011), which ends up in the formation of detrimental insoluble aggregates in the brain and in the eye (Kaarniranta et al., 2010; Bruni et al., 2020), as largely shared both in these neurodegenerative diseases. Impaired or insufficient autophagy activity is implicated in numerous age-related degenerative diseases, including AMD (Kaarniranta et al., 2011) and AD (Rami, 2009; Wong and Cuervo, 2010), since postmitotic, terminally differentiated neurons are largely sensitive to stress in degradative intracellular pathways leading to proteostasis. The neurotoxic intracellular Aβ, present in late endosomes and lysosome compartments of retinal neurons from aged familial AD mice models showing AMD pathology, is more likely to undergo release into cytosolic compartment by provoking destabilization/leaking of the lysosome membrane (Park et al., 2017; Habiba et al., 2020). This further corroborates the finding that an impaired autophagy is involved in Aβ-dependent eye neurodegeneration (Golestaneh et al., 2017). Markers of activated autophagy colocalize with Aβ deposits, especially in aged human RPE cells and specimens from postmortem AMD subjects, and clearly display impairment of an autophagic pathway in the retina (Mitter et al., 2014). In transgenic animal models expressing E693Δ mutation (referred to as the "Osaka" mutation) of amyloid precursor protein (APP), the age-dependent accumulation of intraneuronal Aβ oligomers causes leakage of cathepsin D from endosomes/lysosomes into the cytoplasm, cytochrome c release from the mitochondria, and activation of caspase-3 in the hippocampi of 18-month-old mice (Umeda et al., 2011) as well. Neuroinflammation is considered to crucially take part in neuronal loss and deposition of extracellular aggregates both in AD retina and brain. In particular, microglial cells, which are phagocytes of the CNS, have functional similarities with RPE cells, macrophages, or dendritic cells in AMD, and when activated, all of them secrete similar inflammatory mediators. The phagocytic activity of reactive microglial cells engulfing and degrading Aβ is progressively lost in close proximity to the fibrillary plaque into the brains of AD patients, while chronically activated neuroglia start to release damaging chemokines and cytokines – notably IL-1, IL-6, and tumor necrosis factor α – which further contribute to affect the protein degradation systems and neuronal viability. Likewise, under pathological conditions, microglia migrating into the subretinal space from the inner retina is early activated by Aβ deposited in the drusen with the production of different types of neurotoxic cytokines which drive both the protein aggregation and cell death during development and progression of AMD (Ohno-Matsui, 2011). Astrocyte activation is also present around drusens and in the subretinal space and senile plaques in AMD and AD specimens (Sivak, 2013). In addition to chronic oxidative stress, complement activation is a central mechanism in both pathologies with classical and alternative pathways preferentially involved in AD and AMD, respectively (McGeer et al., 2005; McGeer and McGeer, 2010). Colocalization of Aβ and iC3b immunoreactivity have been detected in the amyloid vesicles within the drusens with markers of chronic complement activation, such as C3d and C4 being elevated in the plasma of both AMD and AD patients (Ohno-Matsui, 2011). Proteins of the acute phase can be found both in the drusens and senile plaques (McGeer and McGeer, 2004; Donoso et al., 2006). Due to oxidative stress and inflammation, secondary neovascularization specifically manifests in exudative AMD with new vessels sprouting from the choroidal capillaries through the Bruch's membrane into the sub-RPE space or into the retinal layer (Miller, 2013; Wang and Mao, 2021). Aβ accumulation, alterations of blood flow dynamics, and an increased endothelial cell apoptosis have been detected in the retina and choroidal vessels of complement factor H knockout mice [Cfh(−/−)], suggested as a valuable model for AMD mice (Aboelnour et al., 2016). Similarly, angiogenesis hallmark is featured in the hippocampus of postmortem human brain tissues from patients with AD by an elevated immunoreactivity of integrin aVb3, a dimeric glycoprotein expressed on the endothelial cell surface markedly increased on angiogenic vessels (Boscolo et al., 2007; Desai et al., 2009). Nevertheless, even though many parallel pathophysiological aspects are shared by both AMD and AD, several concerns have challenged the development of common biomarkers and effective therapies for their clinical management. For instance, the genetic background is completely different between AMD and AD. Besides, even though Aβ is deposited both in the brain and in drusens, the phenotypic traits characterizing AD and AMD are quite dissimilar. Extracellular Aβ-containing deposits are observed as drusens into sub-RPE space in AMD and as senile plaques into the hippocampus and cortex (brain), ganglion cell layer (retina), and around retinal vessels in AD. The main cell types affected in AMD are the RPE cells, whereas in AD, the hippocampal neural cells are primarily damaged. Again, Aβ accumulation occurs outside the outer blood–retinal barrier in AMD and inside the blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier in AD. Collectively, these findings suggest that AMD should be better considered as a subtype of "amyloid disease" with several similarities and differences with AD (Ohno-Matsui, 2011; Wang and Mao, 2021).

NEUROTROPHIN IMPAIRMENT IN AD AND AMD: THE NEUROPROTECTIVE AND REGENERATIVE ACTIONS OF NGF DELIVERY IN THE BRAIN AND EYE

NGF is part, and indeed the pioneer, of neurotrophin family including brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Huang and Reichardt, 2001; Calissano et al., 2010a,b; Cattaneo and Calissano, 2012; Aloe et al., 2015) expressed in mammalian brain and in the neural retina (Garcia et al., 2017). NGF is a pleiotropic factor that promotes the survival, growth, and differentiation of neuronal cells of the CNS and peripheral nervous system (PNS) during development and adulthood. Its "trophic" prosurvival activity has been more recently documented to consist of an

anti-amyloidogenic action keeping under control the apoptotic program (Calissano et al., 2010a,b). NGF also exerts a modulatory role by acting on specific non-neuronal cells of the neuro-immuno-endocrine system (Aloe and Chaldakov, 2013). NGF and its cognate receptors (the high-affinity tyrosine kinase receptor TrkA and the low-affinity pan-receptor p75), in addition to their classical distribution in the cholinergic basal forebrain nuclei and in the cortical and hippocampal regions of the CNS, are also expressed in different ocular fluids and tissues, such as aqueous/vitreous humor, posterior lens, retina, and optic nerve (Micera et al., 2007). An imbalance between the NGF-dependent, TrkA-mediated survival, the growth actions, and the p75NTR-mediated activation of apoptosis/growth inhibitory pathway is causally associated with the onset and/or development of several neurodegenerative diseases of both CNS and PNS (Micera et al., 2004; Cuello et al., 2007, 2010; Garcia et al., 2017), including AD (Sofroniew et al., 2001; Schulte-Herbruggen et al., 2008; Cattaneo and Calissano, 2012) and AMD (Lambiase et al., 2009; Telegina et al., 2019; Esposito et al., 2021). In view of its strong neuroprotective and regenerative actions both *in vitro* and *in vivo*, delivery of NGF to the brain and eye has been always explored with therapeutic purposes for cerebral and extracerebral (retinal) neurodegenerative diseases, including AD and AMD (Gupta et al., 2016). Nevertheless, the clinical benefits of NGF therapy to the brain are restricted by its inability of bypassing the BBB to reach the CSF *via* the brain ventricles, poor pharmacokinetic profile, and nociceptive unwanted side effects due to the broad distribution of its receptors along the intrathecal space (Eriksdotter Jönhagen et al., 1998). All these pharmacological aspects of NGF drastically reduce its efficacy and safety when administered *in vivo*. In order to achieve a cerebral long-term delivery of NGF in biologically active therapeutic doses, multiple approaches have been employed to allow a sustained release/targeting of NGF both in the brain and eye. However, starting from the initial attempts of direct injection, the classical gene- and cell-based routes along with the more innovative nanotechnological strategies – including carrier-mediated release, polymer-based and encapsulated cell (EC) delivery system, nanoparticles, and quantum dots – have encountered several hurdles prior to becoming suitable treatments of cerebral and extracerebral symptoms of patients suffering from AD and, potentially, from AMD. The main therapeutic approaches for NGF delivery will be discussed in the following sections.

Direct Intracerebral Infusion of NGF

To date, local and direct intracerebroventricular (ICV) injections of NGF in close proximity to the cholinergic neuronal soma located in the CNS basal forebrain have been carried out by implantation of fibroblasts or neuronal progenitors genetically modified to secrete NGF or by delivery of NGF-expressing adeno-associated virus both in animal models and in early clinical trials (Fischer et al., 1987; Olson et al., 1992; Seiger et al., 1993; Eriksdotter Jönhagen et al., 1998). Adult cholinergic neurons are successfully rescued from degeneration by NGF ICV delivery, and mnemonic recovery is greatly sustained *in vivo* following infusion of this neurotrophin as well (Tuszynski et al., 1990, 2005, 2015; Markowska et al., 1994, 1996; Martínez-Serrano et al., 1995; Castel-Barthe et al., 1996; Klein et al., 2000; Bishop et al., 2008). An intraparenchymal application of recombinant NGF protein into sites adjacent to degenerating cholinergic cell bodies of rodents is effective and well-tolerated at least up to a 2-week period of treatment (Tuszynski, 2000; Pizzo and Thal, 2004).

Peripheral Administration of NGF Using Nasal and Intraocular Delivery

Even though the initial improvement of cholinergic functions following the direct intracerebral delivery of NGF *in vivo* turned out to be encouraging, the manifestations of an undesirable back pain and weight loss along with a low diffusivity of the drug within the brain have prompted researchers and companies to further discontinue this route of administration for therapeutic reasons (Faustino et al., 2017; Mitra et al., 2019). Thus, peripheral intranasal administration (INS) has provided an alternative approach to target *via* the olfactory path a sizeable amount of NGF directly to the brain, especially in a rodent model (Cattaneo et al., 2008). By taking advantage of the unique anatomic connections of the olfactory and trigeminal nerves linking the nasal mucosa and the CNS, INS delivery provides a high-vascularized absorption area favoring rapid penetration of NGF, but it does not overcome the occurrence of its adverse effects. Moreover, long-term INS of a drug causes damage to the nasal mucosa by altering the mucociliar activity (Malerba et al., 2011; Zhu et al., 2012). On the other hand, more recent studies have underscored the interesting possibility that the ocularly administered exogenous NGF (oNGF) is able to exert neuroprotective and/or regenerative properties on the retina and its brain projections, thus representing an innovative, safe, and non-invasive option for the cure of affected patients in the eye–brain system (Di Fausto et al., 2007; Lambiase et al., 2007; Tirassa et al., 2018). Furthermore, comparative studies carried out on adult and aged anti-NGF AD11 transgenic mice – an animal model characterized by AD-like neuropathology following chronic NGF antibody-mediated deprivation – have clearly demonstrated that ocular administration is less effective than intranasal delivery to protect cholinergic neurons and prevent behavioral deficits, even if used at higher doses (Capsoni et al., 2009; Covaceuszach et al., 2009). Likewise, intranasal delivery of NGF significantly improves the clinical outcome in children with neurological impairment following traumatic brain injury (TBI; Chiaretti et al., 2017).

Gene- and Cell-Mediated NGF Delivery

Different approaches aimed at achieving a direct administration of exogenous NGF to the brain with beneficial outcomes have been recently provided by gene- and cell-based therapies (Faustino et al., 2017; Mitra et al., 2019). In this regard, preclinical and clinical studies have proved that the stereotactic surgical delivery of CERE-110 – an AAV serotype 2-based vector encoding for the human NGF – to the nucleus basalis of Meynert is a reliable and accurate neuroprotective strategy, being largely neurorestorative for a large part of cholinergic neurons localized in the rat fimbria-fornix lesion, in aged monkey models,

and in human patients (Bishop et al., 2008; Mandel, 2010). Unfortunately, preliminary clinical studies designed to evaluate the actual safety and efficacy of the long-term administration of CERE-110 in subjects suffering from early-moderate AD (Rafii et al., 2014) are not followed by encouraging results. As reported in a recently published phase II clinical trial, no significant clinical rescue was detected by using AAV vectors expressing human NGF on 49 enrolled AD-diagnosed patients who underwent intracerebral injections of AAV-NGF or sham surgery, respectively (Rafii et al., 2018). In parallel with these findings, following 1-year lentivirus NGF gene delivery to the cholinergic basal forebrain, neither the systemic leakage of NGF and the occurrence of anti-NGF antibodies nor the activation of detrimental neuroinflammatory response in the brain with back pain or weight loss has been detected in aged non-human primates (Nagahara et al., 2009). Although the gene therapy procedures exploit recombinant AAV in which the viral genes for self-replication and incorporation of genetic material into chromosomal DNA have been deleted, the impossibility of modifying the dosage and/or interrupting the treatment once started along with potential toxicity and immunogenicity has raised concerns regarding the practicability, suitability, and usefulness of its application. To overcome the limitations of direct viral vector-mediated gene delivery, *ex vivo* gene therapy has been also evaluated. Neuronal stem cells (NSCs) transduced with human NGF by using a AAV2 vector, when grafted into the cerebral cortex of cognitively impaired rats undergoing chronic ICV infusion of the serine/threonine protein phosphatase (PP) inhibitor okadaic acid, are proven to successfully integrate into the host brain and to improve cognitive performance after transplantation (Wu et al., 2008). The NSC line overexpressing the human choline acetyltransferase (ChAT) gene recovers the learning/memory deficits and elevates the levels of acetylcholine (ACh) in CSF when transplanted into rat brain of an experimentally induced AD model, in which the application of ethylcholine mustard aziridinium ion (AF64A) specifically inactivates the cholinergic nerves. Interestingly, transplanted ChAT human NSCs migrate to different brain areas including the cerebral cortex, hippocampus, striatum, and septum of AF64A-cholinotoxin-lesioned AD rat model and successfully differentiate into viable and functional neurons and astrocytes as well (Park et al., 2012).

Carrier-Mediated Sustained Release of NGF

Encapsulated cell biodelivery is an innovative strategy involving an *in vitro* genetically engineered human cell line which grows on a polymer scaffold behind a semipermeable filter and continuously releases a low but sufficient amount of therapeutic protein directly into a localized region of brain cells. This method combines the potency of gene therapy with the advantage offered by an implantable and retrievable device, acting as an actual biological micropump. In this regard, grafts of polymer-ECs modified to release NGF delay and/or prevent the degeneration of axotomized cholinergic neurons in the basal forebrain of rodents as well as of aged monkeys (Hoffman et al., 1993; Emerich et al., 1994; Kordower et al., 1994; Lindner et al., 1996). Implantation of encapsulated NsG0202, a clinical device which houses an NGF-secreting cell line (NGC-0295) derived from a human RPE cell line, is well-tolerated into the basal forebrain of Göttingen minipigs up to 12 months leading to an increase of NGF levels in the surrounding tissue (Fjord-Larsen et al., 2010; Wahlberg et al., 2012). EC-NGF biodelivery devices targeting the basal forebrain of implanted AD patients ($n = 6$) are safe in a 12-month study, leading to improvement in cognition assessed by clinical rating scales, EEG, MRI, and positron emission tomography (PET) (Eriksdotter-Jönhagen et al., 2012). Nevertheless, several drawbacks, such as the invasive nature of the procedures requiring hospitalization and neurosurgical intervention and their high costs, have discouraged the pursuing of both gene- and cell-based therapies as interventions to achieve a sustained and controlled release of NGF into the brain. An advancement in the field of NGF therapeutic strategies has been made with the contemporary development of intracerebral implants composed of biocompatible, synthetic, and natural polymers, including poly(ethylene co-vinyl acetate) (EVAc), poly(D,L-lactide-co-glycolic acid) (PLGA), polyanions (e.g., heparin, dextran sulfate, gelatin), and polycationic or polyanionic hydrogels which are endowed with different shapes and surface areas to provide high and localized doses of this neurotrophin into the brain (Hoffman et al., 1990; Powell et al., 1990; Johnson et al., 2008; Yang et al., 2009; Song B. et al., 2012; Hines and Kaplan, 2013). Nanoparticles have been more recently explored due to their multiple advantages, including small size (nanoscale dimensions), high solubility, multifunctionality, improved transport properties and pharmacokinetic profile, enhanced permeability, and retention. These parameters significantly enhance the penetrance of a drug into tissues through capillaries and also enhance its efficient delivery to target sites, thus providing a more powerful medicament for patients. Liposomes, polymeric micelles, dendrimers, magnetic nanoparticles, and quantum dots functionalized with NGF effectively deliver this neurotrophin both in neuronal cell culture and/or in animal model of AD leading to neurite outgrowth, diminution of cerebral Aβ accumulation, rescue in cholinergic functions, improved viability, and neuroprotection (Angelova et al., 2013; Zhao et al., 2016; Faustino et al., 2017; Marcus et al., 2018; Mitra et al., 2019; Tosi et al., 2020).

In summary, the pivotal role of NGF in the development, growth, maintenance, and plasticity of the CNS and PNS, both in normal and diseased conditions, represents a strong rationale and incentive of advancing innovative techniques to provide its localized and finely modulated delivery to target tissues.

OCULAR ADMINISTRATION OF NGF AS A PROMISING THERAPEUTIC APPROACH TO COUNTERACT DEGENERATION OF THE EYE AND BRAIN BOTH IN AMD AND AD PATHOLOGIES

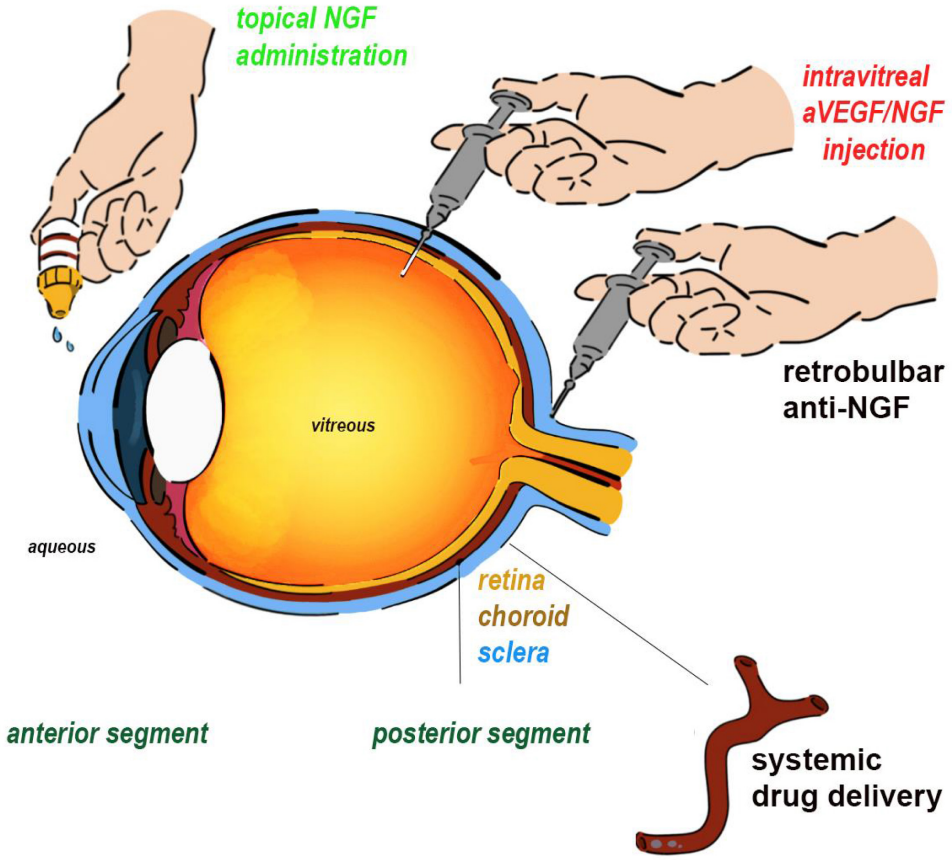
The independent trophic and neuroprotective roles of NGF on the retina, optic nerve, and brain visual area and its

direct and indirect projections to limbic structures, including the cholinergic hippocampus and septum, have been largely documented in different animal models and in paradigms of retinal injury and disease (Micera et al., 2004; Cattaneo and Calissano, 2012; Aloe et al., 2015; Garcia et al., 2017; Esposito et al., 2021).

In particular, under oxidative stress and inflammatory conditions similar to those early occurring both in AMD and AD, the activity of matrix metalloproteinase-7 protease is altered in the eye causing a local accumulation of proNGF and concomitant reduction of mature NGF followed by changes in the expression and function of its receptors (i.e., the survival TrkA receptor and the neurotrophin p75NTR receptor). Thus, even though it is still unclear whether ocular neuronal death is associated with the lack of neurotrophic support or impairment of its signaling events regardless of the level of the growth factor itself, ophthalmological clinical trials have taken advantage of the well-known anti-apoptotic action of NGF to slow and/or stop sight loss resulting from death of injured RGC or photoreceptors (Pardue and Allen, 2018; Hill et al., 2021). Compelling experimental evidence has documented the regenerative role of NGF on retinal ganglion cell survival in different animal models of optic nerve transection, ischemic injury, ocular hypertension, and glaucoma (Roberti et al., 2014). The impressive pharmacological potential of this neurotrophin in ophthalmic diseases has been recently brought to light by Guo et al. (2020) reporting that topical application of recombinant human NGF (rh-NGF) significantly reduces the RGC apoptosis *in vivo* following partial optic nerve transection (pONT) by protecting their soma and axons likely *via* the retrobulbar route (Guo et al., 2020). Intravitreal administration of rhNGF counteracts RGC degeneration occurring within 2 weeks following optic nerve crush (ONC) by reducing p75NTR and proNGF and by enhancing phosphorylation of TrkA and its intracellular signals at the retina level (Mesentier-Louro et al., 2019), in line with previous findings on another animal model of retinal disease, such as inherited retinitis pigmentosa (RP) (Sacchetti et al., 2017). The beneficial effect of NGF on injured eye has been also validated in RP experimental paradigm following retrobulbar injection (Lenzi et al., 2005) or in affected patients after eye drop administration (Falsini et al., 2016b). A randomized double-blind phase II clinical trial, aimed at testing the vision functions in 18 patients aged 2–23 years with stable disease and severe blindness caused by glioma, has proven that ocular instillation of NGF protects the optic pathway glioma-related visual impairment in the absence of any apparent side effects (Falsini et al., 2016a).

More interestingly, a growing body of experimental and clinical studies have shown that administration of NGF in different ocular sites can be a feasible means to convey NGF and/or activate its downstream signals into the brain (Eftimiadi et al., 2021). Thus, the increase of the endogenous NGF availability, following its intraocular (intravitreal or periocular) exogenous injection and/or topic instillation (**Figure 2**), is currently exploited as a safe, non-invasive approach to successfully counteract retina and even brain neurodegeneration underlying clinical signs of AMD and AD phenotypes (Tirassa et al., 2018; Mitra et al., 2019). Consistently, when administered topically in animal models, NGF becomes available to the retina and optic nerve and exerts neuroprotective and/or regenerative properties on projections of the eye–brain system, including the nucleus basalis and septum as well (Lambiase et al., 2005, 2007). The local application of NGF drops using the ocular surface way induces an upregulation in the expression of NGF receptors and ChAT immunoreactivity (cholinergic markers) in chemically injured basal forebrain neurons of adult rats, indicating that eye NGF application somehow affects brain cells (Di Fausto et al., 2007; Lambiase et al., 2007). Along this line, the finding that a single ocular administration of NGF solution is sufficient to enhance the distribution of Ki67-positive cells (marker for proliferative cells) also expressing p75 neurotrophin receptors in the proliferating layer of the subventricular zone (SVZ) further supports the feasibility of ocular application of NGF in upregulating the development and maturation of neuronal progenitors in the CNS (Tirassa, 2011). Immunohistochemical studies have reported that an instillation of NGF as eye drops increases in a time-dependent manner the immunoreactivity of c-fos (markers for neuronal activation) in several areas of the limbic system and in primary visual centers of rats as well (Calza et al., 2011). Furthermore, an improvement in visual acuity and electrofunctional parameters has been found in a 94-year-old female affected with AMD 3 months after initiation of treatment with NGF and in the absence of any side effects up to 5 years of follow-up (Lambiase et al., 2009). Thus, despite intrinsic limitations due to proper physiology of the eye (tear turnover, nasolachrymal drainage, reflex blinking, ocular static, and dynamic barriers) which makes difficult a deeper drug penetration, the chance of exploiting in common clinical practice the ocular route to achieve a non-invasive distribution of several medicaments, including NGF, in therapeutic concentrations for the brain is gaining increasing interest to ocular scientists (Patel et al., 2013; Esposito et al., 2021). The advent of innovative nanotechnologies, new techniques, and devices (nanoparticles, nanosuspensions, liposomes, dendrimers, *in situ* gelling systems, intraocular implants, and microneedles) designed to overcome ocular barriers and side effects linked with conventional topical drops is currently allowing a more sustained drug release and an improved target specificity (Patel et al., 2013). Furthermore, the recent development of a genetically engineered NGF variant endowed with biological activity and reduced pain effect (painless NGF) further opens the expanding panorama of its applications *via* eye drop administration (Malerba et al., 2015; Testa et al., 2021).

Collectively, these recent and promising *in vivo* results underline that the protective and reparative actions of NGF used as eye drops are not only confined to the primary visual areas, but are also extended to other retinal central targets, including the forebrain structure (Calza et al., 2011; Tirassa, 2011). Moreover, these studies encourage investigations on the clinical effects of NGF therapy for the treatment of neurodegenerative diseases characterized by an imbalance in NGF/TrkA signal transduction pathway in the eye and in the brain, including AMD and AD, respectively (Mufson et al., 2008; Aloe et al., 2015; Canu et al., 2017; Esposito et al., 2021).



DELIVERY ROUTE	TYPE OF STUDY: EXPERIMENTAL MODEL	TYPE OF STUDY: HUMAN
TOPICAL ADMINISTRATION	Calza et al., 2011 Di Fausto et al., 2007 Lambiase et al., 2005, 2007 Tirassa et al., 2011	Falsini et al., 2016a/b Lambiase et al., 2009
INTRAVITREAL INJECTION	Mesentier-Louro et al., 2019 Sacchetti et al., 2017	-
PERIOCULAR ADMINISTRATION	Guo et al., 2020 Lenzi et al., 2005	-

FIGURE 2 | All ocular sites tested for experimental NGF delivery are summarized as follows: (1) the topical administration (eye drop instillation and emulsion/gel application) (green labeled), (2) the intravitreal injections (ophthalmic solutions/suspentions, implants, and nanotecnology), and (3) the periocular administration (implants or injections) at the posterior segment. The periocular route includes the subconjunctival, subtenon, peribulbar, and retrobulbar sites of injection. Except for the systemic route, all the others have been tested for NGF and anti-NGF at both experimental and clinical levels. The topical administration is actually a routine procedure for specific eye disorders. Highlighted in red: our future directions on experimental animals, comprising the anti-VEGF/NGF combined intravitreal injection or the use of NGF nanoparticles layered in the vitreal chamber. The table summarizes the different routes of NGF administration in the eye, in an experimental model and human beings.

ESSENTIAL TRACE METALS: DIAGNOSTIC PERSPECTIVES IN AD AND AMD

The common physiopathological aspects linking AD and AMD not only have important therapeutic implications but also can give additional insights into the development of novel biomarkers for both diseases. Besides, since retinal degenerative alterations manifest before the corresponding changes detected in the brain, quantification of ocular biomarkers will facilitate (i) the diagnosis of AD as well as AMD at their earliest stages when therapy is more likely to be effective in halting/slowing down symptomatology

and (ii) a non-invasive monitoring of disease progression (Micera et al., 2019; Ong et al., 2019). In this context, a growing body of evidence has shown that dyshomeostasis of essential trace metals, namely, both depletion and excess of copper (Cu), iron (Fe), and zinc (Zn), causes severe damage to neurons and is causally associated with various neurodegenerative and retinal diseases, including AD and AMD (Bush, 2013; Faller et al., 2013; Micera et al., 2019).

In AD, a Cu imbalance is evident and associated with the misplacement of metal from cellular and tissue compartments: meta-analytic studies reported that Cu levels are decreased in the AD brain while increased in the periphery, suggesting a breakdown of Cu homeostasis control in the disease (Mathys and White, 2017). Elevated values of Cu in serum and decreased Cu values in the brain are associated with increased levels of Cu not bound to proteins and primarily to ceruloplasmin (the main Cu protein in serum) in general circulation: this Cu component, called non-ceruloplasmin Cu, also known as "free" Cu, is an established marker of Wilson disease, the paradigmatic disease of Cu toxicosis/accumulation. Non-ceruloplasmin Cu is an exchangeable, small molecular weight and filterable Cu component in the bloodstream that is toxic above a certain cutoff and can pass the BBB and distribute to brain parenchyma (Choi and Zheng, 2009; Squitti, 2012; Kepp and Squitti, 2019). Aβ40 and Aβ42 are flexible small α-helix and β-sheet structures representing the majority of Aβ forms in the AD brain. They can bind Cu(I/II) with relevant effects in initiating Aβ aggregation processes (Squitti, 2012; Mathys and White, 2017; Kepp and Squitti, 2019). Cu binding confers redox activity to Aβ (Squitti et al., 2017) that affects neuron viability (White et al., 1999). It has been recently hypothesized that a Cu dysfunction in the aging human brain might evolve as a gradual displacement of Cu from pools tightly bound to proteins to pools of loosely bound metal ions, involved in gain-of-function oxidative stress associated with Aβ peptides, a shift that might be provoked by chemical aging (Kepp and Squitti, 2019). The loss of Cu tightly bound to proteins may be associated with the loss of energy production and antioxidant function within neuronal cells, two important drivers of AD neurodegeneration (Kepp and Squitti, 2019). Non-ceruloplasmin Cu fits well in this construct since it is a loosely bound specie of Cu in general circulation and has been demonstrated to be a potential prognostic biomarker for conversion from mild cognitive impairment (MCI) to symptomatic AD, typified by copper imbalance (Squitti et al., 2014; Rozzini et al., 2018; Sensi et al., 2018). In AD, non-ceruloplasmin Cu reaches values similar to Wilson disease (Squitti et al., 2018) and has demonstrated a fair sensitivity in detecting which individuals with MCI will convert to Cu-AD (Squitti et al., 2014) and high specificity (95%) in detecting patients affected with Cu-AD due to copper exposure (Squitti et al., 2017), in line with preclinical models of the disease (Sparks and Schreurs, 2003; Singh et al., 2013; Yu et al., 2018; Hsu et al., 2019). Recently, several studies have shown that non-ceruloplasmin Cu might serve as a stratification biomarker for a subset of AD patients (Kepp and Squitti, 2019). The feature, called Cu-AD (Squitti et al., 2017), appears specific to a subset (50%–60%) of AD patients and is characterized by being a carrier of selective ATP7B gene variants (Squitti et al., 2013) and by having peculiar cortical activity and neuroimaging deficits (Kepp and Squitti, 2019).

Fe dysfunction has been strongly associated with AD pathogenesis in recent years. It is known that Fe imbalance and Aβ metabolism are synergistically regulated in neurodegeneration and can facilitate cellular processes which may result in AD development, pointing out to a central role of Fe dyshomeostasis in AD pathology. Recently, potential biomarkers associated with Fe imbalance have been individuated: ferritin, either in plasma or in CSF, has been shown to have potential in discriminating AD patients in their preclinical stage, categorized into low and high neocortical amyloid-β load, prior to cognitive impairment (Goozee et al., 2018). High ferritin levels suggest an increase of Fe abundance in the CSF and brain which can be associated with ferroptosis, an iron-dependent cell death that results from a buildup of lipid peroxides and is regulated by glutathione (GSH) peroxidase 4 (Acevedo et al., 2019). A recent study provided a very interesting theoretical significance of abnormalities of Fe associated with AD onset and progression (Ayton et al., 2021). Fe and Cu metabolism are intertwined in physiology, and they are certainly tied in AD (Squitti et al., 2010). Ceruloplasmin exemplifies the crosstalk protein between Cu and Fe balance because it is the main Cu protein in the body, and it controls Fe oxidative state: imbalance in one of the two metals has effects on the other (Siotto et al., 2016). Consistent with both Cu and Fe balance abnormalities, ceruloplasmin in the CSF has been shown to predict cognitive decline and brain atrophy in people with underlying Aβ pathology (Diouf et al., 2020). High levels of ceruloplasmin and the activation of the ceruloplasmin:transferrin (Cp:Tf) system, one of the main antioxidant systems acting in general circulation, were associated with worse cognitive performances and a severe medial temporal lobe atrophy, supporting the view that local iron accumulation in brain areas critical for AD might be strictly associated with Fe systemic alterations (Squitti et al., 2010). Recently, another point of conversion between Cu and Fe metabolism has been provided by the evidence of the loss in GSH levels in AD. GSH is one of the main endogenous free radical scavenger systems in the brain, acting as a co-substrate in GSH peroxidase-catalyzed reactions (Pinnen et al., 2011). GSH loss in the brain is linked to cognitive dysfunction (Currais and Maher, 2013) and is associated with Cu imbalance. This has been mainly stressed in preclinical models of AD induced by Cu exposure: high Cu and cholesterol levels trigger Aβ aggregation and plaque formation in the hippocampus and temporal cortex and impair learning and memory, thus recapitulating deficits featuring mental dementia (Yao et al., 2018).

Zinc homeostatic imbalance has been advocated as a driver of neurodegenerative processes, primarily linked to oxidative stress and the aging brain (Capasso et al., 2005; Frazzini et al., 2006). Somewhat reduced levels of zinc have been shown in AD through meta-analyses (da Silva et al., 2014; Ventriglia et al., 2015; Wang et al., 2015). The hypothetical role of this metal in AD pathology has been connected to neurotoxicity induced by mitochondrial production of ROS and by disruption of metabolic enzymatic activity (i.e., Cu, Zn superoxide dismutase activity) eventually

leading to apoptosis and/or neurodegeneration (Li et al., 2017; Kabir et al., 2021). Zinc therapy, used in Wilson disease, effectively reduces non-ceruloplasmin Cu levels: this suggests potential beneficial effects in slowing the progression of cognitive decline in subsets of individuals who show both cognitive disturbances and signs of Cu imbalance (Squitti et al., 2020).

Concerning AMD, there is evidence that heavy metals might be involved in the pathogenesis of AMD (Ugarte et al., 2013; Micera et al., 2019). Beyond the deposition of Fe, Cu, and Zn metal ions in extracellular deposits of AMD, recent studies have shown accumulation of heavy intoxicant metals in eye tissues and in general circulation of AMD patients (Aberami et al., 2019). Major metal changes involve the accumulation of loosely bound Fe in human RPE, in Bruch's membrane, and in photoreceptors located in the macula (Biesemeier et al., 2015; Dalvi et al., 2019). Fe plays a pivotal role in retina physiology as a cofactor of several processes of visual transduction: the expression of transferrin, ferritin, and ferroportin is upregulated in AMD macula (Baumann et al., 2017). A tight Fe control can avoid the accumulation of pro-oxidant and proinflammatory loosely bound species of the metal (Courtois et al., 2020). To contrast the accumulation of Fe in the aged retina of AMD patients, Fe chelation therapy has been proposed (Shu and Dunaief, 2018). Indeed, treatment with the Fe chelator deferiprone has been shown to be useful to prevent retinal degeneration (Song D. et al., 2012; Cui et al., 2020). However, several chelators (deferoxamine B) have been shown to cause adverse events (Micera et al., 2019). Recently, intravitreal injection of transferrin, a natural Fe chelator, has been demonstrated to have neuroprotective effects against oxidative stress in retinal degeneration: after injection in the vitreous, transferrin spans rapidly within the retina and accumulates in photoreceptors and in RPE, protecting retinal function from loosely bound Fe (Picard et al., 2015). Consistent with previous evidence (Micera et al., 2019), new findings show that Cu is increased in choroid-RPE of AMD. In contrast to Fe and Cu trends, Zn appears decreased in the aged retina and Zn supplementation has been proposed for AMD treatment (Gilbert et al., 2019). Zn supplementation has beneficial effects in reducing the progression of AMD according to the Age-Related Eye Disease Study (AREDS; Age-Related Eye Disease Study Research Group (AREDSRG), 2000; Chew et al., 2015; Seddon et al., 2016): daily supplementation with a formulation consisting of 500 mg vitamin C, 400 IU vitamin E, 25 mg zinc, 2 mg copper, 10 mg lutein, and 2 mg zeaxanthin (AREDS and AREDS2) was effective for slowing AMD progression (Kim et al., 2016). Cochrane reviews support the use of Zn for the delay of AMD progression and vision loss (Evans and Lawrenson, 2017; Micera et al., 2019). Beyond studies on essential trace metals, an increasing number of studies have been performed on circulating or local concentrations of intoxicant heavy metals, mainly linked to environmental or lifestyle risk factors. It is acknowledged that environmental and lifestyle factors affect the balance of bodily trace metals, and in particular, this occurs in the retina mainly through oxidative stress that originated from exposure of macular tissues to sunlight and local or systemic exposure to oxidative stressors, including smoke. In this line of research, important results have been gained mainly for cadmium (Cd) that, in most of these studies, has been found to be elevated in AMD patients, especially within the smoking population (Kim et al., 2016; Wu et al., 2014; Güngör et al., 2018). These findings are coherent with the results from histology studies, showing Cd accumulation in the retina and in the RPE, particularly in smokers (Wills et al., 2008). The proposed mechanisms of Cd uptake in the retina involve zinc transporters (ZIP4 and ZIP8) with high Cd affinity (Girijashanker et al., 2008). Studies on circulating intoxicant heavy metals have shown also increased levels of blood lead (Pb) (Güngör et al., 2018). Higher blood Pb and Cd levels in AMD (Güngör et al., 2018; Heesterbeek et al., 2020) together with increased levels of barium (Ba) have been found in neovascular AMD (236 patients with neovascular AMD compared with 236 age-matched controls). Conversely, chromium (Cr) concentration decreased. Interestingly, higher Cd concentrations have been found mostly in smokers. On this basis, preventive strategies targeting decreased Cd exposure have been postulated to reduce the burden of AMD, primarily directed to skip smoking (Heesterbeek et al., 2020).

CONCLUSION

Alzheimer's disease and AMD are brain and retinal degenerative diseases whose incidence is increasing worldwide due to lengthening of life expectancy. AMD and AD share several clinical and pathological aspects, including Aβ accumulation and aggregation, oxidative stress, inflammation, and alterations in local supportive/regulatory actions of NGF. Based on these similarities and in view of the strong evidence that NGF is endowed with regenerative actions on both the brain and the retina, a growing number of opportunities can be offered by the administration of this neurotrophin as a common therapeutic agent and neuroprotective strategy for AD and AMD. Currently, great efforts are being made to enhance the effectiveness of NGF-based therapy by exploring novel, safe, and reliable routes of its delivery to the brain and eye, ultimately assessed in preclinical and clinical trials. Alterations in the systemic and ocular levels of essential trace metals are currently evaluated as diagnostic and/or even predictive biomarkers for future precision medicine of both AD and AMD.

AUTHOR CONTRIBUTIONS

GA, RS, and AM designed and wrote the manuscript. VL, BB, MV, and PC contributed to the data, text, and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported (in part) by Fondo Ordinario Enti (FOE D.M 865/2019) funds in the framework of a collaboration agreement between the Italian National Research Council and EBRI (2019–2021) and Regione Lazio, POR FESR Lazio 2014-2020, "Progetti di Gruppi di Ricerca 2020" (Determinazione

dirigenziale n.G08487 del 19 luglio 2020) to GA. VL is supported by a postdoctoral fellowship by Operatori Sanitari Associati (OSA). AM is supported partially by the Italian Ministry of Health (RC2765949) and 5xMille 2016 to Fondazione Bietti. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

BB and AM are grateful to Fondazione Roma (Italy) for the continuous support. Many thanks to Angelica Napoli for drawing **Figure 2**. We are also grateful to Dott. Egidio Stigliano for critical reading of the manuscript and suggestions.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fphar.2021.680903

Ginsenoside Rb1 Produces Antidepressant-Like Effects in a Chronic Social Defeat Stress Model of Depression Through the BDNF–Trkb Signaling Pathway

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *15 March 2021*
Accepted: *10 August 2021*
Published: *29 September 2021*

Citation:
Jiang N, Huang H, Zhang Y, Lv J, Wang Q, He Q and Liu X (2021) Ginsenoside Rb1 Produces Antidepressant-Like Effects in a Chronic Social Defeat Stress Model of Depression Through the BDNF–Trkb Signaling Pathway. Front. Pharmacol. 12:680903. doi: 10.3389/fphar.2021.680903

Ginsenoside Rb1 (Rb1), an important bioactive ingredient of *Panax ginseng*, has potent neuroprotective effects. The objective of the study is to elucidate the impact of Rb1 treatment on chronic social defeat stress (CSDS)–induced depressive-like behaviors and its related mechanism. According to the obtained results, the daily oral administration of Rb1 (35 and 70 mg/kg) and imipramine (15 mg/kg) for 28 days significantly reversed the social avoidance behavior, anhedonia, and behavioral despair *via* CSDS exposure, as demonstrated by the considerable elevation in the time in the zone in the social interaction test, consumption of sucrose solution in the sucrose preference test, and decrease in immobility time in the forced swim test. Moreover, Rb1 obviously restored the CSDS-induced decrease in the BDNF signaling pathway and hippocampal neurogenesis. Rb1 significantly increased the hippocampal levels of ERK, AKT, and CREB phosphorylation and increased the number of DCX+ cells in DG. Importantly, the antidepressant effects of Rb1 were completely blocked in mice by using K252a (the nonselective tyrosine kinase B inhibitor). In conclusion, our results indicated that Rb1 exerts promising antidepressant-like effects in mice with CSDS-induced depression, and its effects were facilitated by enhancing the BDNF signaling cascade and upregulation of hippocampal neurogenesis.

Keywords: ginsenoside Rb1, depression, CSDS, BDNF, neurogenesis, mice

INTRODUCTION

Stress is substantially involved in many neuropsychiatric complications, such as anxiety, depression, and posttraumatic stress disorders. Depression is a prevalent neuropsychiatric disorder, and its main clinical characteristics are constant and obvious depression of mood. Worldwide, the lifetime prevalence of depression is approximately 15–20%, which means that almost one in five people experience depression in their lifetime, and 15% of cases of major depressive disorder result in suicidal deaths (Malhi and Mann, 2018). During the last few years, the monoamine hypothesis has been one of the most widely studied etiologies of depression. Nearly all currently used antidepressants work by increasing the levels of monoamine

neurotransmitters. However, these antidepressant agents usually take weeks to months to exert their therapeutic effects. Furthermore, more than 30% of patients do not respond to these agents (Licinio and Wong, 2005; Fukumoto et al., 2019). Thus, there is a need to develop more effective and safer antidepressants.

To date, various studies have attempted to explore the pathogenesis of depression and develop antidepressant drugs by using animal models subjected to chronic stress (Ito et al., 2017). Chronic social defeat stress (CSDS) is associated with depression in humans and simulates human emotions, such as fear caused by failure and frustration (Von Frijtag et al., 2000). Indeed, environmental stressors associated with daily life (such as a high-pressure social environment) are more common causes of depression than primary neural circuit impairment (Prudo et al., 1981). In the CSDS paradigm, model animals are subjected to psychological social stress *via* a widely adopted preclinical stress procedure with excellent face, constructive, and predictive validity (Hao et al., 2019; Warren et al., 2020). Long-term CSDS causes changes corresponding to the symptoms of post-stress depression, including elevated social avoidance, behavioral despair, and anhedonia, while these CSDS-stimulated behavioral changes can be ameliorated by long-term (10 days) treatment with traditional antidepressant drugs (Robison et al., 2014; Munshi et al., 2020).

According to recent studies, the BDNF signaling cascade is crucial for depression treatment and pathophysiology (Schmidt and Duman, 2007). Through tyrosine kinase B (TrkB), BDNF signaling induces the phosphorylation and activation of CREB. Furthermore, BDNF and TrkB have also been found to significantly activate signaling cascades associated with downstream signaling of BDNF, that is, the AKT and MAPK-ERK signaling cascades (Jiang et al., 2015; Ghosal et al., 2018). It has been extensively shown that continuous exposure to stress decreases the level of hippocampal BDNF in animal models of depression, and BDNF expression has been found to be decreased in the brains of patients with major depressive disorders (Wang et al., 2016; Jiang et al., 2019a). In animal models and clinical studies, several drugs have been found to exert antidepressant-like effects and regulate the expression of proteins associated with BDNF signaling pathways (such as CREB, AKT, and ERK) (Luo et al., 2015).

Traditional herbal medicines have been used for many years and have been proven effective for the treatment of various neuronal disorders, such as depression (Liu et al., 2016). Recently, the use of medicinal plant–based medicines to treat depression has gained wider scientific attention because these drugs have few side effects (Sun et al., 2014). Ginsenoside Rb1 (Rb1) is the most significant bioactive constituent of the herb *Panax ginseng* C.A. Meyer. In Far East countries, this herb has been widely used as a tonic for more than 2000 years. During the last few decades, *Panax ginseng* C.A. Meyer has also gained scientific recognition as a tonic remedy in Western countries (Helms, 2004). Previous pharmacological studies have revealed that Rb1 exerts multiple biological effects, including antioxidant, antitumor, and anti-inflammatory effects. (Deng et al., 2017; Xin et al., 2019). Rb1 has been demonstrated its protection for the central nervous system and is apparently highly distributed to the brain (Wang GL. et al., 2018).

Previous reports have shown that Rb1 exerts a significant antidepressant-like effect in a chronic unpredicted mild stress model by mediating central neurotransmitters of the noradrenergic, serotonergic, dopaminergic, and aminoacidergic systems (Wang et al., 2017; Wang YZ. et al., 2018). Recent studies have revealed that Rb1 alleviates depressive-like behaviors, suppresses neuroinflammation, and activates the AKT pathway in mice subjected to chronic restraint stress (Guo et al., 2021). However, several studies have demonstrated that Rb1 has therapeutic potential against depression-like conditions and that it exerts an antidepressant-like effect against behavioral abnormalities caused by CSDS; however, its underlying mechanism is not fully understood. In the current study, the antidepressant potential of Rb1 was evaluated in CSDS-exposed mice. To further explore the possible mechanism, the role of the BDNF signaling cascade in the hippocampus was also assessed.

MATERIALS AND METHODS

Animals

The CD1 (12 months old, male) and C57BL/6J (7–8 weeks old, male) mice were provided by the Institute of the Chinese Academy of Medical Science Center, Beijing. Both types of mice were kept in the maintained place according to the standard animal housing conditions, such as 55 percent humidity, 20–22°C temperature, and 12:12 light and dark duration, with free access to water and food. The experimental procedure involving animals was performed with proper approval (approval no. SYXK 2017-0020), following the guidelines provided by the Animal Research Committee of the Institute of Medicinal Plant Development, Peking Union Medical College.

Drugs and Treatments

Ginsenoside Rb1 (HPLC grade, greater than 98% purity) was obtained from Ruifensi Biological Technology Co., Ltd. (Chengdu, China). Imipramine (IMI) was provided by Sigma–Aldrich Co. (St. Louis, MO, United States). All these compounds were administered at a dose of 10 ml per kg of body weight. This study was divided into two experiments.

Experiment 1: Animals were randomly allotted to one of five groups: the control, CSDS-exposed, Rb1-treated (35 or 70 mg per kg of body weight), and IMI-treated groups (15 mg per kg of body weight).
Experiment 2: Animals were randomly allotted to one of five groups: the control, CSDS-exposed, Rb1-treated (70 mg per kg of body weight), IMI-treated (15 mg per kg of body weight), and CSDS + Rb1 (70 mg per kg) +K252a (25 μg per kg)-treated groups.

All mice, except those in the control group, were exposed to CSDS for 38 days. Water, Rb1, or IMI was administered orally to CSDS-exposed mice for 33 consecutive days until the behavioral

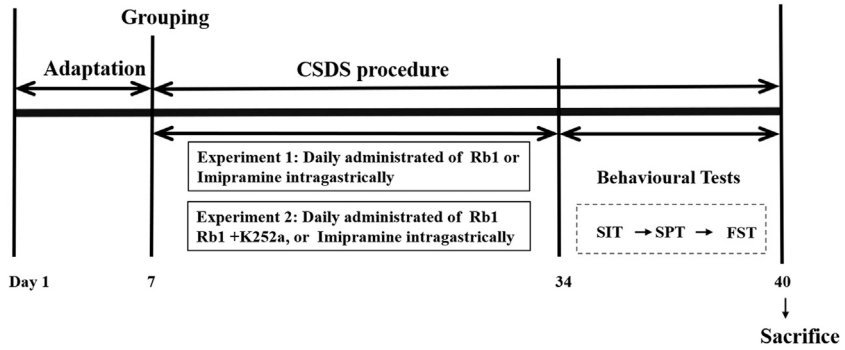

FIGURE 1 | Experimental design. Ginsenoside Rb1, Rb1; chronic social defeat stress, CSDS; social interaction test, SIT; sucrose preference test, SPT; forced swim test, FST.

tests were completed. The experimental procedure is shown in **Figure 1**.

CSDS Procedure

CSDS was performed as previously described with minor modifications (Golden et al., 2011; Monleon et al., 2015; Jiang et al., 2020b). In brief, CD1 mice were used to study the aggressive behavior of the CSDS-exposed mice and to evaluate the tendency of the mice to attack a mouse following intrusion into their home cage (Sial et al., 2016). C57BL/6 mice were subjected to physical defeat by exposing them to aggressor CD1 mice for 5 min daily for 28 days. The C57BL/6 mice were housed in the same cage as an aggressor mouse separated *via* a transparent organic acrylic plate (4 mm thick and porous) the following day and were exposed to chronic psychological stress, such as threatening auditory, olfactory, and visual stimuli, for the subsequent 24 h. The control mice were housed in pairs in the same cage separated by an identical porous transparent organic acrylic plate.

Social Interaction Test

The SIT was carried out according to previously reported methods with slight modifications (Bo et al., 2015). In brief, each mouse was kept in an open-field arena (40 × 40 × 40 cm) containing a perforated transparent acrylic plastic box (7 × 10 × 40 cm) on one side. Social avoidance behavior was evaluated based on a 2-stage SIT (a video recording tool was used for recording). In the first stage ("target-absent"), all C57 mice were placed in the arena under 5 lux and allowed to explore freely for 150 s without a CD1 aggressor mouse in the interaction zone (IZ). At the end of the first phase, the experimental mice were removed for 30 s, and the arena was cleaned. Next, a CD1 mouse was placed in a transparent plastic box, and the test mouse was reintroduced into the arena. Then, the second stage of the test was performed, and the same metrics were measured for 150 s. The time spent in the IZ in the presence and absence of the target was recorded.

Sucrose Preference Test

In the present study, the SPT was conducted, as discussed earlier (Jiang et al., 2020a). On the first 2 days (at 9:00 a.m.), each mouse was concurrently presented with two bottles containing 100 ml of either 1% sucrose solution or tap water. The animals had access to food/liquids ad libitum, and the location of the water bottles was altered every 12 h to minimize potential location bias. On the third day (at 9:00 a.m.), we removed the food and the bottles, followed by 8 h of inaccessibility to water/food. At approximately 17:00 p.m., each mouse was allowed to drink the pre-weighed sugar water and pure water for 16 h in a quiet and peaceful environment. On the next day (at 9:00 a.m.), all the bottles were removed, weighed, and recorded. The sucrose preference was measured by the following equation: sucrose preference index (%) = (sucrose solution consumed/total solution consumed) × 100%.

Forced Swim Test

The FST was carried out according to the previously reported protocol. All mice were restrained to swim for 6 min at a 15 cm depth in an acrylic cylinder (d × h: 14 × 20 cm) at room temperature. Video tracking software (Tail Suspension Real-Time Analysis System 2.0) was used for the recording of immobility time during the last 4 min post-habituation (2 min) in a 6-min test.

Western Blotting Analysis

The analysis was carried out according to the previous method with slight modifications (Kim et al., 2018; Xie et al., 2019). First, the mice were euthanized and then rapid dissection of the bilateral whole hippocampus was carried out on the ice, followed by homogenizing in lysis buffer for 0.5 h. After RIPA lysis buffer (comprising phosphatase/protease inhibitors) homogenization, the centrifugation (12,000 g) of the hippocampal homogenate was carried out at 4°C for 15 min and then the BCA protein assay was used for quantification of proteins. Subsequently, SDS-PAGE (10%) was employed for the separation of protein samples (30 μg), followed by transferring onto a PVDF membrane (Millipore, United States). For the blockage purpose of the membrane nonspecific sites, the membranes were incubated with skimmed milk (5%, dry) for 60 min in TBS-T. Then, for 24 h, the membranes were incubated with primary antibodies (at 4°C) *i.e.,* ERK 1/2 (1:2000; 9107#), phospho-ERK1/2 (*p*-ERK1/2; 1:2000; 4370#), CREB (1:500; 9197#), phospho-CREB-Ser133 (*p*-CREB; 1:500; 9198#), AKT (1:2000; 4691#), phospho-AKT (p- AKT; 1:2000; 4060#), β-actin (1:1000; 4967#) (all from Cell Signaling, United States),

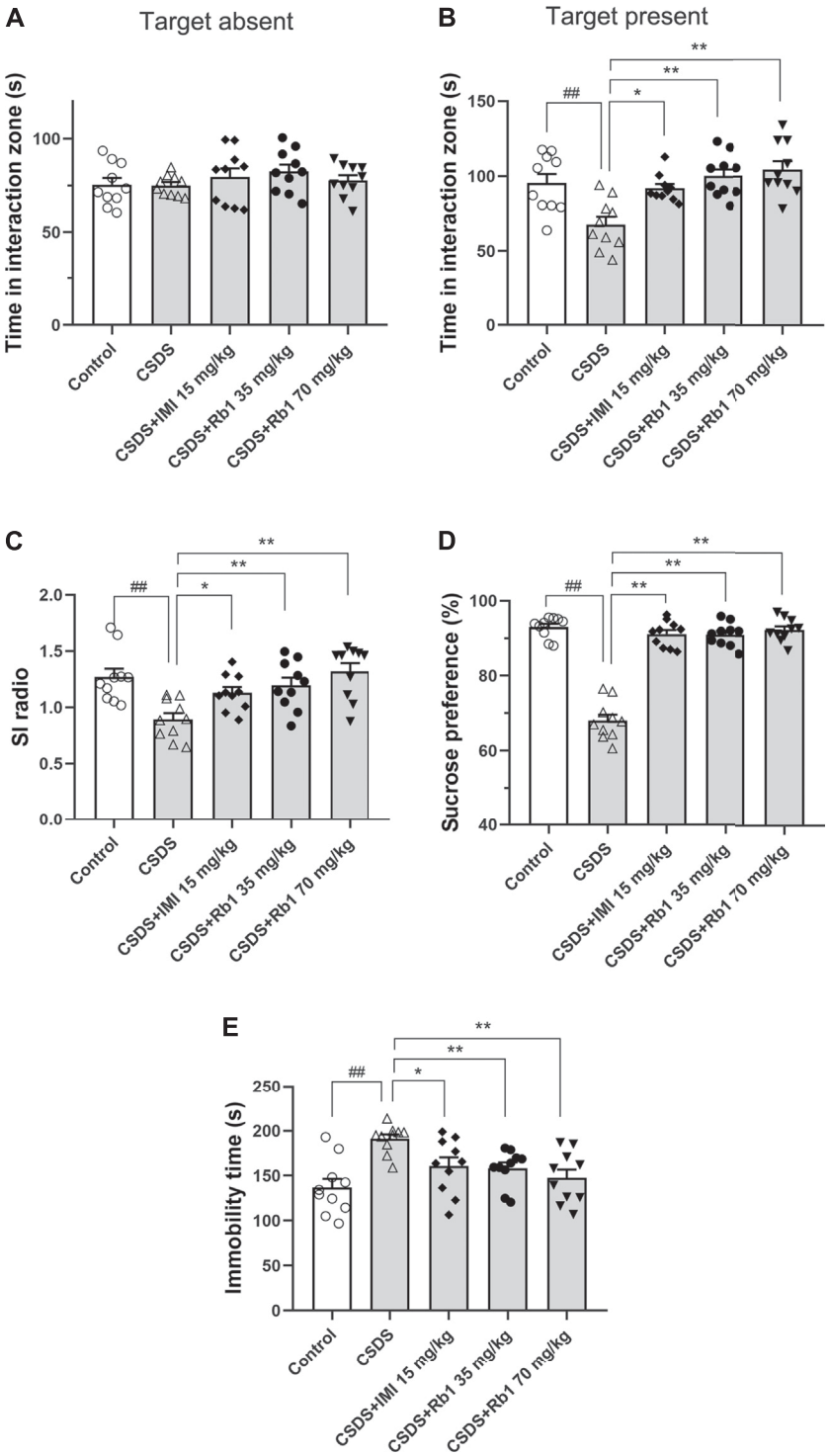

FIGURE 2 | Rb1 exerted antidepressant-like effects in a CSDS-induced depression model **(A–C)** The time spent in the social IZ and the SI ratio in the SIT. **(D)** The intake of sucrose (in percentage) in the SPT. **(E)** Immobility time in the FST. N = 10 in each group; the data are presented as the mean ± S.E.M. $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the group exposed to CSDS; one-way ANOVA.

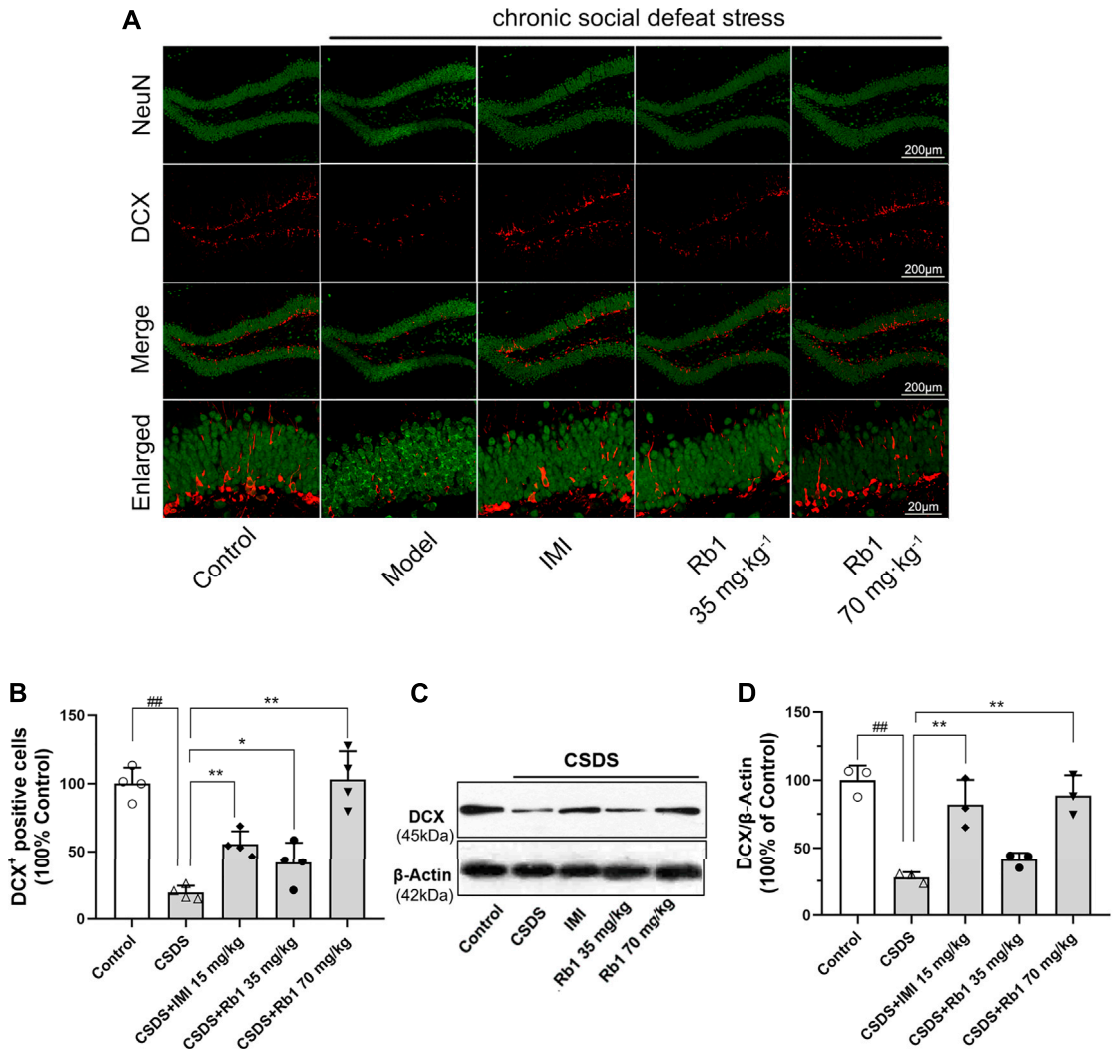

FIGURE 3 | Rb1 treatment improved the hippocampal neurogenesis in the CSDS depression model. **(A)** Representative immunofluorescence images of the dentate gyrus (DG) and costaining of the neuronal nuclear protein (NeuN) and DCX in green. The scale bar is 200 and 20 μm for the representative and the enlarged images, accordingly. **(B)** The number of DCX + cells in the DG. **(C–D)** Represents immunoblotting of DCX coincided with immunohistochemical changes. N = 3–4 per group, the obtained data have been depicted as the mean ± S.E.M. $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the model group.

and BDNF (1:5000; Ab108319). After washing, the membranes were incubated with secondary antibodies (HRP-conjugated) at ~ 25°C for 60 min. The bands were visualized *via* enhanced chemiluminescence and were captured *via* ChemiDoc XRS (Bio-Rad, United States).

Immunofluorescence Studies

Immunofluorescence studies were performed following the reported method with slight modifications [30, 31]. Following the behavioral tests, the animals were anesthetized by injecting pentobarbital sodium, followed by the transcardial perfusion *via* paraformaldehyde (4%) in phosphate buffer (0.01 M) for 24 h after the last session. The removal of the brain was carried out carefully and then the brain was post-fixed with the formaldehyde (4%) fixative and embedded in paraffin, followed by dissecting into thick sections (5 μm).

For double fluorescence staining, the sections were successively exposed to Triton X-100 (0.3%) in PBS (0.01 M) for 0.5 h and BSA (3%) in PBS (0.01 M) for 0.5 h. Next, the sections were incubated with a mouse anti-NeuN antibody (1: 100; Ab77450#) and a rabbit anti-DCX antibody (1:100; Ab177487#) at 4°C for 24 h (Abcam, United Kingdom). The sections were subsequently exposed to fluorescein isothiocyanate–labeled goat anti-rabbit IgG (1:300; GB22303) and rhodamine-labeled goat anti-mouse IgG (1:300; GB22301) for 50 min (Servicebio). PBS (0.01 M) was used for washing the sections, followed by their fixation on slides. After fixation, the slides were subjected to dehydration and were coverslipped. A fluorescence microscope (Leica, Germany Q9) was used for visualizing images with ×400 magnification. ImageJ software (Media Cybernetics, United States) was used for evaluating the fluorescence intensity of each group.

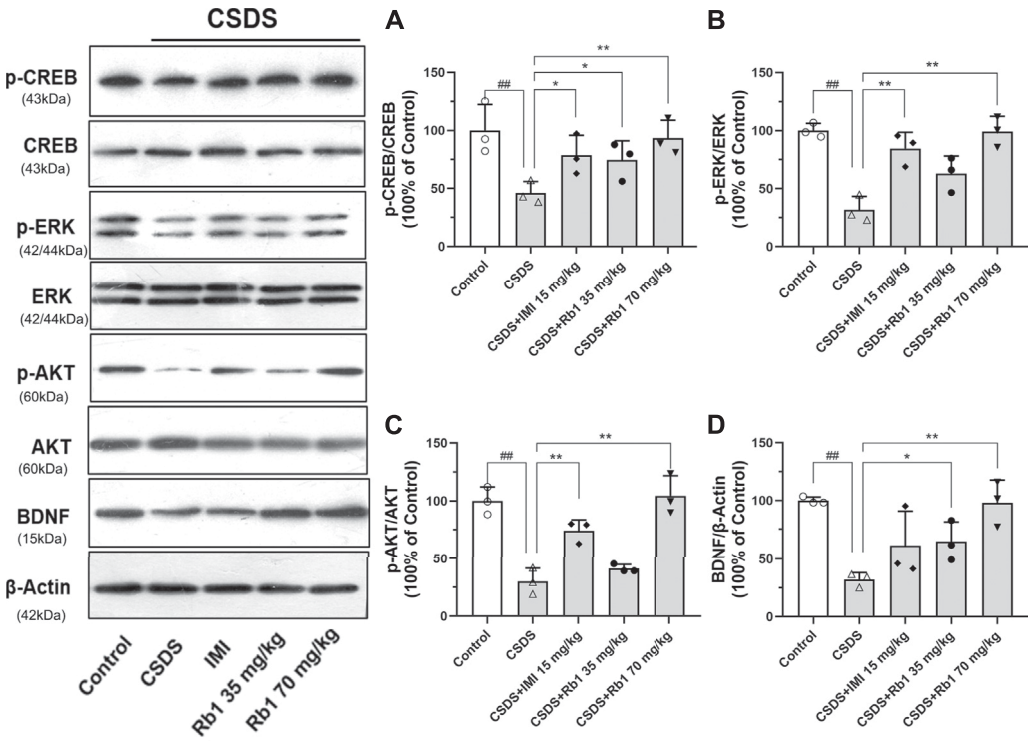

FIGURE 4 | Influence of Rb1 treatment on the expression of the BDNF signal pathway in the hippocampus of mice exposed to CSDS. **(A)** pCREB/CREB, **(B)** pERK/ERK, **(C)** pAKT/AKT, and **(D)** BDNF protein levels. N = 3 per group, the results are indicated as the mean ± S.E.M. $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the group exposed to CSDS, by one-way ANOVA analysis.

Data and Statistical Analyses

The analysis of the obtained results was performed *via* SPSS Statistics version 21.0 (Chicago, United States). The variations between mean values were determined *via* one-way or two-way ANOVA, as appropriate. For all one-way ANOVA analyses, post hoc tests were performed using the least significant difference test. For all two-way ANOVA analyses, Bonferroni post hoc tests were used to assess isolated comparisons. A p-value less than 0.05 was considered statistically considerable, and the obtained values were indicated as mean ± SEM.

RESULTS

Rb1 Treatment Prevents the Development of Depressive-Like Behaviors in CSDS-Exposed Mice

After 28 consecutive days of modeling and drug administration, different depressive-like behaviors were evaluated. These behaviors included anhedonia, as assessed by the sucrose preference test (SPT); social avoidance, as measured by the time spent in the IZ; and behavioral despair, as evidenced by the immobility time in the forced swim test (FST). In the vehicle-treated CSDS-exposed group, significant declines in the time spent in the IZ and social interaction (SI) ratio in the SIT ($F_{4,45} = 5.903$, $p < 0.01$; $F_{4,45} = 6.500$, $p < 0.01$) and sucrose consumption ($F_{4,45} = 31.747$, $p < 0.01$) and an increase in the immobility time in the FST ($F_{4,45} = 6.109$, $p < 0.01$) were observed, as shown in **Figures 2A–E**, respectively. However, the time spent in the IZ ($p < 0.01$ and <0.05) and sucrose intake in the SPT ($p < 0.01$ and 0.05) were considerably increased, and the immobility time in the FST ($p < 0.01$ and <0.05) was decreased in the groups treated with Rb1 (35 or 70 mg/kg) compared with the CSDS-exposed group; similar changes were seen in the 15 mg/kg IMI-treated group ($p < 0.05$). These results reveal that Rb1 exerts a potent antidepressant-like effect based on these behavioral tests.

Rb1 Treatment Counteracts the CSDS-Induced Deficits in Hippocampal Neurogenesis

It has been revealed that chronic stress lowers HN, which contributes to the onset of depression. The immunoreactivity of hippocampal doublecortin (DCX) was determined to evaluate the influence of Rb1 on HN. DCX is a microtubule-associated protein that is transiently expressed in newborn neurons and is used as a marker of neurogenesis (Jiang et al., 2014). Immunofluorescence revealed that the expression level of DCX was decreased in the dentate gyrus (DG) (red fluorescence) after 28 days of

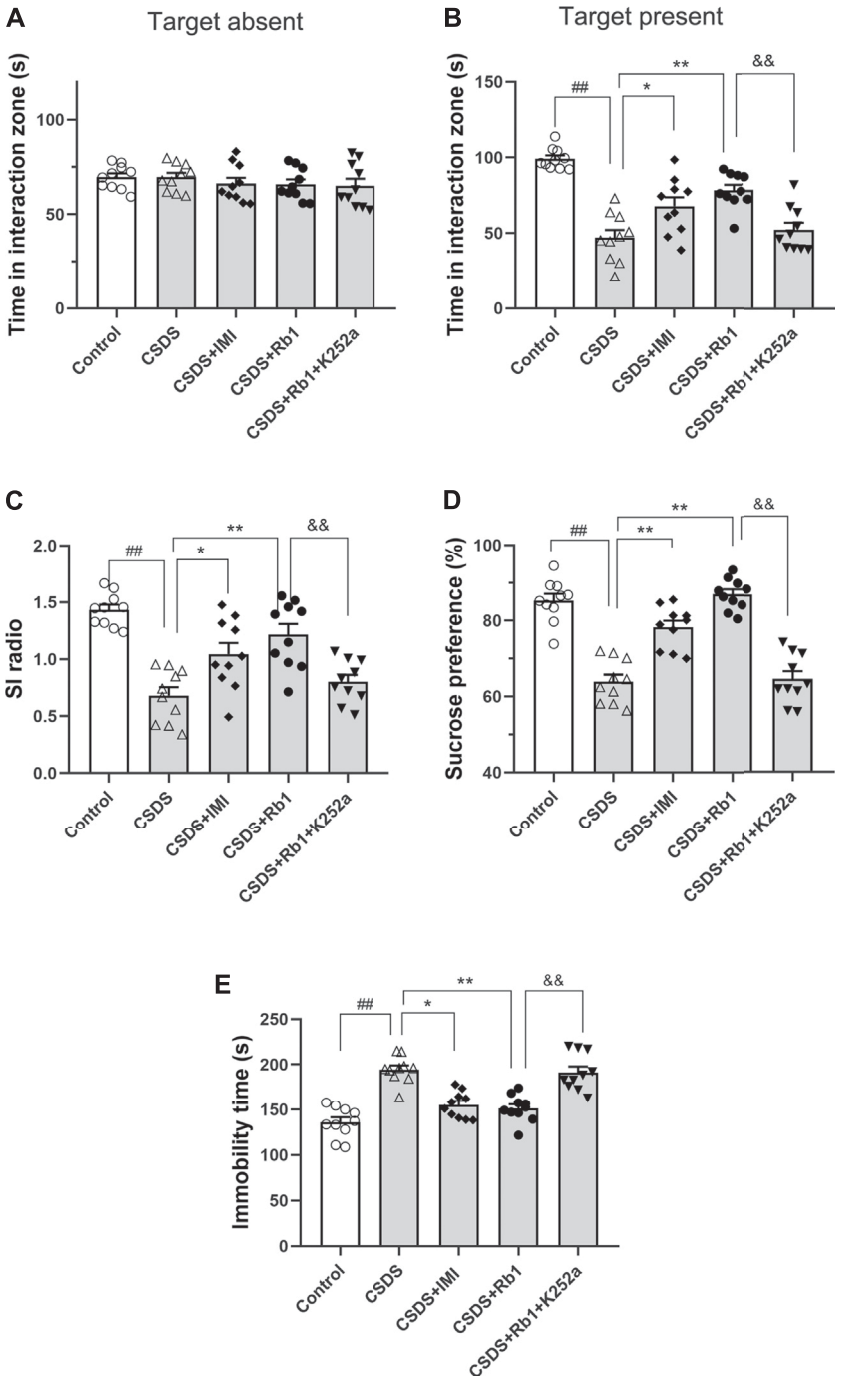

FIGURE 5 | Blockage of BDNF–TrkB signaling by K252a abolished the antidepressant activity of SY. **(A–C)** The time in the social interaction zone and the social interaction (SI) ratio in the SIT. **(D)** The intake of sucrose (in percentage) in the SPT. **(E)** Immobility time in the FST. The obtained results are indicated as means ± SEMs (n = 10). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the model group.

exposure to CSDS and that this change was reversed by Rb1, as presented in **Figure 3A**. Upon exposure to CSDS, the mice showed a marked reduction in the number of DCX^+ cells in the DG ($F_{4,15} = 44.883$, $p < 0.01$), as shown in **Figure 3B**. Moreover, treatment with Rb1 (35 or 70 mg/kg) or IMI (15 mg/kg) for 33 days significantly increased the number of DCX^+ cells in the DG ($p < 0.01$ and 0.01, respectively). Similarly, Western blotting revealed a significant decline in DCX protein expression in the hippocampus of the CSDS-exposed mice ($F_{4,10} = 21.265$, $p < 0.01$), as shown in **Figure 3C**, whereas Rb1 (35 and 70 mg/kg) treatment counteracted the decrease in BDNF expression induced by

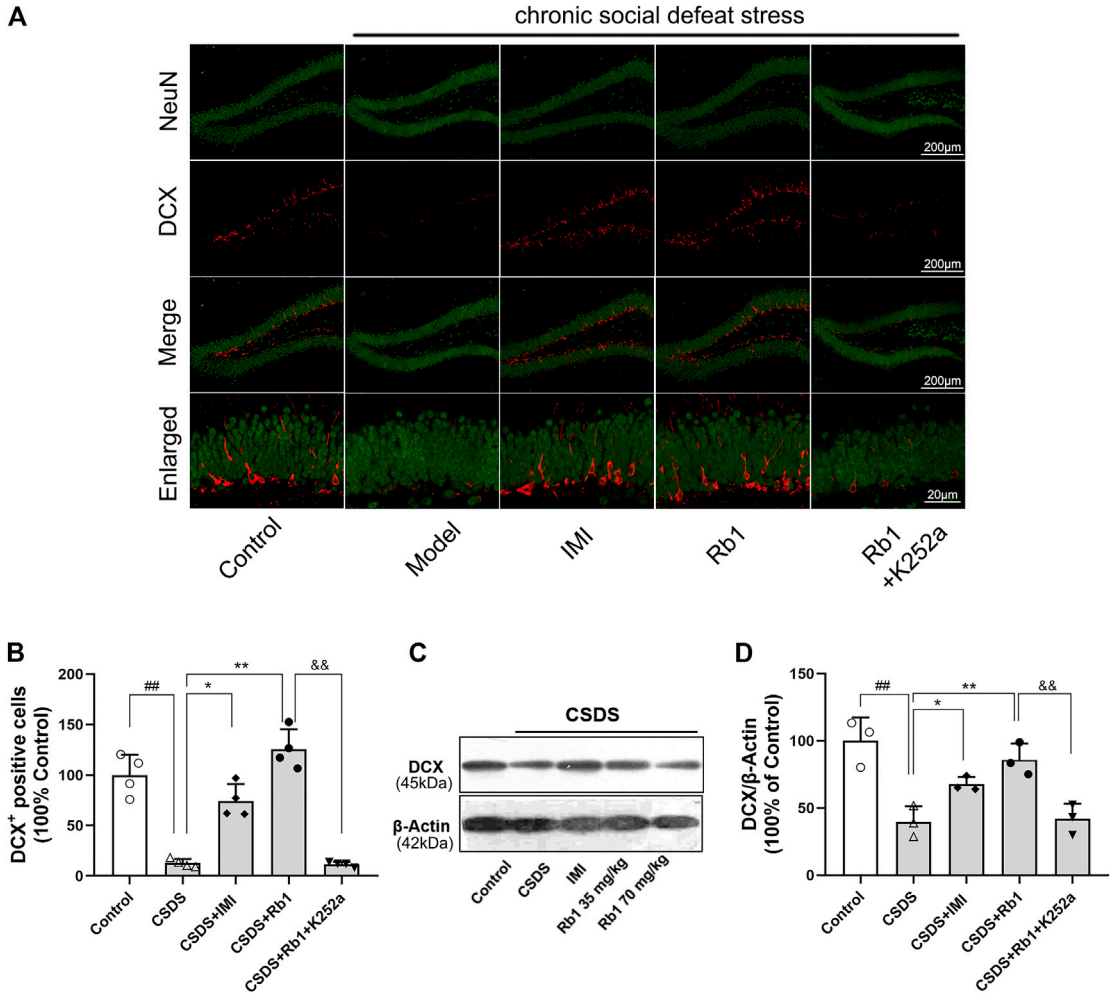

FIGURE 6 | Impact of pretreatment with K252a on the number of DCX-positive cells (Rb1-induced) in CSDS. **(A)** Representative immunofluorescence images of the dentate gyrus (DG) and costaining of the neuronal nuclear protein (NeuN) and DCX in green color. The scale bar is 200 and 20 μm for the representative and the enlarged images, accordingly. **(B)** The number of DCX + cells in the DG. **(C–D)** Represents immunoblotting of DCX coincided with immunohistochemical changes. N = 3–4 per group; the obtained data have been depicted as mean ± S.E.M. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the model group.

CSDS ($p < 0.01$, each). These findings suggest that Rb1 has protective effects on adult HN.

Rb1 Treatment Reverses the CSDS-Induced Inhibition of the BDNF Signaling Cascade

BDNF is a neurotrophic factor (NF) that substantially contributes to neurogenesis in adults and is thus associated with the pathogenesis of depression (Bjorkholm and Monteggia, 2016). In the current study, it was hypothesized that Rb1 may increase the expression of hippocampal BDNF. The Western blotting results revealed that hippocampal BDNF protein expression was considerably increased by exposure to Rb1, particularly at a dose of 70 mg/kg (**Figure 4D**; $F_{4,10} = 7.543$, $p < 0.01$). As indicated in **Figures 4A–C**, Rb1 (70 mg/kg) elevated the levels of the phosphorylated and activated forms of ERK ($F_{4,10} = 16.244$, $p < 0.01$), CREB ($F_{4,10} = 4.660$, $p < 0.01$), and AKT ($F_{4,10} = 24.889$, $p < 0.01$), which have been associated with activation of BDNF signaling.

BDNF–TrkB Signaling May Significantly Contribute to the Antidepressant Effect of Rb1

In the current study, K252a, which potently inhibits the BDNF receptor, that is, TrkB, was used. In brief, K252a (25 μg/kg) was injected into mice exposed to CSDS, Rb1 (70 mg/kg) was administered to these mice, and then behavioral tests were conducted. As presented in **Figure 5**, K252a abolished the antidepressant effects of Rb1 (70 mg/kg) in CSDS-exposed mice in the SIT, SPT, and FST. Similarly, **Figures 6** and **7** show that K252a not only blocked the effects of Rb1 on neurogenesis, as indicated by the fact that Rb1 treatment did not restore the number of DCX^{+} cells in the DGs of the mice

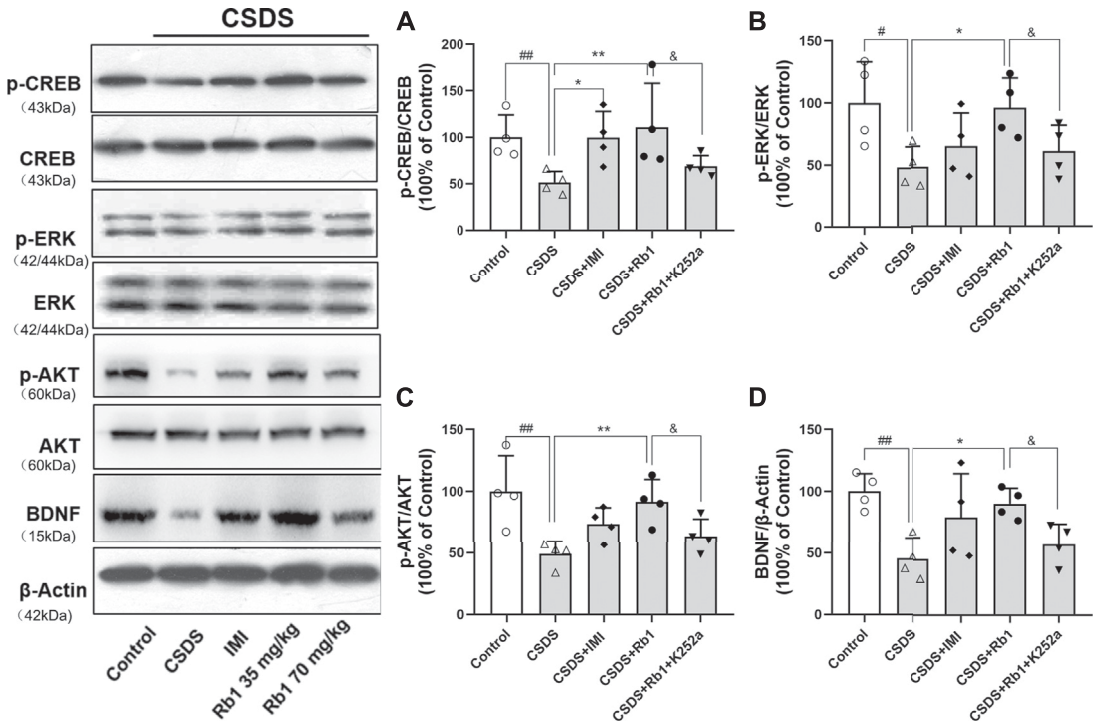

FIGURE 7 | Impact of pretreatment with K252a on Rb1-activated BDNF. The influence of SY on the pCREB/CREB **(A)**, pERK/ERK **(B)**, pAKT/AKT **(C)**, and BDNF **(D)** protein levels in the hippocampus of the rats exposed to CUMS. The results have been indicated as means ± SEMs (n = 4). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. the model group.

(exposed to CSDS), but also prevented the effects of Rb1 on the expression of BDNF, *p*-CREB, *p*-ERK1/2, and *p*-AKT in the hippocampi of the CSDS-exposed mice. Together, these results suggest that the BDNF–TrkB signaling cascade is required for the antidepressant effects of Rb1.

DISCUSSION

In the current study, it was revealed that Rb1 exerts an antidepressant-like effect in a CSDS-induced depression model. Chronic treatment with Rb1 might reverse the reduction in HN and the hippocampal BDNF signaling pathway induced *via* CSDS. By using inhibitors of BDNF–TrkB signaling, we further confirmed that the BDNF signaling cascade is required for the antidepressant-like potential of Rb1. Limitations still exist in the current study. In particular, the results of Western blotting must be considered very preliminary although promising due to the very low number of samples in each experimental group. Therefore, more studies involving more animals are required to confirm the results of Western blots and to understand the mechanisms of the antidepressant effects of Rb1. Moreover, Rb1 has been demonstrated its protection in the central nervous system and is apparently highly distributed to the brain. Previous studies reported that Rb1 could cross the blood–brain barrier (BBB) wnter the brain and the specific distribution of Rb1 in the rat brain (Li et al., 2007). Recent studies indicate that the transport of Rb1 at the BBB is at least partly mediated by the GLUT1 transporter *in vitro* and *in vivo* (Wang GL. et al., 2018). More insights into the pharmacokinetic properties of Rb1, especially for the blood–brain barrier permeability of Rb1, could be useful for its development as a suitable treatment and should be considered in more studies.

Animal models of CSDS-induced depression are useful and effective models that are widely used for studying psychosocial stress–induced depression (Yin et al., 2015). Being consistent with earlier results, the findings of this study showed that CSDS persistently induced a number of depression-like phenotypes characterized by despair, anhedonia, and social-avoidance behaviors, as demonstrated by deficits in the SIT, reduced consumption of sucrose solution in the SPT, and increased immobility time in the FST (Jiang et al., 2019b). These CSDS-induced changes in behavior were ameliorated by long-term treatment with Rb1 (35 or 70 mg/kg), which had a similar effect as the classical antidepressant IMI, suggesting that Rb1 may be a novel candidate for the treatment of depression.

In humans and several other species, neurogenesis in the hippocampal region starts postnatally and continues into adulthood. The development of hippocampal neurons is highly vulnerable to the adverse effects of stress and is involved in the pathophysiology and treatment of mood disorders (Jiang et al.,

2019b). A reduction in neurogenesis may significantly contribute to depressive episodes, and an improvement in HN has been correlated with the use of antidepressants (Sánchez-Vidaña et al., 2019). In the current study, 4 weeks of CSDS exposure suppressed neurogenesis in the mouse DG, as shown by a significant decrease in the number of DCX^+ cells, which is consistent with our earlier studies (Jiang et al., 2020b). However, treatment with Rb1 (35 or 70 mg/kg) elevated the number of DCX^+ cells in the DG and reversed the CSDS-induced suppression of neurogenesis, revealing that Rb1 may be a potential pro-neurogenic drug.

The obtained results also indicated that Rb1 elevated BDNF protein expression in the hippocampi of CSDS mice. BDNF is a key NF that regulates cell survival, substantially contributes to adult neurogenesis, and is important for the pathogenesis and treatment of depression (Warner-Schmidt and Duman, 2006; Bjorkholm and Monteggia, 2016). It was found that BDNF levels are considerably decreased in the hippocampi and cortices of rodents upon exposure to chronic stress; conversely, BDNF expression in these regions is increased by chronic treatment with antidepressants and is required for the effects of drugs on behaviors (Ghosal et al., 2018). Several studies have revealed that Rb1 regulates the expression of BDNF and activates neurogenesis in rats with experimental cerebral ischemia (Gao et al., 2010). Recent studies have shown that Rb1 pretreatment reverses the changes in BDNF/TrkB mRNA and protein expression in the hippocampus in rats exposed to acute immobilization stress (Kang et al., 2019). We thus speculate that Rb1 may promote downstream signaling cascades of BDNF, improve neurogenesis, and exert antidepressant effects. CSDS considerably decreased BDNF expression in the hippocampus, which was consistent with earlier results (Jiang et al., 2017). Conversely, Rb1 prevented the CSDS-stimulated reduction in hippocampal BDNF expression, which corresponded to elevation of neurogenesis. It has been demonstrated that BDNF phosphorylates and activates the protein CREB in the nucleus *via* the TrkB receptor, subsequently promoting the downstream MAPK/ERK and PI3K/AKT signaling cascades, which regulate the growth and survival of neuronal cells in the hippocampus, mediate depression (induced *via* stress), and exert antidepressant effects (Luo et al., 2015; Wu et al., 2018). In the current study, it was revealed that Rb1 treatment enhanced the phosphorylation of AKT and ERK1/2, two downstream regulators of BDNF, in the hippocampus and alleviated depression symptoms in CSDS mice. Moreover, K252a blocked the antidepressant effects of Rb1 in the behavioral tests and enhanced neurogenesis, confirming that the activation of BDNF signaling is required for the antidepressant-like activity of Rb1.

CONCLUSION

In brief, the current study revealed that Rb1 prevents depression-like symptoms in socially defeated mice, which seems to be facilitated *via* activation of the hippocampal BDNF–TrkB signaling pathway. The study explored the pharmacological properties of Rb1 and revealed that Rb1 could be an important candidate molecule in the prevention and treatment of disorders associated with stress, including depression, exerting considerable effects and relatively few adverse effects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved. The experimental procedure involving animals was performed with proper approval (approval no. SYXK 2017-0020), following the guidelines provided by the Animal Research Committee of the Institute of Medicinal Plant Development, Peking Union Medical College.

AUTHOR CONTRIBUTIONS

NJ and XL designed the research. NJ, JL, HH, and YZ conducted the experiments. NJ, HH, and YZ performed the data analysis. NJ, JL, QW, HH, and XL wrote and amended the manuscript. XL and QW supervised the study and contributed to project administration. All authors approved the final version.

FUNDING

This work was supported by the International Cooperative Project of Traditional Chinese Medicine (GZYYG2020023), Ministry of Science and Technology of China (2017ZX09301029) and Space Medical Experiment Project of China Manned Space Program (HYZHXM05003).

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frontiers in Pharmacology

MINI REVIEW
published: 16 November 2021
doi: 10.3389/fphar.2021.754502

Intranasal Delivery of Nerve Growth Factor in Neurodegenerative Diseases and Neurotrauma

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Since the 1980s, the development of a pharmacology based on nerve growth factor (NGF) has been postulated for the therapy of Alzheimer's disease (AD). This hypothesis was based on the rescuing effect of the neurotrophin on the cholinergic phenotype of the basal forebrain neurons, primarily compromised during the development of AD. Subsequently, the use of NGF was put forward to treat a broader spectrum of neurological conditions affecting the central nervous system, such as Parkinson's disease, degenerative retinopathies, severe brain traumas and neurodevelopmental dysfunctions. While supported by solid rational assumptions, the progress of a pharmacology founded on these hypotheses has been hampered by the difficulty of conveying NGF towards the brain parenchyma without resorting to invasive and risky delivery methods. At the end of the last century, it was shown that NGF administered intranasally to the olfactory epithelium was able to spread into the brain parenchyma. Notably, after such delivery, pharmacologically relevant concentration of exogenous NGF was found in brain areas located at considerable distances from the injection site along the rostral-caudal axis. These observations paved the way for preclinical characterization and clinical trials on the efficacy of intranasal NGF for the treatment of neurodegenerative diseases and of the consequences of brain trauma. In this review, a summary of the preclinical and clinical studies published to date will be attempted, as well as a discussion about the mechanisms underlying the efficacy and the possible development of the pharmacology based on intranasal conveyance of NGF to the brain.

Keywords: nerve growth factor, intranasal delivery, pharmacology, neurodegeneration, neurotrauma and neurodegenerative disease

OPEN ACCESS

Edited by:
Bruno Pietro Imbimbo, Chiesi Farmaceutici, Italy

Reviewed by:
William Howard Frey II, HealthPartners Neuroscience Center, United States
Simona Capsoni, Normal School of Pisa, Italy

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *06 August 2021*
Accepted: *01 November 2021*
Published: *16 November 2021*

Citation:
Manni L, Conti G, Chiaretti A and Soligo M (2021) Intranasal Delivery of Nerve Growth Factor in Neurodegenerative Diseases and Neurotrauma. Front. Pharmacol. 12:754502. doi: 10.3389/fphar.2021.754502

INTRODUCTION

The physiological peculiarity of the nerve growth factor (NGF) to regulate the survival and phenotype maintenance of specific neuronal populations in the peripheral and central nervous system (PNS and CNS, respectively) has laid the foundation for a broad line of preclinical and clinical research, aimed at exploring its pharmacological potential for the treatment of neurodegenerative diseases and of the outcomes of neurotrauma (Aloe et al., 2012; Allen et al., 2013). The enormous amount of preclinical research, conducted on a large number of *in vitro* and *in vivo* models, has indicated Alzheimer's disease (AD) as a primary field of intervention (Cattaneo et al., 2008; Cattaneo and Calissano, 2012). The rationale for this therapeutic approach stems from the selective effect of

NGF on the basal forebrain cholinergic neurons (BFCNs) (Hefti et al., 1984; Hefti, 1986) and from the evidence that the circuits connecting BFCNs to the cortex and hippocampus undergo early suffering during the development of AD (Whitehouse et al., 1981; Bartus et al., 1982). This rationale has subsequently been expanded by the accumulation of evidence regarding non cholinergic-specific actions exerted by NGF (Chiaretti et al., 2008; Calissano et al., 2010; Cragnolini et al., 2018; Rizzi et al., 2018). Furthermore, preclinical and clinical data on the pharmacological efficacy of NGF, indicated that this was severely limited by poor permeability of the molecule to the blood-brain barrier (Poduslo and Curran, 1996; Thorne and Frey, 2001) and by the possibility that side effects such as hyperalgesia, myalgias and weight loss, could outweigh the therapeutic benefits (Aloe et al., 2012). This brief review will focus mainly on the clinical experience gained to date, regarding the administration of NGF to the brain of patients suffering from neurodegenerative diseases and from the outcomes of neurotrauma. For a more in-depth discussion of the preclinical studies that have supported the clinical trials conducted so far, the reader is referred to more extensive reviews (Colafrancesco and Villoslada, 2011; Aloe et al., 2012; Cuello et al., 2019; Mitra et al., 2019).

AN EXTENDED RATIONALE FOR THE USE OF NGF IN DISEASES OF THE CENTRAL NERVOUS SYSTEM

NGF is the first discovered growth factor and a member of the neurotrophin family (Levi-Montalcini, 1952, 1987). It is synthesized as a pro-peptide (proNGF) starting from two splicing variants currently identified in humans (Scott et al., 1983; Ullrich et al., 1983; Edwards et al., 1986; Soligo et al., 2020a). The intracellular and/or extracellular processing of proNGFs generates a C-terminal mature fragment of 118–120 aminoacids (Seidah et al., 1996; Bruno and Cuello, 2006), which is the molecule currently under investigation for its pharmacological potential. NGF activates the tropomyosin receptor kinase A (TrkA) (Klein et al., 1991) and/or the p75 pan-neurotrophin receptor (p75NTR) (Johnson et al., 1986). The interaction between the two receptors, whether or not associated in hetero-complex, greatly increases the affinity (kd = 0.03 nM) for the binding of NGF to TrkA (Barker, 2007; Wehrman et al., 2007).

In the CNS, NGF is primarily neurotrophic for cholinergic neurons of the basal forebrain (Hefti, 1986) and for both healthy developing and damaged adult cholinergic interneurons in the striatum (Gage et al., 1989). During adult life, NGF, produced by BFCN-targets of innervation (Korsching et al., 1985), controls the maintenance of the cholinergic phenotype regulating the expression of choline-acetyltransferase (ChAT) (Gnahn et al., 1983; Pongrac and Rylett, 1998). The synthesis and release of NGF could be in turn regulated by the cholinergic activity and the release of acetylcholine (Knipper et al., 1994; Bruno and Cuello, 2006). Once released, NGF is internalized by the cholinergic endings and retrograde transported to the neuronal Soma (Seiler and Schwab, 1984). Thus, the canonical rationale for the treatment of AD patients with NGF is based on reported defective retrograde transport of NGF toward BFCN (Mufson et al., 1995) and on the accumulation of proNGF, that may have neurotoxic action (Lee et al., 2001), in the brain of AD patients (Fahnestock et al., 2001).

Preclinical and clinical studies have also demonstrated a pharmacological value of NGF in the treatment of neurotrauma outcomes (Kromer, 1987; Cacialli, 2021). The rationale behind these studies does not necessarily include the effect of NGF on cholinergic neurons, but extends to other peculiarities of the biological action of NGF. An extension of the therapeutic mechanisms triggered by NGF has been proposed based on the relationship between NGF, its receptors and the metabolism of the amyloid precursor protein (APP) and the protein tau (Cattaneo et al., 2008) (**Figure 1**). Altered metabolism of APP and tau are reported in a wide spectrum of neurological diseases (Gasparini et al., 2007; Hellewell et al., 2010; Zhang et al., 2018; Lim et al., 2019; Edwards et al., 2020). Described hallmarks of both neurodegenerative diseases and neurotraumas are altered processing of APP, the formation of 40–42 aminoacids-long peptides (amyloid-β: Aβ-40, Aβ-42) and their aggregation in the β-amyloid plaques, as well as the excessive phosphorylation and truncation of the tau protein, its aggregation and loss of function as a stabilizer of microtubules (Walsh and Selkoe, 2004; Gong and Iqbal, 2008; Xu et al., 2021). A direct interaction between APP and TrkA has been demonstrated, which, if disturbed by the presence of the Aβ peptides, is correlated to the induction of apoptosis (Canu et al., 2017a; 2017b). Furthermore, NGF binding to TrkA may route the APP metabolism toward the non-amyloidogenic processing, by modulating the interaction of APP with secretases (Canu et al., 2017a; 2017b). It is known that the amyloidogenic cascade is activated following NGF deprivation (Capsoni et al., 2000; Matrone et al., 2008; Latina et al., 2017) and in transgenic mice overexpressing proNGF (Tiveron et al., 2013). Such deprivation of NGF and/or increased proNGF/NGF ratio, both *in vitro* and *in vivo*, also leads to increased phosphorylation of tau and its abnormal cleavage (Nuydens et al., 1997; Capsoni et al., 2000; Shen et al., 2018; Mufson et al., 2019). Overall, these evidences suggest that NGF-based therapy could improve neurological outcomes that are related to dysfunctions of the central cholinergic system, both in neurodegenerative diseases (Mufson et al., 2019) and after TBI (Shin and Dixon, 2015), normalizing APP and tau metabolism in TrkA-expressing cells.

NGF regulates the functions of astrocytes and microglia (Pöyhönen et al., 2019) (**Figure 1**), modulating the glial response especially in conditions of suffering and/or trauma of the nervous system. NGF may modulate astrogliosis by arresting the cell cycle of astrocytes (Cragnolini et al., 2012). It may also act in anti-amyloidogenic way by regulating the inflammatory response of microglia (Capsoni et al., 2017; Rizzi et al., 2018) and decreasing the pro-inflammatory response through the reduction of microglial glycolysis (Fodelianaki et al., 2019). Moreover, NGF treatment leads to a modulation of microglia motility, micropinocytosis and degradation of Aβ deposition (Rizzi et al., 2018). This may account for non-TrkA-mediated, indirect action of NGF on the clearance of oligomers and

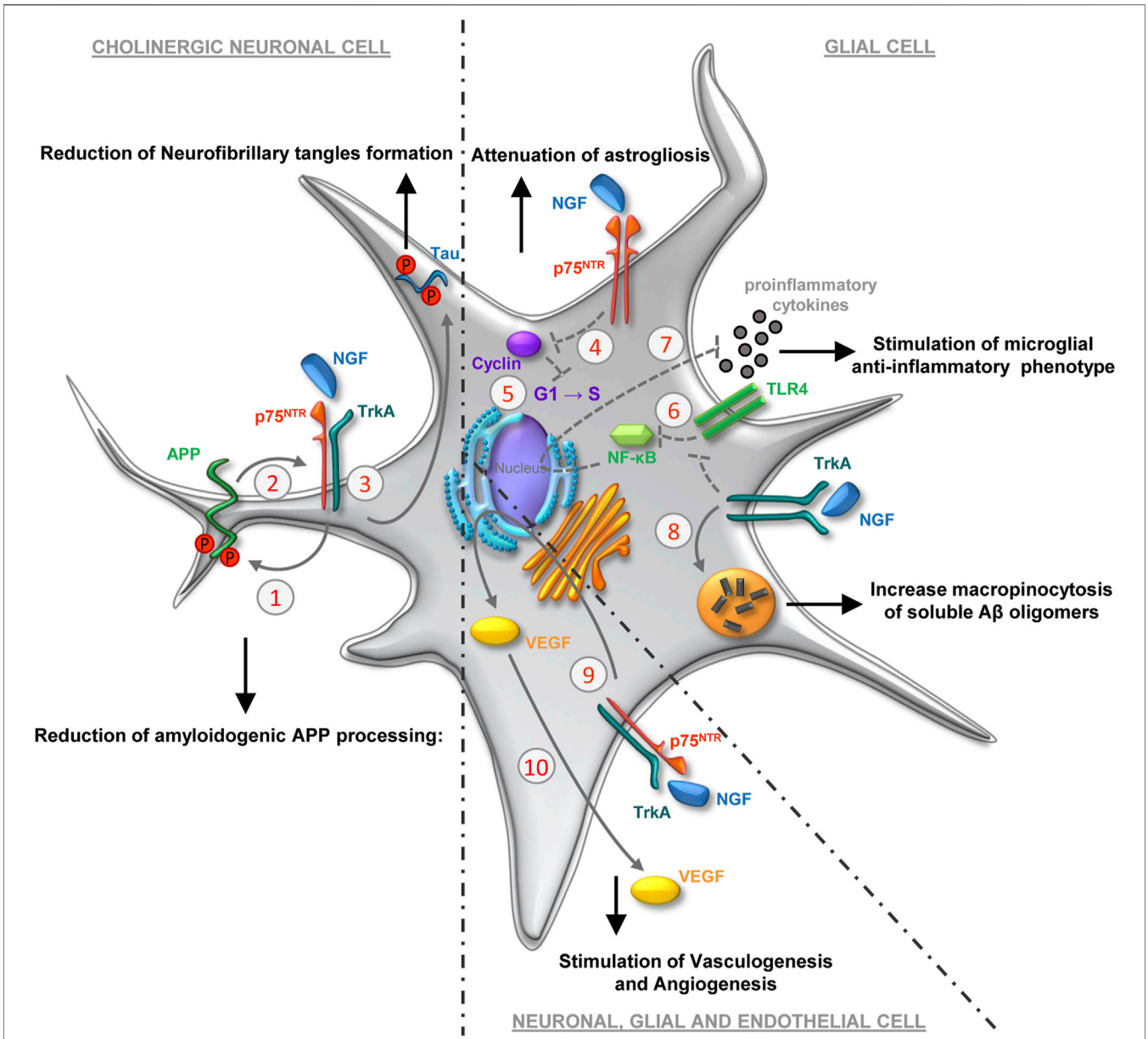

FIGURE 1 | Molecular and cellular mechanisms underlying the therapeutic effect of NGF in the central nervous system. Other than the canonical effects on the phenotypic maintenance of basal forebrain cholinergic neurons, several mechanisms have been proposed to explain the outcomes elicited by the conveyance of exogenous NGF to the brain. The phosphorylation of APP (1) is regulated by the rate of APP/NGF-receptor association (2), in turn modulated by the interaction of NGF with the homodimer TrkA/TrkA and/or heterodimer TrkA/p75NTR (Canu et al., 2017b). The APP/NGF-receptor association makes APP less prone to be processed by β- and γ-secretases, resulting in decreased Aβ oligomerization in neurons expressing NGF receptors. The binding of NGF to its receptor complex reduces tau neurofibrillary tangles formation (3), regulating the post-translational modification of tau (phosphorylation, cleavage, and ubiquitination) (Canu et al., 2017b). In astrocytes, NGF/p75NTR interaction attenuates the induction of cyclins (4), thereby promoting the withdrawal of astrocytes from the cell cycle (5) attenuating astrogliosis (Cragnolini et al., 2012). NGF, inhibiting early TLR4-mediated activation of the NFκB (6) and JNK pathways, attenuates pro-inflammatory cytokines release in microglia (7) and may thereby contribute to regulation of microglia-mediated neuroinflammation (Fodelianaki et al., 2019). NGF-TrkA binding modulates microglia motility, macropinocytosis and degradation of Aβ deposition (8) (Rizzi et al., 2018). NGF initiates signaling (9) that supports the production and release of VEGF (10), in turn involved in both vasculogenesis and angiogenesis (Samii et al., 1999).

aggregates in the brain of AD or TBI patients. The description of the complex glial function during neurological diseases goes beyond the scope of this work, (for recent reviews on the topic see: Rasband, 2016; Meyer and Kaspar, 2017; Stevenson et al., 2020). Nevertheless, it is important to underline that through its modulation of glial function, NGF may promote the establishment of a *milieu* advantageous to the processes of neuroprotection and neurorepair.

Strengthening this last consideration, is the positive effect on brain perfusion observed after administration of NGF to the

brain of both laboratory animals and humans (Raychaudhuri et al., 2001; Cantarella et al., 2002; Dolle et al., 2005; Chiaretti et al., 2008; Jadhao et al., 2012). NGF has a pro-angiogenic activity. Inducing the production of vascular-endothelial growth factor (VEGF) (Samii et al., 1999; Graiani et al., 2004; Manni et al., 2005), a growth factor expressed either by neurons, glia and endothelial cells (Ogunshola et al., 2000; Nag et al., 2002), NGF may promote the proliferation and migration of endothelial cells (Chiaretti et al., 2002; Emanueli et al., 2002; Graiani et al., 2004; Salis et al., 2004) (**Figure 1**). Moreover, NGF stimulates the production of vasodilating agents, such as nitric oxide (Nizari et al., 2021). Furthermore, intranasal NGF is able to stimulate neo-angiogenesis following cerebral infarction in rats by activating PI3k/Akt signaling (Li et al., 2018). Overall, these mechanisms may underlie the observed increase in brain perfusion after NGF delivery to the human brain (Olson et al., 1992; Eriksdotter-Jönhagen et al., 1998; Tuszynski et al., 2005; Chiaretti et al., 2008, 2017, 2020; Fantacci et al., 2013; Rafii et al., 2018). Finally, the indirect action exerted by NGF on cerebral perfusion by stimulating the innervation of the cerebral vasculature (Isaacson et al., 1990) and the possible role of NGF-modulated glial regulation of brain perfusion and metabolism (Rasband, 2016), should not be underestimated.

Intraparenchymal and Intracerebroventricular Delivery of NGF to the Human Brain

Since 1991, the administration of NGF to the human brain has been pursued through delivery to the brain parenchyma (intraparenchyma: IP) or cerebral ventricles (intracerebroventricular: ICV). The rationale was based on the action of NGF on NGF-responsive cells, therefore on BFCN in AD patients (Olson et al., 1992; Eriksdotter-Jönhagen et al., 1998, 2012; Tuszynski et al., 2005; Bishop et al., 2008; Rafii et al., 2014; Karami et al., 2015; Eyjolfsdottir et al., 2016; Machado et al., 2020) or on catecholaminergic cells of adrenal origin transplanted into the brain of Parkinson's patients (Olson et al., 1991). Only in some compassionate studies NGF has been administered ICV to pediatric patients suffering from severe hypoxic-ischemic trauma, not aiming at stimulating selectively the cholinergic function (Chiaretti et al., 2005, 2008; Fantacci et al., 2013). The delivery systems, whether purified NGF was delivered, whether it was the inoculation of adenovirus for gene therapy or those of NGF-producing cells, involved invasive, relatively risky surgical procedures for administration/implantation, difficult to configure in view of the need for large-scale treatments. For detailed description and methodological consideration about the delivery of NGF to CNS in the above-cited clinical studies the reader is referred to more comprehensive reviews (Thorne and Frey, 2001; Aloe et al., 2012; Wahlberg et al., 2012; Mitra et al., 2019; Eftimiadi et al., 2021).

Here, it is important to underline some aspects that integrate the clinical experiences related to the delivery of NGF to the brain, so far reported in clinical trials (some of which are accessible on https://clinicaltrials.gov/: NCT00017940, NCT01163825, NCT00087789, NCT00876863). In AD patients, most studies reported increased activity of nicotinic receptors, measured through the incorporation of ^{11}C-nicotine (Olson et al., 1992; Eriksdotter-Jönhagen et al., 1998, 2012; Tuszynski et al., 2005; Karami et al., 2015). The data from a specific trial (NCT01163825) also highlighted, albeit only on patients defined as "responders", increase in the activity of ChAT and AChE in the CSF following delivery of NGF mediated by cellular implants in the basal forebrain. These outcomes showed positive correlation with a slower cognitive decline, increased glucose uptake, increased ^{11}C-nicotine binding, decreased Aβ-42 and phospho-tau levels in CSF (Eriksdotter-Jönhagen et al., 2012; Karami et al., 2015; Eyjolfsdottir et al., 2016; Mitra et al., 2019). The assessments of cognitive status, although not consistently showing effects related to NGF treatments, indicate the possibility of slowing the progression of AD through the supplementation of NGF to BFCNs (Tuszynski et al., 2005; Eyjolfsdottir et al., 2016). The improvement in the fast-to-slow waves ratio recorded in the EEG in several of the studies mentioned so far, also indicates the potential efficacy of NGF in correcting neurophysiological deficits observed in AD patients.

One of the most relevant and common effects of NGF in the aforementioned studies, was the increase in cerebral perfusion and ^{18}F-FDG uptake, an index of increased glucose metabolism found in various brain areas. The mechanisms underlying the effect of NGF on cerebral perfusion have already been addressed above. The augmented metabolism could be linked to the increased availability of nutrients, the rise in the septum-cortical circuits activity (Tuszynski et al., 2005) or also to specific effects of NGF on the metabolism of NGF-responsive neurons and glia (Rasband, 2016; Colardo et al., 2021).

As for the development of side effects, in the clinical studies conducted so far (Eriksdotter-Jönhagen et al., 1998) they have been mainly related to the insurgence of hyperalgesia and allodynia (back pain, myalgia) and to the onset of an anorectic effect (Lapchak and Araujo, 1994) with consequent weight loss. It should be noted that these effects are generally reversible and dose-dependent (Eriksdotter-Jönhagen et al., 1998) and that they occurred following ICV, but not after IP delivery.

Although characterized by encouraging indication about the safety and tolerability of some of the procedures used to deliver NGF to the brain parenchyma (Tuszynski et al., 2005; Eriksdotter-Jönhagen et al., 2012; Rafii et al., 2014; Eyjolfsdottir et al., 2016), these studies have not yet laid the foundation for the development of a NGF pharmacology based on invasive neurosurgical procedures. Indeed, a recent post-mortem study revealed the failure of targeting BFCN after virus-mediated NGF gene delivery, due to the limited spread of the vector from the injection site (Castle et al., 2020). On the other hand, the encapsulated cells biodelivery of NGF appears to be in an early stage of development, still being hampered by variations in the levels of NGF-release between implants, inconsistent cells viability and inflammatory reactions due to surgical procedures (Mitra et al., 2019).

Intranasal Delivery of Nerve Growth Factor to the Brain

The non-invasive, intranasal delivery of biomolecules, aimed at bypassing the blood-brain barrier and reaching the brain

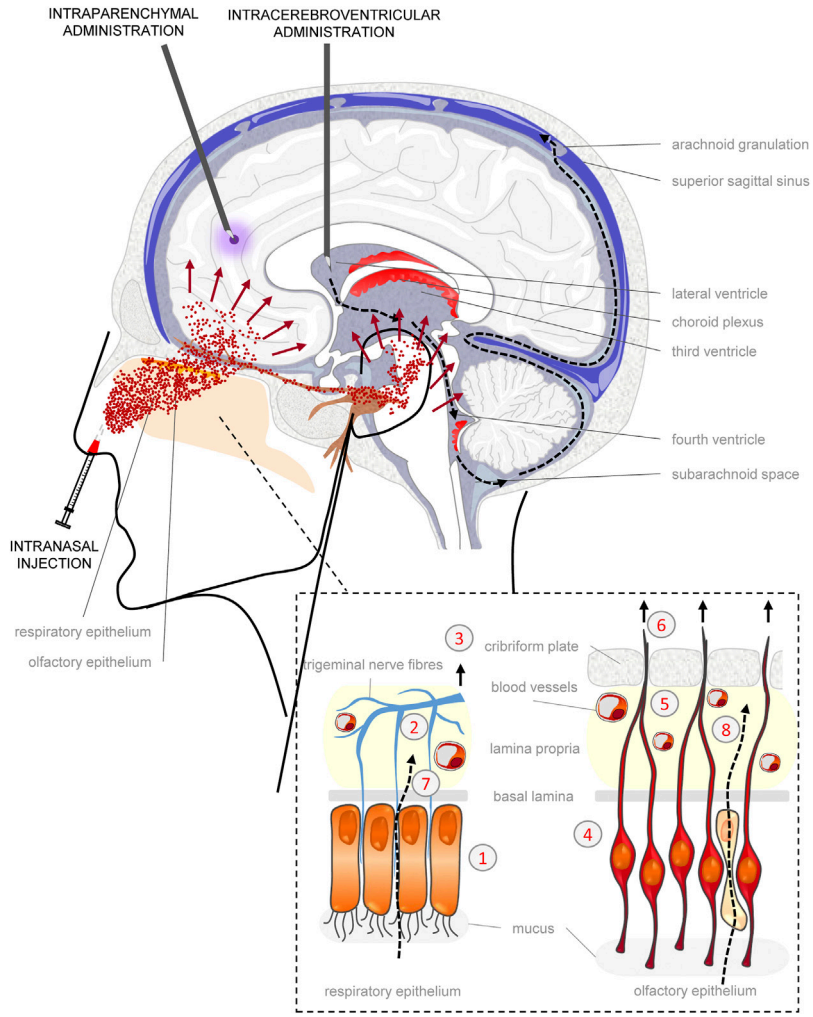

FIGURE 2 | Mechanisms of exogenous biomolecule distribution to the brain tissue. Intraparenchymal (IP) administration allows the local delivery of biomolecules in CNS tissue, limiting distribution to an area no more than about 2 mm from the site of introduction (violet spread). Intracerebroventricular (ICV) administration may deliver biomolecules to wide areas of CNS as a result of circulation within cerebrospinal fluid (CSF) (black dotted arrows). From the lateral ventricle, through the fourth ventricle biomolecules can reach the subarachnoid space where the CSF is filtered by arachnoid granulations in the bloodstream of the superior sagittal sinus. However, many limitations affect ICV administration: penetration into the underlying parenchymal tissue (about 2 mm), rapid clearance (NGF half-life < 1 h, in the 150 ml of CSF, which is replaced entirely within 8 h), sequestration because of binding to protein component of CSF. Intranasal (IN) injection is a non-invasive alternative to both IP and ICV administrations that permits direct delivery to CNS bypassing the BBB (Lochhead and Thorne, 2012). Nose-to-brain passage of biomolecules (red spray) may occur either by intracellular or extracellular pathways both in the respiratory and olfactory epithelium (enlarged box at the bottom right), allowing drugs to reach in almost every brain region. The intracellular transport occurs through: endocytosis across the respiratory epithelium (1), toward the peripheral trigeminal nerve (2) and transport to brainstem (3); endocytosis into olfactory sensory neurons (OSN) (4) that extend across the basal lamina and converge with axons from other OSN to form nerve bundles (5) projecting to the olfactory bulbs, piriform cortex, amygdala and entorhinal cortex (6); transcytosis to the lamina propria across other cells of the respiratory epithelium (7) and sustentacular cells (8) of olfactory epithelium (Lochhead and Thorne, 2012). The extracellular pathways consist of paracellular diffusion within perineural, perivascular or lymphatic channels associated with trigeminal and olfactory fibres that enter the brain (Lochhead and Thorne, 2012), and is the preferential route of diffusion of NGF into the brain, due to the lack of TrkA expression on the olfactory epithelium that limit the intracellular entry and transport (Frey et al., 1997).

parenchyma, has been extensively explored since the end of the last century (Frey et al., 1995, 1997; Thorne et al., 1995; Chen et al., 1998) and several patents by Frey et al. claim intranasal delivery of drugs to the brain along the olfactory neural pathway (Frey, 1997), and the trigeminal neural pathway (Jogani et al., 2008).

The transport of drugs from nose to brain occurs after conveyance to the olfactory epithelium, the uppermost part of the nasal cavity that contains the olfactory sensory neurons (Dhuria et al., 2010; Lochhead and Davis, 2019). The transport to the brain (**Figure 2**) can occur by extracellular and intracellular pathways and through diffusion in the perivascular and perineural spaces of the olfactory and trigeminal nerves (Dhuria et al., 2010; Lochhead and Davis, 2019). Once it reaches the brain, rostrally via the olfactory pathways and caudally via the trigeminal nerve, the drug rapidly diffuses into the cerebral perivascular spaces, potentially distributing itself throughout the whole cerebral parenchyma (Lochhead and Davis, 2019).

Intranasally-delivered iodinated NGF (IN-NGF), unlike NGF administered intravenously, was able to rapidly (within 20 min from inoculation) spread in the brain (Frey et al., 1997). IN-NGF was mainly found in the olfactory bulbs and the brainstem, albeit distributed in the whole brain region between them. (Frey et al., 1997). Of note, only 0.3% of exogenous NGF was found in the bloodstream after IN-NGF (Frey et al., 1997). These results, obtained after delivery of iodinated NGF, were confirmed by subsequent studies, in which native murine NGF (Chen et al., 1998) or a "painless" human NGF mutein (Capsoni et al., 2017) were IN delivered and detected by ELISA. Based on these findings, on the linear relationship between the intranasal dose and resulting brain concentration and on the known absence of TrkA receptors on the olfactory epithelium, an extracellular and perineural/perivascular pathway of diffusion was hypothesized (Frey et al., 1997; Chen et al., 1998). Interestingly, despite diffusion of IN-NGF in the CSF was predictable, due to the connection between perineural and perivascular spaces and nasal lymphatics with the subarachnoid space (Dhuria et al., 2010) (**Figure 2**), low levels of IN-NGF were found in the CSF by ELISA (Chen et al., 1998).

IN-NGF has been extensively studied in preclinical models of AD, using NGF brain-deprived mice (AD11 mice), or multiple-transgenic models, co-expressing mutated forms of APP and presenilin 1 (APPxPS1) or comprising five familial Alzheimer's disease mutations (5xFAD). IN-NGF, delivered in its native form or as a "painless" mutein, improved neurodegenerative symptoms (Capsoni et al., 2002) by ameliorating cholinergic deficits (Covaceuszach et al., 2009; Capsoni et al., 2012), decreasing tau phosphorylation (Capsoni et al., 2009, 2012; Covaceuszach et al., 2009), APP metabolism and Aβ plaque deposition (Covaceuszach et al., 2009; Capsoni et al., 2012, 2017; Yang et al., 2014), at the same time rescuing both recognition-spatial memory deficits (De Rosa et al., 2005; Capsoni et al., 2012, 2017), hippocampal and LTP deficits (Capsoni et al., 2017). IN-NGF also counteracted microglia and astrocytes activation, Aβ presence in both cellular types and the production of pro-inflammatory cytokines (Capsoni et al., 2012, 2017).

Other relevant models of neurological pathologies in which the efficacy of IN-NGF has been attempted, include cerebral ischemia, traumatic lesions of the brain and spinal cord, epilepsy, amyotrophic lateral sclerosis, hypogonadism related to premature aging, and depression. Also in these models, IN-NGF improved selective behavioral performances (Cheng et al., 2009; Shi et al., 2010; Bianchi et al., 2012; Tian et al., 2012; Aloe et al., 2014; Zhong et al., 2017), Aβ plaque deposition and tau phosphorylation (Tian et al., 2012; Lv et al., 2014), and promoted anti-inflammatory response (Lv et al., 2013). It also decreased seizure onset (Lei et al., 2017), counteracted disease-induced apoptosis (Cheng et al., 2009; Lv et al., 2013; Lei et al., 2017), enhanced VEGF and endothelial cell migration (Li et al., 2018), enhanced neurogenesis (Cheng et al., 2009; Zhu et al., 2011), regulated hypothalamic gonadotropin releasing hormone production (Luo et al., 2018). Only one study found IN-NGF not effective in ameliorating motor functions impaired by brain trauma (Young et al., 2015).

A total number of four patients have so far been treated with IN-NGF. In the first case-report (Chiaretti et al., 2017) murine NGF was intranasally delivered in a 4-years-old boy suffering for the consequences of a severe TBI. The patient received one cycle (100 μg/kg twice a day for 10 consecutive days) each month for 4 months of IN-NGF. This regimen progressively improved brain perfusion and brain metabolism, increased EEG fast/slow waves ratio, reduced ventricular dilatation and parenchymal lesions and normalized the size of subarachnoid spaces. No side effects related to NGF therapy were reported, related to nociceptive hyper-response or autonomic abnormalities, despite a modest increase of NGF content in the CSF. A second clinical study (de Bellis et al., 2018) reported IN-NGF in two adult patients affected by frontotemporal dementia associated with corticobasal syndrome. Patients received 2 μg/day of murine NGF for a 1-year period. A dose escalation to 4 and 6 μg/day was attempted and the insurgence of reversible side effects (rhinitis, rigidity, moderate psychomotor agitation) recorded. Significant reduction in the mini-mental state examination score was observed and returned to pre-treatment conditions within 1 year after stopping NGF treatment. PET-scans revealed a progressive and significant increase in FDG-uptake in several cortical and subcortical brain areas, which was also reverted to pre-treatment levels after IN-NGF interruption. In a third case-report (Chiaretti et al., 2020) a 7-weeks-old infant with persistent wakefulness syndrome due to late-onset group-B *Streptococcus* meningitis was treated with commercial human recombinant NGF (Oxervate®, Dompè Farmaceutici). The infant received five monthly cycles of intranasal NGF (20 μg/day for seven consecutive days). IN-NGF promoted a progressive improvement of brain hypometabolism, increasing glucose uptake in cortical and subcortical regions. Clinical scales for assessment of comatose and cognitive states all improved after the study protocol was completed.

A EU-registered therapeutic exploratory (phase II) clinical trial (https://www.clinicaltrialsregister.eu/ctr-search/trial/2019-002282-35/IT) on five children aged between 6 months and 5 years, and affected by severe neurosensory, cognitive and motor deficits after traumatic brain injury, is actually ongoing, aiming at producing evidence of changes in clinical and neurological conditions after treatment with 50 μg/kg of IN-rhNGF (Oxervate®, Dompè Farmaceutici).

DISCUSSION

The pharmacology of IN-NGF seems to be heading towards promising development, based on the ease of administration, the efficiency of drug distribution to the brain parenchyma and the efficacy demonstrated in a number of preclinical studies. Some points, in addition to those already discussed, deserve to be deepened, such as the role of exogenous NGF in modifying the proNGF/NGF ratio in the brain, the possible synergistic effects of other therapies to be associated with IN-NGF, the potential development of side effects and the development of proper IN delivery devices/strategies. Furthermore, some considerations should be made regarding the strategies for future research aimed at optimizing treatments protocols for IN-NGF, to be translated into clinical practice.

The delivery of NGF to the brain may change the balance between endogenous proNGF and mature NGF (mNGF), which if shifted toward the former, can itself promote the development of functional dysfunctions and neurodegenerative events. ProNGF is the prevalent form of NGF in the brains of AD patients (Fahnestock et al., 2001) and its processing into mature NGF may be impaired in neurological diseases (Cuello et al., 2010). The biological effect of proNGF and NGF may be opposite (Hempstead, 2014), especially when the neuronal distress increases p75NTR/TrkA ratio (Chakravarthy et al., 2012), favoring the binding of proNGF to p75NTR and the activation of the apoptotic cascade (Hempstead, 2014). Therefore, further investigation of these mechanisms after IN-NGF deserves attention and future work.

By being a facilitator of metabolism and perfusion, IN-NGF may impact a broad neuro-pathological spectrum. It is worth noting that, similarly to IN-NGF, intranasal insulin was able to enhance brain energy levels, to improve memory loss and to reduce white matter degeneration in MCI and AD patients (Craft et al., 2012; Jauch-Chara et al., 2012; Kellar et al., 2021). A synergistic combination of these intranasal growth factors may deserve, therefore, a specific investigation. The possible recovery of the physiological phenotype promoted by NGF in neurons and glia produced functional improvements in patients with established deficits and disabilities, but has been proven reversible (de Bellis et al., 2018). Therefore, it might be useful that IN-NGF be associated with physical therapies (e.g., transcranial direct current stimulation, vagal stimulation, electroacupuncture, physiotherapy) (Cheng et al., 2009) or stem cell transplantation (Zhong et al., 2017; Wang et al., 2020). These may selectively stimulate the recovery of the connectivity and plasticity of the damaged areas, being synergic in their action with the effects of IN-NGF and irreversibly consolidating the functional changes promoted by IN-NGF alone.

Until now, the pharmacology of NGF has been severely limited by the onset of side effects after systemic (Apfel, 2002) or intracerebroventricular (Eriksdotter-Jönhagen et al., 1998) delivery and by the difficulty of identifying a "therapeutic window" in which the therapeutic target is reached, maximizing the efficacy and minimizing or avoiding altogether the onset of adverse events (Cattaneo et al., 2008; Aloe et al., 2012). In the preclinical studies mentioned above, IN-NGF dosages and duration of administration were very heterogeneous. Furthermore, only in few cases were assessments on the safety of the treatment carried out. In particular, it has been found that at least up to a dose of 0.48 μg/kg of IN-NGF delivered three times a week for 2 weeks, there were no physiological and molecular indications for the development of painful symptoms (Capsoni et al., 2009). In clinical studies, at much higher doses than this latter, no side effects were found, attributable to the action of NGF, after IN delivery in TBI children (Chiaretti et al., 2017, 2020). This aspect will be further and specifically investigated, as a secondary endpoint, in the ongoing clinical trial mentioned in a previous section (https://www.clinicaltrialsregister.eu/ctr-search/trial/2019-002282-35/IT). When delivered at low daily dosage for a long period of time in adult patients (de Bellis et al., 2018), any effects on nociceptive or autonomic systems have been recorded, while other reversible and dose-dependent side effects were noticed (rhinitis, rigidity, moderate psychomotor agitation). The possibility that IN-NGF may, at therapeutic doses, partly diffuse into the CSF (Thorne and Frey, 2001), sensitizing spinal neurons, cannot be ruled out (see **Figure 2**). However, one must take into account the short half-life of NGF (less than 1 h after ICV) (Lapchak et al., 1993), and that the percentage of IN-NGF spreading by perivascular and perineural space into the subarachnoid space (Dhuria et al., 2010) instead of in the parenchyma, may not be sufficient to reach the spinal cord neurons in relevant concentrations, especially after a delivery regimen limited to a few days. Nevertheless, in order to avoid potential development of side effects after IN-NGF, such as those related to pro-nociceptive function or loss of body weight, while inducing neurotrophic outcomes, the delivery of NGF-variants that target specifically the p75NTR (Manni et al., 2019; Soligo et al., 2019, 2020b) or that do not promote the phosphorylation of residue Tyr490 on TrkA, with subsequent activation of PLC-1 (Capsoni et al., 2011; Cattaneo and Capsoni, 2019), have been attempted. It should be noted, however, that these pharmacological approaches currently seem to be more suitable for systemic delivery of NGF, yet described as inducing side effects (Apfel, 2002).

Finally, much remains to be explored regarding the delivery technology. The physical and metabolic barriers that potentially hinder the penetration of IN-NGF into the cerebral parenchyma concern the anatomy of the human nasal cavity (Lochhead and Thorne, 2012; Gänger and Schindowski, 2018) and the rate of muco-ciliary clearance (Gänger and Schindowski, 2018). Regarding the latter, preclinical testing is underway on formulations that provide for the protection and increased absorption of NGF (Vaka et al., 2009; Vaka and Murthy, 2010; Luo et al., 2018), which can be obtained through lipid carriers, surfactants or polysaccharides (Erdő et al., 2018; Gänger and Schindowski, 2018). Also, the possibility exists of delivering NGF-mRNA through exosomes (Yang et al., 2020). As for physical obstacles to nose-to-brain delivery, the development of devices that maximize the deposition of drugs to the upper part of the nasal cavity, avoiding dispersion in the airways, or passage of the drug from the nasal mucosa to the blood circulation is underway but still not used to administer NGF (Djupesland et al., 2014; Gänger and Schindowski, 2018).

AUTHOR CONTRIBUTIONS

LM and MS wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

FUNDING

This work was funded by the Italian Ministry of Health Grant: RF-2018-12366594 "Nerve growth factor in pediatric severe traumatic brain injury: translational and clinical studies on a candidate biomarker and therapeutic drug".

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frontiers in Neuroscience

SYSTEMATIC REVIEW
published: 07 March 2022
doi: 10.3389/fnins.2022.847572

TrkB Truncated Isoform Receptors as Transducers and Determinants of BDNF Functions

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Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family of secreted growth factors and binds with high affinity to the TrkB tyrosine kinase receptors. BDNF is a critical player in the development of the central (CNS) and peripheral (PNS) nervous system of vertebrates and its strong pro-survival function on neurons has attracted great interest as a potential therapeutic target for the management of neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS), Huntington, Parkinson's and Alzheimer's disease. The TrkB gene, in addition to the full-length receptor, encodes a number of isoforms, including some lacking the catalytic tyrosine kinase domain. Importantly, one of these truncated isoforms, namely TrkB.T1, is the most widely expressed TrkB receptor in the adult suggesting an important role in the regulation of BDNF signaling. Although some progress has been made, the mechanism of TrkB.T1 function is still largely unknown. Here we critically review the current knowledge on TrkB.T1 distribution and functions that may be helpful to our understanding of how it regulates and participates in BDNF signaling in normal physiological and pathological conditions.

Keywords: TrkB.T1, BDNF, TrkB truncated, splicing, neurodegeneration

OPEN ACCESS

Edited by:
Viviana Triaca,
National Research Council (CNR), Italy

Reviewed by:
Luigi Manni,
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Specialty section:
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

Received: *02 January 2022*
Accepted: *10 February 2022*
Published: *07 March 2022*

Citation:
Tessarollo L and Yanpallewar S (2022) TrkB Truncated Isoform Receptors as Transducers and Determinants of BDNF Functions. Front. Neurosci. 16:847572. doi: 10.3389/fnins.2022.847572

INTRODUCTION

In mammals, the neurotrophin family is comprised of four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5). Two types of receptors mediate their actions, the Trk family of tyrosine kinase receptors and the p75 NGF receptor, a member of the tumor necrosis factor receptor superfamily. Binding experiments with cell lines were used to determine the ligand-receptor relationship between neurotrophins and their receptors. NGF binds to TrkA, whereas BDNF and NT4/5 bind to TrkB. NT-3 signals mainly through TrkC but can also bind with lower affinity to TrkA and TrkB (Bothwell, 1995; Chao and Hempstead, 1995; Friedman and Greene, 1999; Kaplan and Miller, 2000; Huang and Reichardt, 2003). The phenotypes observed in mice with targeted mutations in neurotrophins and their Trk receptors have demonstrated the specificity of these interactions, particularly in the developing peripheral nervous system (PNS; Snider, 1994; Tessarollo, 1998). Trk receptors, upon ligand binding, activate well-known intracellular signaling cascades such as the Ras/MAPK pathway, phosphoinositide 3 kinase and phospholipase Cγ (PLCγ) pathways. In the early experiments of gene targeting of the Trk genes in mice, the mutations were directed toward the tyrosine kinase domain region of the full-length receptors because of the established biological activities of these isoforms. However, Trk genes by alternative splicing can produce a wide array of other isoforms. For many of these isoforms the pattern of expression, the conservation among species, and their

physiological abundance has not yet been elucidated (Tsoulfas et al., 1993; Luberg et al., 2010, 2015). However, the TrkB and TrkC genes, by alternative splicing generate isoforms that lack tyrosine kinase activity, are conserved among species and are expressed at high levels (Klein et al., 1990; Middlemas et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner and Large, 1994; Luberg et al., 2010, 2015). Though they were first discovered about 30 years ago, only recently we have begun to learn about the function of these types of receptors. This was in part due to their lack of tyrosine kinase activity, unclear signaling and no obvious pro-survival effect on neurons. The lack of pro-survival functions on different neuronal populations was confirmed *in vivo* with the second generation of mouse models targeting all Trk genes isoforms showing only minimal phenotypic differences compared to the initial mouse models targeting the kinase domain (Klein et al., 1993, 1994; Smeyne et al., 1994; Tessarollo et al., 1997; Liebl et al., 2000; Luikart et al., 2005). Thus, for a long time, the main functions attributed to these truncated receptor isoforms were of acting as dominant-negative inhibitors of the full-length receptors or limiting ligand availability (Biffo et al., 1995; Eide et al., 1996; Palko et al., 1999). In 2003 (Rose et al., 2003), the report that TrkB.T1 can signal independently by inducing calcium release from the intracellular stores, and in 2006, the generation of a new mouse model targeting specifically the exon encoding the TrkB.T1 isoform without affecting the spatio-temporal pattern of expression of TrkB full-length (TrkB.FL; Dorsey et al., 2006) started a new research effort into the functional role of these enigmatic receptors. As outlined below this research has led to new exciting and unanticipated functions for TrkB.T1 in mammalian physiology. Truncated TrkC isoforms signal by binding the scaffold protein Tamalin which in turn activates the Rac1 GTPase through the adenosine diphosphate-ribosylation factor 6 (Esteban et al., 2006). Moreover, it has also been shown that TrkC.T1 can lead to neural differentiation in collaboration with p75NTR (Hapner et al., 1998). However, the generation of a suitable mouse model to study TrkC truncated receptors *in vivo* has been hampered by the fact that targeting of the exons encoding specifically the truncated TrkC.T1 (also known as TrkC.NC2) leads to dysregulation of the TrkC.FL receptor expression (Bai et al., 2010). Therefore, the lack of a proper mouse model has limited our ability to study the precise function of TrkC.T1 *in vivo*. In this review, we focus on the current knowledge on truncated TrkB.T1, the most studied truncated Trk receptor to date. We discuss the many *in vivo* functions that have been uncovered so far and its relevance in the pathophysiology of neuronal disorders in both animal models and human diseases.

ALTERNATIVE SPLICING AS A MECHANISM TO DIVERSIFY BRAIN-DERIVED NEUROTROPHIC FACTOR/TrkB SIGNALING

The finding that the mouse and human genome contain fewer genes than previously thought have underlined the importance of alternative splicing as a mechanism to build more complex organisms (Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002a,b). Importantly, alternative splicing in mammals (Clancy, 2008; Donaldson and Beazley-Long, 2016) is particularly prominent and highly conserved in the brain (Raj and Blencowe, 2015). Functionally, alternative splicing can result in protein isoforms that are inactive or exhibit similar, different, or even opposing actions. Moreover, alternatively spliced transcripts and their protein isoform products show dynamic changes in expression and function that are dependent on cell, tissue, age, and context (physiological vs. pathological). BDNF and its receptor TrkB are good examples of genes whose function is tightly associated with alternative splicing. The BDNF gene consists of at least 8 different promoters that can generate 18 separate transcripts. While all transcripts generate the same BDNF polypeptide, the presence of different promoters allows for the differential regulation of BDNF expression. For example, in the cortex, promoter IV-dependent transcription is responsible for activity-induced BDNF expression (Timmusk et al., 1993; Timmusk and Metsis, 1994; Tao et al., 1998; Aid et al., 2007; Hong et al., 2008). In addition, the different mRNA variants can be transported to differential subcellular locations for local translation of BDNF leading to selective morphological remodeling of dendrites (Baj et al., 2016). The human TrkB gene, spanning about 400 kbp, consists of 24 exons that through a complex pattern of alternative splicing can generate up to 30 TrkB isoforms (Stoilov et al., 2002; Luberg et al., 2010). However, despite the abundance of potential TrkB isoforms the most highly expressed isoforms in the mammalian brain are the TrkB.FL and TrkB.T1 receptors (Tomassoni-Ardori et al., 2019). These TrkB receptors are differentially expressed within the diverse areas of the nervous system in a spatial and temporal fashion (Stoilov et al., 2002; Luberg et al., 2010). Developmentally, TrkB.FL is predominantly expressed in the embryonic and early postnatal CNS whereas TrkB.T1 expression is very low in the embryo but increases gradually postnatally and peaks in adulthood (Escandon et al., 1994). At the cellular level, in the nervous system, expression of TrkB.FL is restricted to neurons whereas TrkB.T1 is expressed in both neurons and glia cells (Sommerfeld et al., 2000; Dorsey et al., 2006; Holt et al., 2019; Tomassoni-Ardori et al., 2019). Interestingly, outside the nervous system, TrkB.T1 appears to be the main isoform as it is expressed in the adult heart, kidney, lung, and pancreas (Stoilov et al., 2002; Fulgenzi et al., 2015, 2020).

REGULATION OF TrkB.T1 RECEPTOR ISOFORM EXPRESSION

As mentioned above, the TrkB locus produces many different TrkB receptor isoforms by alternative splicing. However, very little is known about the mechanisms regulating their spatial and temporal expression. TrkB.T1 is the major isoform expressed in the adult mammalian brain and the best characterized among all known truncated isoforms. It includes the exons encoding the extracellular, transmembrane and the juxtamembrane domain that are common to the TrkB.FL receptor up to exon 15. After

exon 15, instead of including exon 17 which is the first exon specific to the TrkB.FL isoform, the splicing machinery uses exon 16 which encodes a short intracellular 11 amino acid tail (FVLFHKIPLDG) that is unique to the TrkB.T1 isoform (**Figure 1**). This sequence lacks obvious homology to any known protein motifs but is 100% conserved between rodents, human and chicken (Klein et al., 1990; Middlemas et al., 1991; Biffo et al., 1995; Luberg et al., 2010). In addition to the 11 aa tail, exon 16 includes a stop codon and has its own 3′UTR sequence with a number of polyadenylation sites. The mechanism underlying the coding of TrkB.T1 versus the TrkB.FL isoform by alternative splicing is unknown and deserves further study. So far, only mechanisms regulating the levels of expression of the different isoforms have been described. For example, TrkB.T1 downregulation in the cortex of suicide victims appears to result from combined epigenetic mechanisms, including methylation of the promoter and the 3′UTR DNA sequence, histone modifications, and microRNA binding (Ernst et al., 2009, 2011; Maussion et al., 2012). More recently, the RNA binding protein RbFox1 has been shown to increase the levels of TrkB.T1 in the hippocampus by direct binding to the TrkB.T1 mRNA. In turn, TrkB.T1 upregulation impairs BDNF-dependent LTP which can be rescued by genetically restoring TrkB.T1 levels strongly suggesting that TrkB.T1 regulates important brain functions (Tomassoni-Ardori et al., 2019).

MECHANISMS OF TrkB.T1 SIGNALING

Dominant-Negative Regulation of TrkB.FL Signaling

The most studied mechanisms of TrkB.T1 signaling have been a dominant-negative role on TrkB.FL function and a BDNF scavenging action by limiting availability of the neurotrophin to activate TrkB.FL (**Figure 2A**). Small changes in TrkB.T1 levels can influence TrkB.FL activity because TrkB tyrosine kinase receptors can signal in response to extremely low concentrations (nano- to picomolar) of BDNF (Eide et al., 1996). The TrkB.T1 extracellular domain is identical to the TrkB.FL which allows it to engage BDNF with the same affinity of TrkB.FL and/or heterodimerize with a TrkB.FL monomer (**Figures 1**, **2**; Biffo et al., 1995). Therefore, the formation of TrkB.T1 homodimers that sequester BDNF or TrkB.T1 heterodimers with the TrkB.FL isoform both impair BDNF signaling (Ohira et al., 2001). Several studies have supported these mechanisms. For example, co-expression of TrkB.T1 isoform with full-length receptor in xenopus oocytes prevents BDNF-induced activation of phospholipase C-γ pathway as measured by calcium efflux (Eide et al., 1996). Moreover, co-culture of the neuroblastoma cell line SY5Y expressing TrkB.FL, with NIH3T3 cells expressing TrkB.T1, inhibited neurite outgrowth in SY5Y cells under limiting concentration of BDNF (Fryer et al., 1997). Also, transfection of TrkB.T1 in a line of PC12 cells expressing TrkB.FL reduced its survival in the presence of BDNF and inhibited TrkB.FL autophosphorylation and kinase activity (Haapasalo et al., 2001), and pre-synaptic expression of TrkB.T1 in cultured hippocampal neurons prevented synaptic potentiation induced by BDNF (Li et al., 1998). Similarly, transfection of increasing amounts of TrkB.T1 in a cell line stably expressing TrkB.FL impairs BDNF-dependent TrkB.FL phosphorylation and signaling in a way that is directly proportional to TrkB.T1 levels. Conversely, TrkB.T1 knockout primary hippocampal neurons have higher basal, as well as BDNF-stimulated levels of p-TrkB.FL and p-ERK compared to control hippocampal neurons (Tomassoni-Ardori et al., 2019). These functions have also been supported by *in vivo* data as well showing that the level of expression of TrkB.T1 isoform has direct significant pathophysiological consequences. For example, in a mouse model with overexpression of TrkB.T1in postnatal cortical and hippocampal neurons, the induction of transient focal cerebral ischemia by middle cerebral artery occlusion causes significantly more neuronal damage as compared to controls. The increased damage occurs despite a BDNF mRNA upregulation in the peri-infarct region suggesting that increased TrkB.T1 limits BDNF function (Saarelainen et al., 2000). In addition, in the trisomy 16 (Ts16) mouse model there is increased apoptosis in the cortex and accelerated cell death of hippocampal neurons that cannot be rescued by administration of BDNF (Dorsey et al., 2006). This phenotype was mechanistically linked to increased TrkB.T1 expression since restoration of the physiological level of this isoform by gene targeting rescued Ts16 cortical cell and hippocampal neuronal death (Dorsey et al., 2006). Lastly, *in vivo* reduction of TrkB.FL signaling by removal of one BDNF allele could be partially rescued by TrkB.T1 deletion, which was revealed by an amelioration of the enhanced aggression and weight gain associated with BDNF haploinsufficiency (Carim-Todd et al., 2009).

Regulation of Brain-Derived Neurotrophic Factor Signaling by TrkB.T1 Trafficking

TrkB.T1 also appears to have a distinct intracellular trafficking pattern compared to TrkB.FL. Binding of BDNF to TrkB.FL receptor results in rapid dimerization, transphosphorylation, and endocytosis. After internalization, the BDNF-TrkB complex is transported to various intracellular compartments which determines the type, strength, amplification, and duration of the downstream signaling cascades (Barford et al., 2017). Upon internalization, the receptors either go to the lysosomes for degradation or recycle back to the cell surface. TrkB.FL receptors predominantly get sorted to the degradative pathway resulting in downregulation of BDNF upon ligand-binding. In contrast, TrkB.T1 is predominantly recycled back (Sommerfeld et al., 2000; Chen et al., 2005) suggesting that the recycled TrkB.T1 can further sequester additional BDNF to regulate the duration of TrkB.FL mediated sustained activation of downstream MAP-kinase signaling (Huang et al., 2009). This function has been reported mainly for TrkB.T1 expressed in neurons (Sommerfeld et al., 2000; Chen et al., 2005). Additionally, TrkB.T1 expressed in astrocytes also appears to play a distinct role. TrkB.T1 in astrocytes isolated from rat hippocampi has been shown to mediate storage of endocytosed BDNF in a stable intracellular pool that can be used to release BDNF back into the extracellular

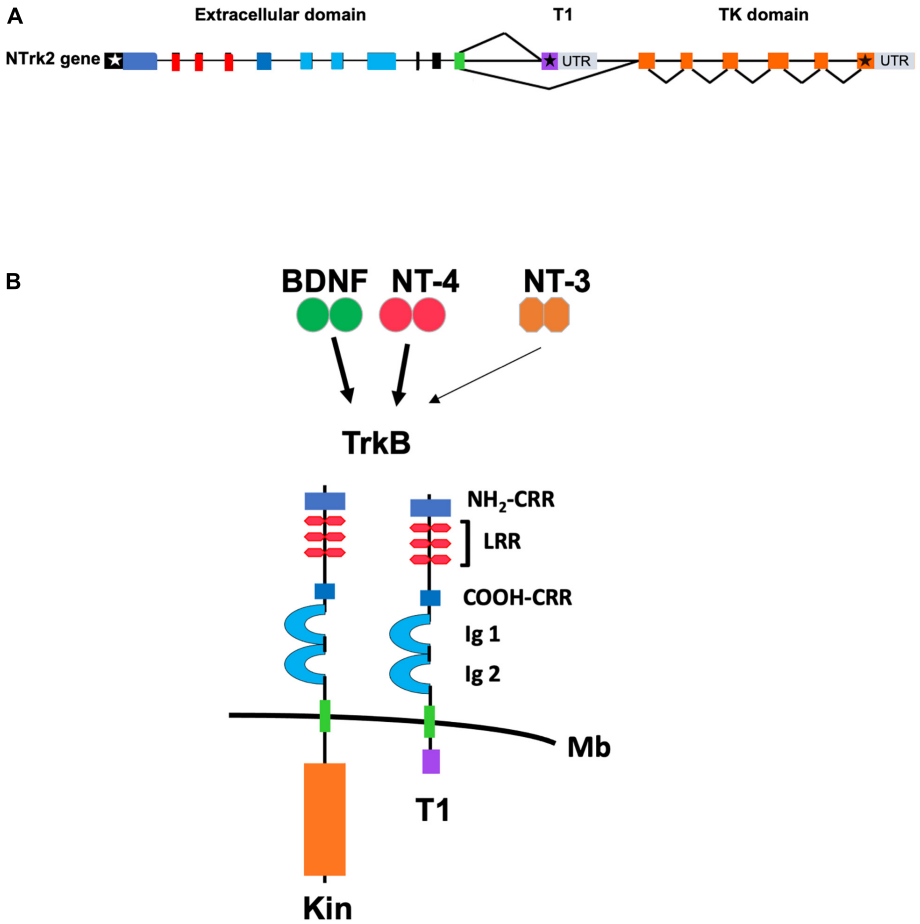

FIGURE 1 | (A) Schematic representation of the genomic structure of the murine TrkB gene. Exons are shown as boxes and introns are shown as lines. White and black stars indicate the start and stop condons, respectively. Color of exons indicate the domains of the TrkB protein isoforms shown in panel **(B)**. Gray boxes indicate the 3′ untranslated region (UTR) of the TrkB transcripts. T1 and TK (Tyrosine Kinase) domain indicate the exons encoding, respectively, the TrkB.T1 and the TrkB.FL isoform that are produced by alternative splicing. **(B)** Schematic representation of the TrkB tyrosine kinase and TrkB.T1 isoform receptors. The extracellular TrkB protein domains include the amino (NH_2-CRR) and carboxy (COOH-CRR) cysteine rich region; the leucine rich region (LRR) and the IG like, immunoglobulin like-domain (Ig1 and Ig2). The intracellular tyrosine kinase (Kin) and T1 domain are indicated below the cell membrane (Mb). BDNF and Neurotrophin-4 (NT-4) are the TrkB ligands binding with high affinity (solid arrow) whereas Neurotrophin-3 (NT-3) binds to the extracellular domain with lower affinity (thin arrow).

environment over time (Alderson et al., 2000). Although the details regulating the sequestering and storage of BDNF mediated by TrkB.T1 endocytosis have not been fully elucidated, this represents another potential mechanism used for the fine-tuning of BDNF signaling.

TrkB.T1 Independent Signaling

The first suggestion that TrkB.T1 could elicit signaling entirely independent of the full-length isoform was suggested by the Feinstein lab. Baxter et al. (1997) showed that TrkB.T1 is capable of mediating BDNF-induced signal transduction. More specifically, BDNF activation of TrkB.T1 increases the rate of acidic metabolite release from the cell, a common physiological consequence of many signaling pathways, and these changes occur with kinetics distinct from those mediated by TrkB.FL. Importantly, mutational analysis demonstrated that the specific intracellular domain of TrkB.T1 is essential for signaling (Baxter et al., 1997). Another key study showed that astrocytes that express TrkB.T1 respond to brief applications of BDNF by releasing calcium from intracellular stores. The finding that the calcium transients are insensitive to the tyrosine kinase blocker K-252a and persist in mutant mice lacking TrkB.FL strongly suggested a direct TrkB.T1 signaling role. While the study did not identify the downstream signaling mediators, pharmacologically, TrkB.T1-induced calcium release was found to be mediated by inositol-1,4,5-trisphosphate (Rose et al., 2003). Subsequently, it has been demonstrated that TrkB.T1 mediates BDNF-induced calcium release in cell types that exclusively express TrkB.T1 namely, cardiomyocytes and pancreatic β-cells (Fulgenzi et al., 2015, 2020). Lastly, there are a few isolated reports that have identified novel TrkB.T1 interactions and signaling mechanisms. It has been reported that the Rho GDP dissociation inhibitor 1 (GDI1), a GDP dissociation inhibitor of Rho small G-proteins, associates with TrkB.T1. This interaction

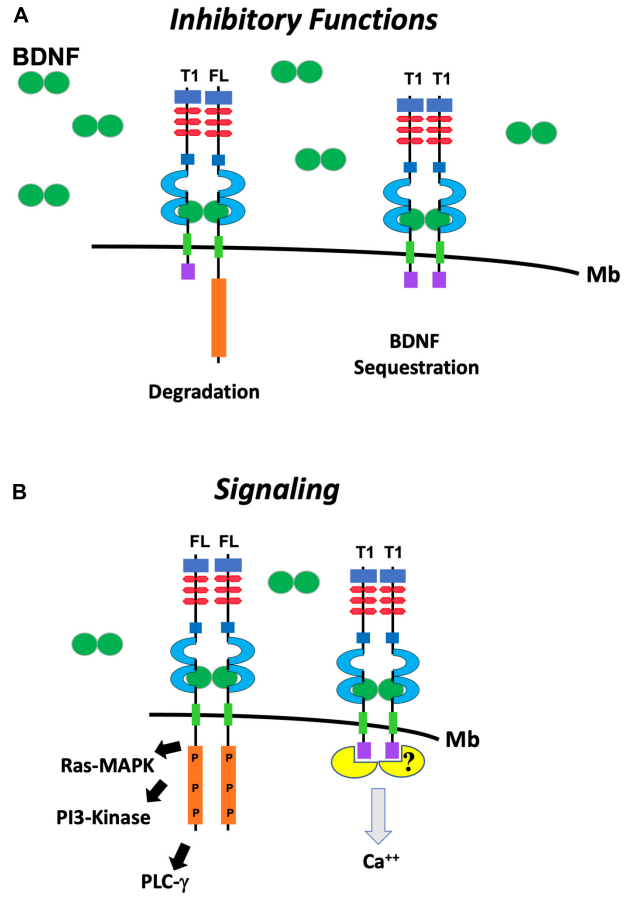

FIGURE 2 | Schematic representation of the putative functions of TrkB.FL (FL) and TrkB.T1 (T1). **(A)** TrkB.T1 has inhibitory functions when it heterodimerizes with one isoform of TrkB.FL in response to BDNF (green ovals) binding and leading to internalization and degradation of the heterodimer (left). It also inhibits BDNF signaling by binding and sequestering it when it homodimerizes (right). **(B)** TrkB.FL (FL) homodimers in response to BDNF signal lead to auto-phosphorylation the kinase domain (P) and activation of the Ras/MAP Kinase (Ras-MAPK) pathway, phosphoinositide 3 kinase (PI3-Kinase) and phospholipase Cγ (PLCγ) pathways (left). TrkB.T1 homodimers can induce calcium (Ca++) release from the intracellular store through the binding and activation of a still unknown pathway (right).

appears to be constitutive but is disrupted by BDNF binding to TrkB.T1 (Ohira et al., 2005). Additionally, another 61 kDa protein has been reported to interact with TrkB.T1 but it has never been isolated (Kryl and Barker, 2000). In contrast to its role on calcium release, follow-up molecular studies on proteins directly interacting with TrkB.T1 have been lacking. Therefore, the characterization of the precise molecular pathways activated by TrkB.T1 is still largely unknown. It is possible that the small TrkB.T1 intracellular domain is unable to form stable interactions with proteins thus precluding their isolation and identification by mass-spectrometry.

Physiological Roles of TrkB.T1

To prove a direct signaling function of TrkB.T1 *in vivo* has been challenging because the initial mouse model with a gene targeted mutation in the TrkB gene was generated by targeting the region encoding the tyrosine kinase domain (Klein et al., 1993). Although, this mouse model retained expression of the TrkB.T1 isoform because the TrkB.T1 encoding exon is upstream of the tyrosine kinase domain (see schematic in **Figure 1A**), it did not make it possible to determine a potential function of this truncated receptor due to the early postnatal lethality of the mutant. Other mouse models with either a complete deletion of all isoforms or selective inactivation of the kinase domain by a chemical genetic approach also did not allow determination of specific functions of TrkB.T1 because TrkB.FL exerts most BDNF pro-survival functions (Luikart et al., 2005; Johnson et al., 2008). To circumvent this problem, our laboratory targeted the exon specific to TrkB.T1 in mouse, generating a mouse model retaining the correct spatio-temporal pattern of expression of TrkB.FL. This mouse is viable but shows increased anxiety-like behavior accompanied by reduced length and complexity of dendritic arbors in basolateral amygdala (Carim-Todd et al., 2009). TrkB.T1 deletion also causes late onset cardiomyopathy because of defects in BDNF-induced calcium signaling and its role in cardiac contractility (Fulgenzi et al., 2015). Furthermore, loss of TrkB.T1 causes impaired glucose tolerance and insulin secretion due to its function in TrkB.T1-expressing pancreatic β-cells (Fulgenzi et al., 2020). In astrocytes, a cell type expressing high levels of TrkB.T1, its deletion causes immature morphology and reduced cellular volume as well as dysregulated expression of perisynaptic genes associated with mature astrocyte function (Holt et al., 2019). The lack of maturity by TrkB.T1 astrocytes may be the cause of slower *in vitro* migration in response to BDNF and reduced *in vivo* proliferation that has been associated with increased neuropathic pain and neurological dysfunction in TrkB.T1 KO mice following spinal cord injury (Matyas et al., 2017). The *in vivo* phenotypes caused by deletion of TrkB.T1 in cardiomyocytes, pancreatic β-cells and astrocytes, all cell types expressing this receptor isoform exclusively, provide definitive evidence of the independent signaling capability of TrkB.T1. One common feature of TrkB.T1 signaling in all these cell types is the activation of release of calcium from intracellular stores that, at least in glia cells, appears through a signaling pathway that involves an as yet unidentified "G protein" (Rose et al., 2003; Carim-Todd et al., 2009; Fulgenzi et al., 2015, 2020). It will be important to define whether the recorded calcium changes are indeed caused by TrkB.T1 association to a common G-protein or a specific G-protein unique to each cell type in which TrkB.T1 is expressed. Alternatively, TrkB.T1 could associate to some other adaptor or signaling proteins. Unbiased genome-wide targeting using the CRISPR/Cas9 system to inactivate the genes downstream of TrkB.T1 signaling may help identify all the components of this pathway/s. Lastly, the partial rescue by TrkB.T1 deletion of the enhanced aggression and weight gain associated with BDNF haploinsufficiency provides definitive *in vivo* evidence for a TrkB dominant/negative or BDNF sequestering role of TrkB.T1 (Carim-Todd et al., 2009). Taken together, over the last two decades the above studies have identified a variety of physiological roles for TrkB.T1 showing that it regulates a wide range of processes that extend beyond the nervous system.

ROLE OF TrkB.T1 IN DISEASE

Function in Cancer

Activation of the TrkB tyrosine kinase receptor has long been implicated in human tumorigenesis especially in the context of gain of function mutations associated with translocations and gene fusions (Eggert et al., 2001; Khotskaya et al., 2017). For these reasons, pan-Trk inhibitors of Trk tyrosine kinase receptors have been developed and were recently approved for cancer patients with NTRK fusion-positive solid tumors (Marcus et al., 2021). Whether TrkB.T1 plays a role in cancer biology has been less clear, mostly because of the lack of evidence of activation of specific pathways involved in cancer development and/or progression. Some studies, however, have reported a possible role for TrkB.T1 in tumorigenesis. Specifically, it has been shown that TrkB.T1 overexpression induces liver metastasis of pancreatic cancer and invoked the signaling mechanism by which TrkB.T1 sequesters GDI leading to activation of RhoA signaling (Li et al., 2009). In another study, a similar mechanism was proposed to cause morphological changes in C6 rat glioma cells (Ohira et al., 2006). More recently, the Holland lab experimentally tested *in vivo* whether expression of TrkB.T1 plays a role in gliomas. This study stems from the observation that TrkB.T1 is the predominant TrkB isoform expressed across a range of human gliomas and, surprisingly, that high transcript expression of TrkB.FL is associated with better, not worse, prognosis for both glioblastoma multiforme (GBM) and low grade gliomas (LGG; Pattwell et al., 2020). Using the elegant experimental paradigm that employs the RCAS-tv/a technology, they demonstrated that TrkB.T1 enhances PDGFB-driven tumors in mice, and the perdurance of PI3K and STAT3 signaling pathways (Pattwell et al., 2020). Together, these results demonstrate a previously unidentified role for TrkB.T1 in gliomas although how TrkB.T1 influences PI3K and STAT3 signaling is still unclear.

Relevance of TrkB.T1 in Neurological Disorders

Diverse and region-specific dysregulation of BDNF-TrkB signaling has been observed in many neuropsychiatric disorders such as Alzheimer's disease (AD), Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease, epilepsy, stroke, mood disorders and schizophrenia. The consistent identification over the years of changes in the expression of truncated TrkB isoforms in these diseases has suggested that these isoforms are important transducers and determinants of dysfunctional BDNF signaling and possibly a critical causative factor underlying neuronal damage.

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis is caused by rapidly progressing degeneration of the upper and lower motor neurons leading to muscle paralysis and death generally within 5 years of onset (Mitchell and Borasio, 2007). Two FDA approved agents, riluzole and edaravone, provide only a small benefit and are not curative. Lack of neurotrophic support is believed to be one of the causative factors leading to motor neuron loss (Bruijn et al., 2004). The initial findings that BDNF rescues injury-induced motor neurons death and motor dysfunction in the wobbler mouse model of motor neuron disease (Sendtner et al., 1992; Yan et al., 1992; Ikeda et al., 1995) provided the rationale for the clinical use of BDNF in ALS patients. However, in all clinical trials BDNF has failed to show any meaningful therapeutic effect (Group, 1999; Ochs et al., 2000; Kalra et al., 2003; Beck et al., 2005). While the lack of efficacy of BDNF has been attributed to its poor pharmacokinetics and pharmacodynamics properties, a number of observations in both human as well as rodent models of ALS have suggested that one of the causes of the underlying pathology in ALS is not the lack of BDNF supply but rather a defect in downstream TrkB signaling. Indeed, muscle of ALS patients has increased levels of BDNF (Kust et al., 2002). Moreover, total TrkB levels were either increased or unaltered in the postmortem analysis, yet there was a decrease in TrkB phosphorylation (Seeburger et al., 1993; Kawamoto et al., 1998; Mutoh et al., 2000). Similar results, i.e., increased BDNF levels associated with decreased TrkB phosphorylation, were observed in the plantaris muscle in the SOD1 G93A mutant mouse model of ALS (Just-Borras et al., 2019).

Based on these findings and the facts that in lumbar spinal cord of WT as well as SOD1 G93A mice, the levels of truncated TrkB.T1 receptors increase with age while that of TrkB.FL decreases we hypothesized that the presence of TrkB.T1 limits the pro-survival functions of BDNF -TrkB.FL signaling (Yanpallewar et al., 2012). This suggestion was supported by experiments in which TrkB.T1 deletion in mutant SOD1 mice delays the onset of the disease, slows down the motoneuron loss and improves mobility test results at the end stage of the disease compared with normal mutant SOD1 mice. Although, the increase in TrkB.T1 could be secondary to the increased astrogliosis in mutant spinal cord (cell types expressing only TrkB.T1) the findings that TrkB.T1 deletion does not change the SOD1 spinal cord inflammatory state suggests that this receptor does not influence microglia or astrocyte activation (Zhang and Huang, 2006; Yanpallewar et al., 2012, 2021; Just-Borras et al., 2019). These observations strongly suggest a role for TrkB.T1 in the pathophysiology of ALS and alternative ways to activate TrkB.FL may be needed to overcome the insufficient or defective TrkB signaling.

Alzheimer's Disease

Alzheimer's Disease is the most common type of dementia (Querfurth and LaFerla, 2010) with cognitive impairments associated with loss of cholinergic neurons and formation of plaques due to the deposition of beta-amyloid and neurofibrillary tangles formed by hyper-phosphorylated tau protein. Because loss of cholinergic neurons is a major feature of AD it was suggested that diminished NGF-mediated neurotrophic support is a major cause of disease. However, there is also strong evidence that dysregulation of BDNF-TrkB signaling can be implicated in AD. For example, BDNF mRNA and protein levels as well as protein levels for the TrkB.FL isoform have been found to be reduced in postmortem brain samples of AD patients, while TrkB.T1 is increased (Phillips et al., 1991; Ferrer et al., 1999). Moreover, in a separate study of AD brains, a

specific increase in the truncated TrkB.Shc isoform has been reported in the hippocampus. This isoform, found mainly in humans and not in mouse, can, like TrkB.T1, inhibit TrkB.FL function (Wong et al., 2012). Although the mechanistic role of TrkB receptors isoform dysregulation in AD is still largely unknown, in neuronal cultures, amyloid beta has been found to increase TrkB.T1 levels while decreasing TrkB.FL. Parallel to this finding, TrkB.T1 overexpression in the APPswe/PS1dE9 transgenic mouse model of AD exacerbated spatial learning and memory impairment whereas overexpression of TrkB.FL improved it (Kemppainen et al., 2012). Importantly, the relevance of BDNF/TrkB signaling in AD has been further validated by the findings that BDNF exerts neuroprotective effects in rodent and primate models of AD (Nagahara et al., 2009).

Stroke and Neuronal Trauma

Stroke is the leading cause of disability and death in developed countries. Ischemic (caused by blockade of a blood vessel) or hemorrhagic (caused by a ruptured vessel) stroke leads to reduced supply of oxygen and nutrients ultimately resulting in neuronal death. In ischemic stroke, levels of BDNF are decreased in the infarct core while there is a rapid and sustained upregulation in the peri-infarct area. This increase in BDNF is believed to be a compensatory mechanism to provide trophic support to neurons (Lindvall et al., 1992, 1994, 1996). Analysis of TrkB expression in human stroke necropsy samples and in animal models of ischemia shows a significant up-regulation of TrkB.T1 that appears neuro-specific, while the levels of TrkB.FL decreases (Vidaurre et al., 2012). One of the mechanisms contributing to the altered TrkB isoforms expression appears related to alternative mRNAs splicing favoring the expression of TrkB.T1 over TrkB.FL. A second mechanism appears related to the calpain-dependent degradation of TrkB.FL. While it is unclear why there is a change in the splicing of TrkB isoforms, it is possible that an alteration in the expression of specific RNA binding proteins caused by the excitotoxic insult could lead to such dysregulation (Tomassoni-Ardori et al., 2019). The *in vitro* rescue of neurons from excitotoxic death by restoration of the TrkB-FL/TrkB-T1 balance suggests that dysregulation of TrkB isoform expression may be one of the causes leading to neuronal cell-death during stroke (Vidaurre et al., 2012; Tejeda and Diaz-Guerra, 2017). A better understanding of this pathophysiological mechanism may lead to improved therapies for this brain injury.

Truncated TrkB.T1 levels are also increased following traumatic brain (Rostami et al., 2014) and spinal cord injury in the area surrounding the damaged tissues, suggesting a role in limiting neurotrophin availability at the lesion site (King et al., 2000; Wu et al., 2013). Up-regulation of TrkB.T1 mRNA is not just limited to the acute phase of the damage but is sustained up to 8 weeks in both a rat model of penetrating traumatic brain injury as well as a mouse model of spinal cord contusion injury (SCI; Rostami et al., 2014; Matyas et al., 2017). Moreover, deletion of TrkB.T1 in astrocytes not only reduces inflammatory response but also improves impaired motor function and neuropathic pain after SCI (Matyas et al., 2017). Curiously, microarray analysis of spinal cord tissue after injury in WT and TrkB.T1 KO mice has also found a differential regulation of cell cycle associated genes although the significance of this changes is still unclear (Wu et al., 2013; Wei et al., 2019). Further studies are needed to investigate if modulating TrkB.T1 expression can indeed influence the recovery after traumatic injuries of the nervous system and whether it can be targeted to develop treatments to improve the outcome of trauma in patients.

Parkinson's Disease

Abundant data have suggested a role for impaired BDNF/TrkB signaling in the etiology of Parkinson's disease, at least from *in vivo* animal models. For example, ablation of the BDNF gene impairs the survival and/or maturation of substantia nigra (SN) dopamine (DA) neurons during development (Baquet et al., 2005); loss of one copy of TrkB leads to an age-dependent increase in the levels of α-synuclein in the SN (von Bohlen und Halbach et al., 2005), and in a mouse model with a chronic reduction in TrkB signaling (∼30% of WT) there is an age-dependent and selective degeneration of SN DA neurons and increased vulnerability of these neurons to neurotoxins (Baydyuk and Xu, 2014). Because it has also been reported that neurons of PD patients have an increase in TrkB.T1 expression (Fenner et al., 2014) and neurons derived from iPSCs of PD patients have elevated RBFOX1 (Lin et al., 2016) it will be of interest to investigate whether TrkB.T1 upregulation in PD is among the determinants of this pathology.

Schizophrenia

Patients with schizophrenia show cognitive and perception deficits that are linked to abnormalities in the function of the dorsolateral prefrontal cortex. Interestingly, in this brain region of deceased schizophrenic patients, BDNF and TrkB.FL are significantly decreased, while TrkB.Shc and TrkB.T1 isoforms are upregulated (Durany et al., 2001; Weickert et al., 2003, 2005; Hashimoto et al., 2005; Wong et al., 2013). These findings have, once again, suggested an imbalance in neurotrophic BDNF signaling in these patients (Yuan et al., 2010). The mechanism underlying dysregulation of TrkB isoform receptor is unknown but the recent report of alternative splicing characteristics of a growing number of schizophrenia risk genes suggests that the changes in TrkB splicing may be part of wider genome alterations in the splicing associated with schizophrenia pathogenesis (Zhang et al., 2021).

Stress Disorders

A role for BDNF-signaling in the pathophysiology of stress disorders such as depression has been extensively studied. Stress, one of the major risk factors underlying mood disorders, decreases BDNF and its downstream signaling whereas antidepressant therapies exert their therapeutic effect, at least in part, through promotion of this signaling pathway (Yang et al., 2020). Specifically, there is altered expression of TrkB.T1 in postmortem brains of suicide victims (Dwivedi et al., 2003; Ernst et al., 2009). Importantly, the observed TrkB isoform imbalance is believed to be due to microRNA Hsa-miR-185* mediated modulation of TrkB.T1 levels (Maussion et al., 2012).

Taken together, these data strongly implicate altered expression of truncated TrkB isoforms in many neurological

disorders. Validation of these findings in more animal models should lead to a better understanding of whether an imbalance in Trk receptor isoform expression is a major factor underlying the pathogenesis of these neural diseases.

THERAPEUTIC RELEVANCE AND FUTURE PERSPECTIVE

Dysregulation of homeostatic BDNF signaling has been associated with a number of neuropsychiatric disorders. Most therapeutic strategies aimed at restoring or enhancing BDNF "trophic" support have focused on exogenous administration of BDNF (e.g., in ALS) and the development of BDNF agonists binding to the TrkB extracellular domain such as TrkB activating antibodies (Lin et al., 2008; Perreault et al., 2013; Guo et al., 2019). To date, the search for small molecule agonists has been mainly unsuccessful, or questionable at best (Todd et al., 2014; Boltaev et al., 2017; Pankiewicz et al., 2021). The delivery of BDNF has universally failed as a therapeutic agent probably due to its inability to cross the BBB, the short half-life, and the difficulties of delivery to specific brain areas in a precise spatio-temporal fashion (Miranda-Lourenco et al., 2020). A variety of approaches are currently being pursued to address the issue of BDNF delivery, including the use of biomaterials and nanoparticles, viral mediated gene delivery and transplantation of neurotrophin-producing cells (Houlton et al., 2019; Jarrin et al., 2021). Unfortunately, most of these approaches ignore the possibility that changes at the level of TrkB receptor, including TrkB.T1 upregulation, may influence the downstream BDNF signaling and therefore the therapeutic outcome. Indeed, upregulation of TrkB.T1 in a trisomic mouse model renders hippocampal neurons completely unresponsive to BDNF both *in vitro* and *in vivo*. Importantly, restoration of TrkB.T1 to physiological levels rescues neurons sensitivity to BDNF and neuronal cell death *in vivo* (Dorsey et al., 2002, 2006). These observations have critical implications for the therapeutic use of TrkB agonist antibodies that bind selectively to TrkB. While these antibodies do not engage p75NTR and have a longer half-life (days instead of minutes to hours seen with BDNF) allowing for better diffusion into the neural tissues (Guo et al., 2019; Han et al., 2019), they still bind to TrkB.T1 with the risk of being sequestered and neutralized. The data presented in this review therefore warrants a critical reexamination of these approaches because neurons may not suffer from a deficit of neurotrophin supply but rather an intrinsic block of signaling at the receptor level. Interestingly, transactivation of TrkB receptors seems to circumvent this issue. Use of adenosine A2A receptor agonists that transactivate TrkB intracellularly have been shown to enhance survival of lesioned facial motoneurons (Wiese et al., 2007) and lead to a delay in disease progression in a mouse model of ALS (Domeniconi and Chao, 2010; Yanpallewar et al., 2012). A similar transactivation of TrkB by glucocorticoids has been shown to promote downstream signaling and exert neurotrophic effect (Jeanneteau et al., 2008). Since, the TrkB transactivation mechanism occurs independently of BDNF and is not influenced by the presence of TrkB.T1 at the membrane, it is possible to increase the ability of diseased neurons to respond to TrkB signaling. One caveat is that TrkB.T1, as described earlier, can independently affect calcium signaling and imbalances in this isoform levels may influence neuronal survival by altering intracellular calcium levels. Indeed, dysregulation of calcium homeostasis in spinal motoneurons of SOD1 mutant mice has already been reported (Damiano et al., 2006; Guatteo et al., 2007). This scenario calls for the consideration of multiple strategies to identify the therapeutic potential of BDNF-TrkB signaling: one to reduce TrkB.T1 to correct its dominant-negative function and neuronal calcium levels and the other to increase activation of TrkB.FL by transactivation. An attractive approach to regulate aberrant TrkB isoforms expression is by targeting TrkB alternative splicing (AS). By targeting AS it is possible to regulate the alternate exon inclusion or block exon skipping to enhance read-through of full length isoform or even blocking a splice site to promote formation of a particular splice variant (Graziewicz et al., 2008; Geib and Hertel, 2009; Lin et al., 2015). This approach has now become reality as a therapeutic antisense oligonucleotide (ASO) that binds to the survival motor neuron 2 (SMN2) messenger RNA has been approved by the FDA for the treatment of spinal muscular atrophy (SMA). Treatment with the steric block ASO Nusinersen, that binds to the SMN2 mRNA, was found to promote exon 7 inclusion resulting in increased full length SMN protein. This ultimately leads to the increased survival and motor function in patients of SMA (Wood et al., 2017). A similar approach could be used to correct TrkB.T1 upregulation since even limited efficacy in correcting aberrant splicing levels of TrkB.T1 may lead to significant therapeutic benefits and may even be more desirable considering that TrkB.T1 is important for the normal function of other organs such as the heart and the endocrine pancreas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LT and SY wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the intramural research program of the National Cancer Institute, NIH.

ACKNOWLEDGMENTS

This review is dedicated to Dionisio Martin-Zanca (1952–2021), a great mentor and scientist. He first cloned TrkA and then was part of the team discovering that TrkA is the receptor for NGF, discoveries that revolutionized the neurotrophin field. We thank, Jodi Becker, Eileen Southon, Francesco Tomassoni-Ardori, and Zhenyi Hong for critical reading of the manuscript.

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frontiers in Neuroscience

PERSPECTIVE
published: 09 March 2022
doi: 10.3389/fnins.2022.773347

Getting Into the Brain: The Intranasal Approach to Enhance the Delivery of Nerve Growth Factor and Its Painless Derivative in Alzheimer's Disease and Down Syndrome

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The neurotrophin Nerve Growth Factor (NGF) holds a great potential as a therapeutic candidate for the treatment of neurological diseases. However, its safe and effective delivery to the brain is limited by the fact that NGF needs to be selectively targeted to the brain, to avoid severe side effects such as pain and to bypass the blood brain barrier. In this perspective, we will summarize the different approaches that have been used, or are currently applied, to deliver NGF to the brain, during preclinical and clinical trials to develop NGF as a therapeutic drug for Alzheimer's disease. We will focus on the intranasal delivery of NGF, an approach that is used to deliver proteins to the brain in a non-invasive, safe, and effective manner minimizing systemic exposure. We will also describe the main experimental facts related to the effective intranasal delivery of a mutant form of NGF [painless NGF, human nerve growth factor painless (hNGFp)] in mouse models of Alzheimer's disease and compare it to other ways to deliver NGF to the brain. We will also report new data on the application of intranasal delivery of hNGFp in Down Syndrome mouse model. These new data extend the therapeutic potential of hNGFp for the treatment of the dementia that is progressively associated to Down Syndrome. In conclusion, we will show how this approach can be a promising strategy and a potential solution for other unmet medical needs of safely and effectively delivering this neuroprotective neurotrophin to the brain.

Keywords: nerve growth factor, down syndrome, intranasal, neurogenesis, inflammation, microglia, astrocytes

OPEN ACCESS

Edited by:
Viviana Triaca,
National Research Council (CNR), Italy

Reviewed by:
Iosif Pediaditakis,
Flagship Pioneering, United States
Yoshiki Koriyama,
Suzuka University of Medical Sciences, Japan

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *09 September 2021*
Accepted: *10 February 2022*
Published: *09 March 2022*

Citation:
Capsoni S and Cattaneo A (2022) Getting Into the Brain: The Intranasal Approach to Enhance the Delivery of Nerve Growth Factor and Its Painless Derivative in Alzheimer's Disease and Down Syndrome. Front. Neurosci. 16:773347. doi: 10.3389/fnins.2022.773347

INTRODUCTION

The neurotrophin Nerve Growth Factor (NGF) (Levi-Montalcini, 1952) has been suggested to play a neuroprotective factor in several neurological diseases and has been a matter of numerous basic and clinical research studies.

Nerve growth factor is produced from a gene located on chromosome 1 (Francke et al., 1983) as a precursor proNGF which exists in two distinct isoforms of 27 and 35 kDa (Edwards et al., 1988). ProNGF is then cleaved to mature NGF by a group of enzymes including furin and metalloproteases (Cuello et al., 2019). Recently, it has been found that both proNGF and mature

NGF can trigger biological responses (Lee et al., 2001), most often with an opposite sign. First, it has been demonstrated that proNGF is the major species of this neurotrophin both in the Central Nervous Systems (Fahnestock and Shekari, 2019). Secondly, proNGF can bind $p75^{NTR}$, the receptor common to all neurotrophins. But it can also bind to the tropomyosin-related kinase receptor TrkA, although with a lower affinity than mature NGF (Fahnestock et al., 2004). Thus, a subtle balance between the amount of proNGF, mature NGF and their receptors can lead to either a neuroprotective or a pro-neurodegenerative outcome (see below).

NERVE GROWTH FACTOR IN ALZHEIMER'S DISEASE AND ITS LIMITS TO CLINICAL APPLICATION

Alzheimer's disease (AD) neuropathology is distinguished by deposits of misfolded proteins, mainly consisting of hyperphosphorylated tau and β-amyloid (Aβ) (Selkoe, 2001). Another prominent feature of the neurodegenerative process characterizing AD is the occurrence of cholinergic deficit (Whitehouse et al., 1982), which put the theoretical basis of the pharmacological therapy available for AD patients (use of cholinesterase inhibitors) (Giacobini, 2006). Basal forebrain cholinergic neurons (BFCNs) were identified as the most significant NGF-sensitive population inside the CNS. These neurons express both NGF receptors TrkA and $p75^{NTR}$ (Hefti, 1986; Holtzman et al., 1992), are able to retrogradely transport NGF from their cortical projections up to their cell bodies (Seiler and Schwab, 1984) and respond to administration of exogenous NGF, in terms of increase of cholinergic phenotypical markers (Gnahn et al., 1983; Mobley et al., 1986). Most importantly, NGF is able to prevent BFCN death or atrophy, following axotomy (Hefti, 1986; Williams et al., 1986; Kromer, 1987) or linked to aging (Fischer et al., 1987). In AD, a selective decrease in the expression of TrkA, and not $p75^{NTR}$, occurs in BFNCs and hippocampus and it correlates with the severity of the disease (Mufson et al., 2019). The distinctive cholinergic deficit in AD, together with the BFCN being NGF target neurons, has led to propose the use of NGF as a treatment for AD (Tuszynski et al., 2005; Mitra et al., 2019).

Work with the anti-NGF AD11 mouse model (Ruberti et al., 2000), in which the expression of antibodies against mature NGF in the adult brain causes a progressive neurodegeneration which is similar to that observed in AD brains, provided the first demonstration that deficits in NGF signaling may lead to a Alzheimer-like neurodegeneration (Capsoni et al., 2011), which is broader than a pure cholinergic deficit. This comprehensive neurodegeneration phenotype suggested that other cells in the brain, in addition to BFCNs, might respond to NGF deficits and, conversely, might represent targets for NGF therapeutic actions. Indeed, triggered by this neurodegeneration picture, we found that microglia are NGF target cells and respond to NGF by activating a potent and broad neuroprotective and anti-inflammatory action (Rizzi et al., 2018).

Deficits in NGF processing or transport could be causally linked to the onset of AD neurodegeneration. Whilst in the AD11 model the NGF deficit is determined by interference with an anti-NGF antibody expressed in the brain, different pathological mechanisms could result in a reduced NGF bioactivity. Thus, a reduced NGF bioactivity might result either by a defect in NGF retrograde transport system (Mufson et al., 1995) or by an unbalance of proNGF vs. mature NGF signaling (Fahnestock et al., 2001; Podlesniy et al., 2006). Indeed, experimentally increasing proNGF in transgenic mice also induces a progressive neurodegeneration, despite concomitant higher-than-normal mature NGF levels (Tiveron et al., 2013; Fasulo et al., 2017).

We can therefore formulate an NGF hypothesis for AD neurodegeneration, whereby a common link behind AD neurodegeneration is a failure or an insufficient NGF signaling, leading to inadequate neurotrophic support (Capsoni and Cattaneo, 2006; Cattaneo et al., 2008; Cattaneo and Calissano, 2012). The failure or unbalance of NGF support could be due to different causes in the overall cascade(s) of events involving NGF bioactivity: (1) decreased NGF synthesis, (2) unbalanced or altered processing, (3) alterations in receptor expression and/or activity or expression ratios, and (4) altered retrograde transport. These events would be "located" upstream of the "amyloid cascade," which is the central core of AD neurodegeneration, as currently described (Selkoe, 2000), and would be part of a negative feedback loop that involves several steps (e.g., links between APP, tau, and axonal transport). On the other hand, the intrahippocampal injection of Aβ oligomers in naïve rats is sufficient to induce a proNGF/NGF unbalance (Bruno et al., 2009).

Thus, an initial deficit in NGF signaling or processing or a reduction of TrkA receptors will result in a feed-forward pathological cycle leading to increased accumulation of Aβ and propagation of proNGF/NGF homeostasis deficits (Cattaneo and Capsoni, 2019).

Within this theoretical frame, any therapy aimed at re-establishing the correct balance between ligands (and receptors) of the NGF pathway appears to have a clear rationale. The most direct therapeutic approach along these lines would be, therefore, to exploit NGF itself. However, the viable clinical application of NGF requires providing a solution to major obstacles, namely finding a more effective NGF delivery to the CNS and limiting adverse effects deriving from undesired NGF actions, most notably, pain.

PAST AND ONGOING NERVE GROWTH FACTOR CLINICAL TRIALS IN ALZHEIMER'S DISEASE

One approach to overcome the limits of NGF administration might be the use of small molecules that could cross the blood brain barrier and mimic NGF action, improving survival of target cells [Pediaditakis et al. (2016a,b) and reviewed in Gascon et al. (2021)]. Currently one of these small-molecule NGF mimetics, the $P75^{NTR}$ binding molecule LM11A-31, is under evaluation in clinical trials in Alzheimer's disease (Yang et al., 2008, 2020).

However, this approach might represent limitations due to a more restricted pharmacological profile with respect to that of the NGF protein.

To achieve a therapeutic concentration of NGF in the brain, while also avoiding systemic exposure, a first clinical has been performed in which an intracerebroventricular infusion was performed in three patients (Eriksdotter Jonhagen et al., 1998). Despite an increase in nicotinic receptor expression and an amelioration in cognitive function, the trial had to be stopped due to the onset of unbearable back pain linked to the diffusion of NGF in the CSF irrorating the spinal cord. For this reason, subsequent clinical trials were performed using cells engineered to secrete NGF or adenoviruses carrying the sequence encoding for NGF, stereotaxically implanted by neurosurgery close to the basal forebrain. In 2005 a clinical trial targeting the BFCNs was performed in 8 patients in which autologous fibroblasts were engineered to produce NGF. Using this approach, slowing down of the cognitive decline, associated with an amelioration of cortical glucose uptake, was found (Tuszynski et al., 2005, 2015). Lately, an NGF-encoding adeno-associated viral vector also injected in the basal forebrain has been used (Rafii et al., 2014) but the treatment did not lead to clinical efficacy, most likely because of the failure to accurately engage the target cells (Rafii et al., 2018).

More recently, clinical trials using the encapsulated cell biodelivery (ECB) have been started. The ECB cells engineered to secrete NGF are located at the tip of a catheter formed by a semipermeable membrane to allow the exchange of NGF and nutrients in the extracellular fluid (Lindvall and Wahlberg, 2008). These catheters have been implanted in the basal forebrain of AD patients and allow to achieve an increase in choline acetyltransferase activity and glucose content in the brain, and amelioration in memory tests (Mitra et al., 2019). However, despite the encouraging results, the trials have been slowed down because of the variability of results due to degeneration of the engineered cells (Mitra et al., 2019).

THE ADVANTAGE OF INTRANASAL DELIVERY VS. OCULAR DELIVERY

To bypass the blood brain barrier, intranasal delivery is an alternative solution that has been proposed for several proteins (Dhuria et al., 2010; Malerba et al., 2011). As far as NGF is concerned, in 1997 Frey's group used radioactive labeled NGF to demonstrate that the intranasal delivery allows to obtain NGF in therapeutic concentrations in several regions of rat brain (Frey et al., 1997). Several hypotheses have been formulated concerning the pathways through which the protein can reach the brain. These include nerves (olfactory and trigeminal) connecting the nasal passages to the brain, vasculature, cerebrospinal fluid (CSF) and lymphatic system [reviewed in Dhuria et al. (2010) and Malerba et al. (2011)]. Our laboratory first applied this technique in anti- NGF AD11 mice, and we showed that intranasally delivered NGF could reduce memory deficits and the accumulation of Aβ deposits, hyperphosphorylated tau and cholinergic deficiency (Capsoni et al., 2002; De Rosa et al., 2005). In a subsequent study, intranasal delivery of NGF was compared to the administration of NGF eye-drops. It was found that the ocular delivery of NGF was less efficient than nasal delivery in rescuing tau-related neurodegeneration in AD11 mice, since a ten times higher dose than the one used for intranasal delivery was necessary to obtain the same effect (Capsoni et al., 2009).

MICROGLIA AS A NEW TARGET FOR THE ACTIONS OF INTRANASAL PAINLESS NERVE GROWTH FACTOR (HUMAN NERVE GROWTH FACTOR PAINLESS)

Intranasal delivery allows not only to reach brain regions, but it also reduces the possibility to have a systemic leakage of the protein in blood circulation, thus reducing the possibility to trigger side effects such as pain. To increase the therapeutic index and to reduce the possibility to trigger nociceptor sensitization, a mutation in the human NGF gene, inspired by a rare human disease, the Hereditary Sensory and Autonomic Neuropathy type V (HSAN V), was introduced. HSAN V patients carry a mutation from arginine 100 to tryptophan (R100W) and suffer of pain insensitivity without having cognitive deficits (Einarsdottir et al., 2004). After screening different amino acid substitutions, we selected the mutation R100E because of (i) its similarity to the R100W mutation in selectively altering TrkA signaling, (ii) in abolishing the binding to $p75^{NTR}$ receptor and because of (iii) a more efficient production in *Escherichia coli* (Covaceuszach et al., 2010). In addition to the R100E mutation, a second one (P61S) was introduced to make the protein detectable against the endogenous human NGF (Covaceuszach et al., 2009). The mutant NGFP61SR100E [painless NGF or human nerve growth factor painless (hNGFp)] was shown to have the same neurotrophic potency as wildtype NGF, in several bioassays, while showing a greatly reduced pain sensitization potency, in a number of pain assays, with respect to wild type NGF (Malerba et al., 2015). From the pharmacological point of view, hNGFp is a TrkA-biased agonist, with a greatly reduced ability to bind and activate $p75^{NTR}$ (Cattaneo and Capsoni, 2019).

In a first study, hNGFp was used to treat AD11 and APPxPS1 mice (Capsoni et al., 2012). We showed that the intranasal delivery was able to improve memory in both transgenic models, as assessed by novel object recognition and in Morris water maze tests. Moreover, in both mouse models Aβ deposition was lowered in both transgenic mice. In AD11 mice, also tau hyperphosphorylation and cholinergic deficit were decreased.

A second paper in which the treatment was performed in 5xFAD mice allowed us to uncover the neuroprotective mechanisms through which hNGFp acts to reduce the neurodegeneration and to compare the effectiveness of intranasal delivery vs. a local delivery to cholinergic neurons, mimicking the approach used in clinical trials (Capsoni et al., 2017). First, we demonstrated that intranasal hNGFp can be detected at 6 and 24 h after the administration in the hippocampus and cerebral cortex, respectively, two areas highly affected by the neurodegeneration. We found that the local delivery of hNGFp to cholinergic neurons of the nucleus basalis was

not decreasing the number of plaques in 5xFAD mice, despite the sprouting of cholinergic fibers. On the contrary, with the intranasal delivery we obtained a reduction in the plaque load because of a reduced pro-amyloidogenic processing of APP and a clearance of deposited Aβ by microglia. Indeed, we found that microglia are the first cellular target of hNGFp, being the only cellular type, beside BFCNs, which express TrkA in 5xFAD mice. Thus, mechanisms through which intranasal hNGFp affects APP processing does not go through BFCNs but involve a modulation of fine cytokines, including Interleukin1α and CXCL12 which we demonstrated to be upregulated in neurons after hNGFp administration as a consequence of the blockade of Tumor Necrosis Factor α (TNFα) by its soluble receptor type 2 (Capsoni et al., 2017). The data on phagocytosis of Aβ oligomers were confirmed in a parallel study performed on primary microglia cells in which it was demonstrated that NGF can increase their micropinocytosis, thus preventing the decrease in neuronal spines and the onset of deficit in long term potentiation (LTP) (Rizzi et al., 2018). LTP was also improved in the entorhinal cortex of 5xFAD mice after intranasal treatment and this correlates also with an amelioration of memory deficits (Capsoni et al., 2017).

In conclusion, a therapeutic effect able to prevent or clear Aβ deposition in the brain of the 5xFAD mouse model required a broad hNGFp biodistribution, such that could be achieved by the intranasal delivery of hNGFp, but not by the local delivery to the basal forebrain. Thus, the intranasal, but not the local, delivery of hNGFp appears to be necessary to permeate the brain with hNGFp, reach microglia which are widely distributed in the brain and provide neuroprotection and anti-neurodegenerative effects (Rizzi et al., 2018).

EFFICACY OF INTRANASAL hNGFp IN DOWN SYNDROME MICE

The fact that microglia is a target cell of NGF in the brain (Rizzi et al., 2018) and that is a primary target of intranasal hNGFp in the 5xFAD Alzheimer's model (Capsoni et al., 2017) suggests that the microglia-mediated broad neuroprotective actions of hNGFp might be exploited in other disease states, in addition to AD. We tested this hypothesis by investigating the efficacy of hNGFp in a mouse model of Down Syndrome (DS). A progressive dementia is a common age-related clinical aspect of DS patients (Hartley et al., 2015), the neurodegeneration including the deposition of β amyloid, neurofibrillary tangles and cholinergic deficit in BFCNs (Hefti, 1986). Abnormal levels of the amyloid precursor protein APP found in Ts65Dn mice (Choi et al., 2009) lead to an impaired transport of NGF to BFCNs (Salehi et al., 2006) and the local infusion of NGF rescues the cholinergic deficit in these mice (Cooper et al., 2001). More recently, an increase of the ratio between the precursor of NGF, proNGF, and mature NGF, and imbalance in TrkA/$p75^{NTR}$ ratio has been found in the brain and plasma from DS patients (Iulita et al., 2014; Iulita and Cuello, 2016; Miguel et al., 2021). This imbalance is known to trigger neurodegeneration (Capsoni and Cattaneo, 2006; Fahnestock and Shekari, 2019) and to contribute to neuroinflammation (Capsoni et al., 2011; Iulita et al., 2016). Indeed, similarly to AD, an activation of astrocytes and microglia, the main mediators of inflammation, has been reported in DS subjects (Wilcock and Griffin, 2013). Given these data, therapies aimed at re-establishing the correct balance between ligands of the NGF pathway appear to have a clear rationale (Cattaneo et al., 2008; Iulita and Cuello, 2014) also for DS.

We therefore tested the effect of intranasally delivered hNGFp in Ts65Dn mice at an early stage (4 months of age), prior to overt accumulation of APP and neurodegeneration [which in this model starts at 6 months of age (Choi et al., 2009; **Figures 1K,L**)]. We started by investigating whether morphological alterations in microglia are found at this early stage. Microglia was reported to be dystrophic in human DS brains (Streit et al., 2009), but was never studied in TS65Dn. By single cell morphologies from confocal images, we found that, despite a similar number of microglial cells (**Figure 1A**) in 4 months old Ts65Dn mice microglia (identified by Iba1 immunohistochemistry) is dystrophic, with a reduction in area, volume, length and number of ramifications (**Figures 1C,F,H**) with respect to euploid mice (**Figures 1B,E,H**). The intranasal administration of hNGFp significantly restored the morphology of microglia (**Figures 1D,G,H**). This is highly relevant, since the morphology of microglia is directly related to its functional state.

Then we measured the levels of markers of microglia activation and cytokines. As might be expected from the fact that we analyzed brains at an age in which Alzheimer-like neurodegeneration had not yet started, we did not find a differential expression among groups for CD68, TNFα, IL-1β, and INFγ (**Figures 1I,J**). On the contrary, we found that hNGFp increased the expression of IL-10 while decreasing IL-6 levels (**Figures 1I,J**). We found that IL-1α, which we know to be decreased in Alzheimer mouse models after hNGFp treatment (Capsoni et al., 2017), was decreased. IL-1α is produced as a precursor, proIL-1α, which is cleaved to an active, lower molecular weight molecule by calpain (Kobayashi et al., 1990; Carruth et al., 1991). We observed a reduction of proIL-1α in the brain extracts from Ts65Dn mice treated with hNGFp with respect to PBS-treated mice, although not statistically significant (**Figures 1K,L**, $P > 0.05$). On the contrary, a significant reduction of mature IL-1α (**Figures 1K,L**) was observed after hNGFp treatment, similarly to previous findings in hNGFp-treated 5xFAD mice (Capsoni et al., 2017).

Concerning astrocytes, we found no difference in their density among the treatment groups(**Figure 2A**). In PBS-treated 4 months old Ts65DN mice we found a significant reduction in volume, surface area, length and number of ramifications (**Figures 2C,F,H**) with respect to euploid mice (**Figures 2B,E,H**) in the hippocampus. These changes resemble the asthenic phenotype which precedes the astrogliosis observed in mouse models of AD and in early human AD (Verkhratsky et al., 2015). The intranasal delivery of hNGFp restores the characteristic shape of astrocytes in the brain of control mice, by increasing all parameters taken into consideration (**Figures 2D,G,H**). The characteristic shape of astrocytes is determined also by the expression the cytoskeletal protein GFAP.

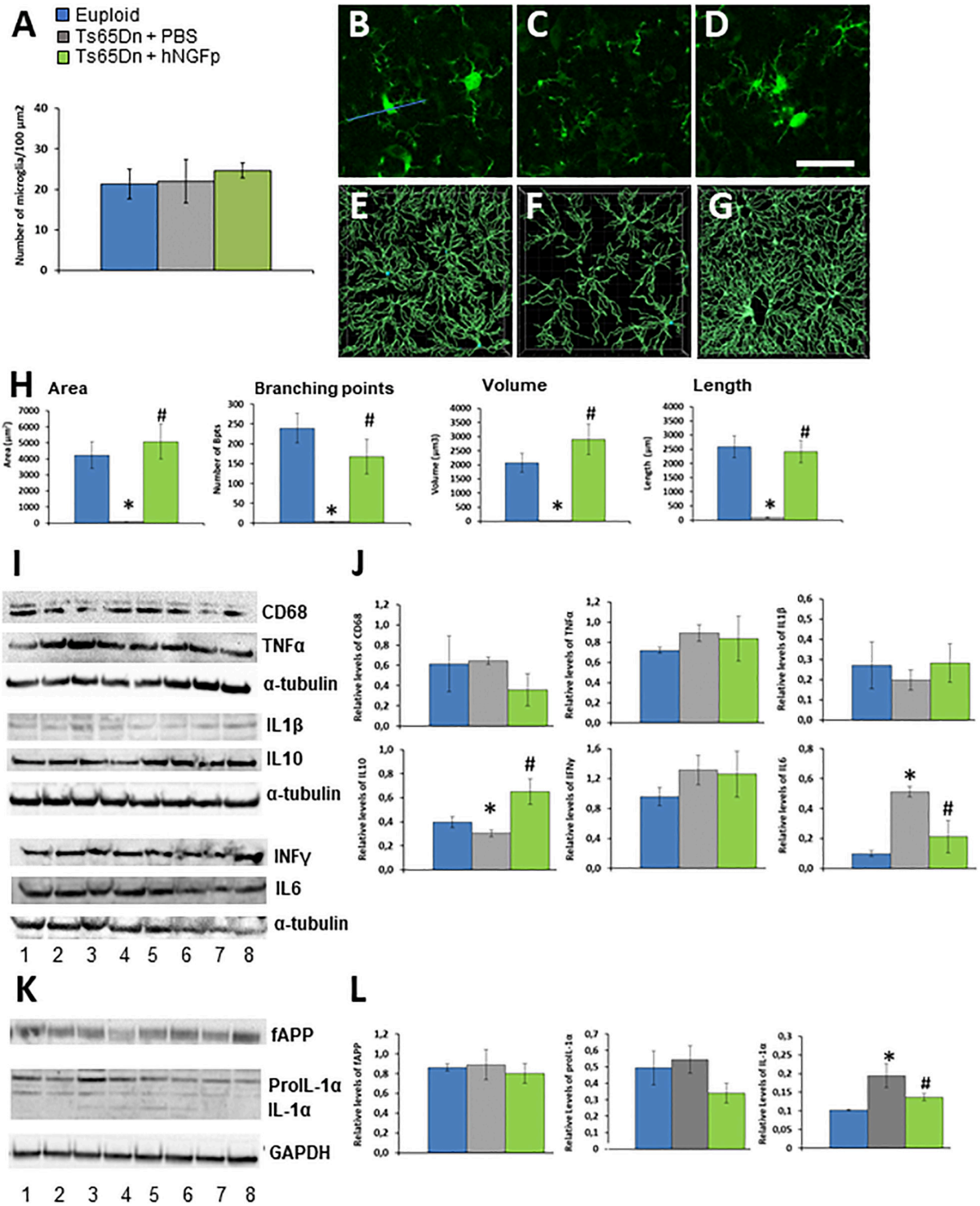

FIGURE 1 | Intranasal hNGFp ameliorates microglial dystrophic morphology and reduces IL-1α levels in Ts65Dn mice. **(A)** Density of microglia cells in euploid, Ts65Dn mice treated with PBS or hNGFp. Immunohistochemistry for IBA-1 in cerebral cortex revealed morphological changes in panel **(C)** Ts65DN microglia with respect to panel **(B)** euploid mice. **(D)** hNGFp treatment rescues these morphological changes. Reconstruction of microglia cells by IMARIS: **(E)** euploid **(F)** Ts65Dn **(G)** NGFp-treated Ts65Dn mice. **(H)** Quantification of microglial morphological parameters. Bars are representative of mean ± SEM. $*P < 0.001$ vs. euploid mice, $\#P < 0.001$ vs. Ts65Dn mice. $N = 6$/group. **(I)** Representative western blots and **(J)** densitometric analysis for CD68, TNFα, IL-1β, IL-10, and IL-6. **(K)** Representative western blots for APP and IL-1α species. Lanes 1–2 = euploid mice; 3–5 = Ts65Dn mice treated with PBS; 6–8 = Ts65Dn mice treated with hNGFp. **(L)** Densitometric analysis of APP, proIL-1α (graph on the left) and mature IL-1α (right panel) levels. Values have been normalized to GAPDH values. Bars are representative of mean ± SEM. $*P < 0.001$ vs. euploid mice, $\#P < 0.001$ vs. Ts65Dn mice. $N = 6$/group. Scale bar = 10 µm.

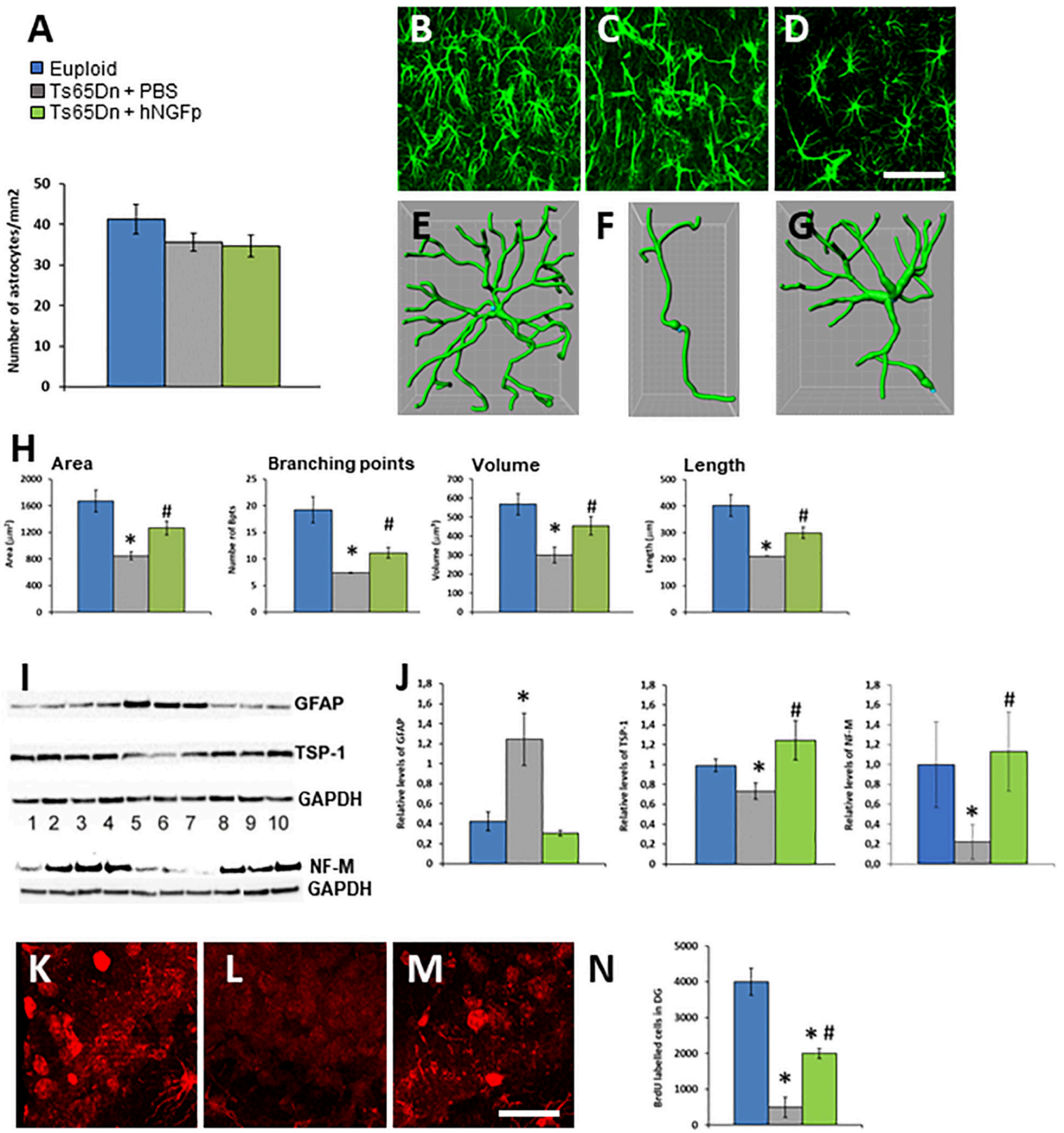

FIGURE 2 | Intranasal hNGFp rescues astrogliopathy and neurogenesis deficit in Ts65DN mice. **(A)** Density of astrocytes in euploid, Ts65Dn mice treated with PBS or hNGFp. Immunohistochemistry for GFAP in hippocampus revealed morphological changes in panel **(C)** Ts65DN astrocytes with respect to panel **(B)** euploid mice. **(D)** hNGFp treatment rescues these morphological changes. Reconstruction of astrocytes by IMARIS: **(E)** euploid **(F)** Ts65Dn **(G)** NGFp-treated Ts65Dn mice. **(H)** Quantification of astrocytic morphological parameters. Bars are representative of mean ± SEM. $^*P < 0.05$ vs. euploid mice, $\#P < 0.05$ vs. Ts65Dn mice. $N = 6$/group. Scale bar = 35 μm. **(I)** Western blot for GFAP, TPS-1 and NF-M. Lanes 1–4 = euploid mice; 5–7 = Ts65Dn mice treated with PBS; 8–10 = Ts65Dn mice treated with hNGFp. **(J)** Densitometric analysis of GFAP, TPS-1, and NF-M levels. Values have been normalized to GAPDH values. Adult hippocampal neurogenesis is deficient in Ts65Dn mice compared to littermates and it is partially rescued by treatment with hNGFp. **(K–M)** examples from panel **(K)** euploid, **(L)** Ts65Dn, and **(M)** Ts65Dn dentate gyrus. **(N)** Stereological quantification of BrdU-labeled cells. Bars are representative of mean ± SEM. $^*P < 0.05$ vs. euploid mice, $\#P < 0.05$ vs. Ts65Dn mice. Scale bar = 200 μm.

We found that, despite the reduction in volume, and consistently with what reported in literature for human DS (Jorgensen et al., 1990), there is an increase of GFAP levels in Ts65Dn mice compared to control mice (**Figures 2I,J**). This increase is completely reverted by the intranasal administration of hNGFp (**Figures 2I,J**).

In DS and in Ts65Dn mice cognitive deficits have been associated to structural abnormalities in dendritic spines. A critical factor for spine development is the production of thrombospondin 1 (TPS-1) by astrocytes. Indeed, decreased levels of TPS-1 have been found in the conditioned medium of cultured DS astrocytes and hypothesized to contribute to the reduced synaptogenesis (Torres et al., 2018). We found that also in the brain of 4 months old Ts65DN mice there is a decrease in TPS-1 protein, which was reverted to normal by the intranasal administration of hNGFp (**Figures 2I,J**).

Astrocytic homeostatic functions in the maintenance of neurogenesis are impaired in DS (Sloan and Barres, 2014). Moreover, we recently found that proNGF/NGF imbalance determines a reduced adult neurogenesis in the hippocampus dentate gyrus (Corvaglia et al., 2019). Also, adult neurogenesis in the dentate gyrus of young Ts65Dn mice showed markedly fewer BrdU-labeled cells than euploid animals (Clark et al., 2006). Based on these data, and on the fact that the intranasal administration of hNGFp increases the production of the chemokine CXCL12 (Capsoni et al., 2017) which is a pro-neurogenesis factor (Li et al., 2012), we measured the number of BrDU-immunoreactive cells in the dentate gyrus of 5 months old TS65Dn mice. We found a dramatic reduction of neurogenesis in the dentate gyrus of TS65Dn mice (**Figures 2K–N**), which was partially but significantly recovered by intranasal hNGFp (**Figures 2K–N**). Consistent with the increased neurogenesis with hNGFp, we found that hNGFp restored the levels the neuronal marker Neurofilament-M (**Figures 2I,J**), which was decreased in Ts65Dn mice.

In conclusion, we found that hNGFp treatment rescues astrogliosis, dystrophic microglia and neurogenesis deficits in the brain of 4 months old TS65Dn mice.

GENERAL CONCLUSION AND NEW PERSPECTIVES

From the data described in this perspective paper, we conclude that the links between deficits or alterations in the NGF system and AD go well beyond the long-established neurotrophic actions of NGF on BFCNs. Indeed, the intranasal delivery studies allowed to uncover that the cellular targets for NGF actions in the brain are more widespread than envisaged so far, including broadly distributed microglia and astrocytes. Given this finding, we conclude that intranasal hNGFp can be applied to other neurodegenerative and neurodevelopmental diseases in which cholinergic neurons are not the primary target of hNGFp action and neuroinflammation plays a relevant role. Thus, we conclude that the spectrum of neurodegenerative diseases that are amenable to be treated by hNGFp is very broad. In line with this conclusion, we presented new data demonstrating that intranasally delivered hNGFp has a potent neuroprotective action on the early phenotypic deficits in the TS65Dn mouse model of Down Syndrome and we propose that hNGFp could be used for the treatment of the progressive dementia affecting DS patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

SC designed the research, performed the research, and analyzed the data and wrote the manuscript. AC designed the research and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Jerome LeJeune Foundation grant to AC entitled "Toward an NGF-based therapy for Down Syndrome."

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Neuroscience

MINI REVIEW
published: 25 March 2022
doi: 10.3389/fnins.2022.846681

Neurotrophins as Therapeutic Agents for Parkinson's Disease; New Chances From Focused Ultrasound?

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Magnetic Resonance–guided Focused Ultrasound (MRgFUS) represents an effective micro-lesioning approach to target pharmaco-resistant tremor, mostly in patients afflicted by essential tremor (ET) and/or Parkinson's disease (PD). So far, experimental protocols are verifying the clinical extension to other facets of the movement disorder galaxy (i.e., internal pallidus for disabling dyskinesias). Aside from those neurosurgical options, one of the most intriguing opportunities of this technique relies on its capability to remedy the impermeability of blood–brain barrier (BBB). Temporary BBB opening through low-intensity focused ultrasound turned out to be safe and feasible in patients with PD, Alzheimer's disease, and amyotrophic lateral sclerosis. As a mere consequence of the procedures, some groups described even reversible but significant mild cognitive amelioration, up to hippocampal neurogenesis partially associated to the increased of endogenous brain-derived neurotrophic factor (BDNF). A further development elevates MRgFUS to the status of therapeutic tool for drug delivery of putative neurorestorative therapies. Since 2012, FUS-assisted intravenous administration of BDNF or neurturin allowed hippocampal or striatal delivery. Experimental studies emphasized synergistic modalities. In a rodent model for Huntington's disease, engineered liposomes can carry glial cell line–derived neurotrophic factor (GDNF) plasmid DNA (GDNFp) to form a GDNFp-liposome (GDNFp-LPs) complex through pulsed FUS exposures with microbubbles; in a subacute MPTP-PD model, the combination of intravenous administration of neurotrophic factors (either through protein or gene delivery) plus FUS did curb nigrostriatal degeneration. Here, we explore these arguments, focusing on the current, translational application of neurotrophins in neurodegenerative diseases.

Keywords: neurotrophins, movement disorders, neurodegeneration, MRgFUS, BBB

OPEN ACCESS

Edited by:
Bruno Pietro Imbimbo, Chiesi Farmaceutici, Italy

Reviewed by:
Allegra Conti, University of Rome Tor Vergata, Italy
Gaetano Barbato, Inno-Sol SRL, Italy

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *31 December 2021*
Accepted: *31 January 2022*
Published: *25 March 2022*

Citation:
Stefani A, Pierantozzi M, Cardarelli S, Stefani L, Cerroni R, Conti M, Garasto E, Mercuri NB, Marini C and Sucapane P (2022) Neurotrophins as Therapeutic Agents for Parkinson's Disease; New Chances From Focused Ultrasound? Front. Neurosci. 16:846681. doi: 10.3389/fnins.2022.846681

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder of aging, caused by the depletion of dopaminergic neurons in the substantia nigra pars compacta with consequent dopamine (DA) deficiency. The pathological trademark of PD is the eosinophilic cytoplasmic neuronal inclusions termed Lewy bodies, whose main component is abnormally folded α-synuclein (α-syn) protein aggregations (Wakabayashi et al., 2007). However, recent uncertainties and failures of protocols based on anti–α-syn antibodies (NCT03100149, NCT03318523, Antonini et al., 2020) reinforced

the skepticism of those who rather emphasize the centrality of energy failure as the primum movens of disease pathogenesis; hence, the opportunity once more to validate neurorescue-based therapy including neurotrophins. Significantly, our group is actively challenging the metabolic production/accumulation of lactate as reliable biomarker for the progression of different neurodegenerative conditions (Liguori et al., 2015, 2016).

Consensus prevails on some seminal concepts:

- It is believed that PD pathology is a consequence of the interaction between genetic susceptibility and toxic environmental factors, but the factors responsible for the initiation of the pathophysiological cascade remain still largely unknown (for a review Henderson et al., 2019).

- In this scenario, no effective disease-modifying therapy is presently available for neurodegenerative disease such as PD and related sinucleinopaties. Current treatments for PD are symptomatic and, although effective in the management of motor symptoms, they do not counteract the progression of neurodegeneration and disease time-course; moreover, complications such as motor fluctuations dyskinesias are unescapable after years of replacing DA therapies (LeWitt, 2015).

- Albeit we are handling complex therapies as routine, and public sensibility of stakeholders drive new multidisciplinary approaches (Bloem et al., 2021), the unmet needs maintain their severe burden.

That said, the increasing understanding of PD pathophysiology led to refuel at considering the potential role of several molecules with neuroprotective and neurorestorative properties as rescue therapy, together with questioning on solutions, which may overcome the pitfalls encountered in previous trials.

Over the last decades rigorous and extensive research has tried to devise disease-modifying therapies for neurodegenerative extrapyramidal diseases. In this area, neurotrophic factors (NTFs), due to their action on promoting neuronal survival and neurite outgrowth, emerged as compounds potentially able to limit, or even stop, cell degeneration, improving functionality of stressed neurons (Chmielarz and Saarma, 2020). In fact, there is a converging view that insufficient neuronal reserve of NTFs may be, in part, involved in synaptic plasticity deficit and neurodegenerative diseases onset (Baquet et al., 2005; Zuccato and Cattaneo, 2009).

This brief review will examine the evidence collected in experimental models; hence, we will discuss the current limitations detected when trying to transfer those results in meaningful clinical success, finally questioning on the potential utilization of low-intensity focused ultrasound (LIFU) as non-routine key approach for NFTs delivery.

A BRIEF EXCURSUS AROUND NEUROTROPHINS IN EXPERIMENTAL MODELS OF PARKINSON'S DISEASE

NTFs are important regulators of neural survival, development, function, and plasticity. NTFs are classified into three main groups: (i) the neurotrophins, including the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4); (ii) the glial cell line–derived neurotrophic factor (GDNF) family of ligands, including GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN); (iii) the neurokines, including ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), and cardiotrophin (CT-1) (Bothwell, 2014).

Given the fundamental role played by neurotrophins in regulating neuronal functions and their distribution in critical neuronal areas, it is not surprising that a significant number of psychiatric and neurodegenerative disorders is associated with altered NTFs' levels and with changes in the expression of their receptors.

The interest in NTFs applied to PD dates back to the 1990s. Early studies in rat mesencephalic cultures found that BDNF prevents death of dopaminergic neurons, supporting their survival in the ventral tegmental area and medial substantia nigra (SN), also promoting their differentiation into dopaminergic cells (Hyman et al., 1991; Beck et al., 1993; Murer et al., 1999; Baquet et al., 2005). Besides the well-established neurotrophic action, BDNF also possesses other neuroprotective activities, such as anti-apoptosis, anti-oxidation, suppression of autophagy, and restoration of mitochondrial dysfunction (Chen et al., 2017; Miller et al., 2021).

Multiple lines of pre-clinical evidence documented abnormal expression of BDNF in different PD animal models proving that neurotrophins become downregulated, although the results of different studies were not always in complete agreement, probably for the different experimental settings (Collier et al., 2005; Mocchetti et al., 2007; Berghauzen-Maciejewska et al., 2015; Sampaio et al., 2017).

Silencing the gene encoding BDNF in mice resulted in the loss of dopaminergic neurons and in motor impairments, which confirms the role of BDNF in the deterioration of motor and cognitive abilities in PD protecting neurons against degeneration (Baker et al., 2005; Baquet et al., 2005). However, controversial results on neuronal dopaminergic protection and recovery of DA levels were obtained from different animal models of PD (Levivier et al., 1995; Lucidi-Phillipi et al., 1995; Galpern et al., 1996; Klein et al., 1999). A fairly recent study by Hernandez-Chan et al. (2015) used a delivery method called neurotensin-polyplex that utilized the neurotensin receptors for the internalization of nano vesicles specifically in dopaminergic neurons. The delivery of BDNF after the 6-hydroxydopamine (6-OHDA)–induced lesion showed substantial improvement in parkinsonian behavior related to a recovery of striatal DA. Moreover, the significant sprouting of dopaminergic fibers and the absence of recovery of DA level and number of surviving tyrosine hydroxylase (TH)–positive cells in the SN support the conclusion that BDNF was unable to stimulate neurogenesis but can sustain neuritogenesis both in the SN and striatum, in line with previous studies (Klein et al., 1999; Somoza et al., 2010).

Apart from the studies focused on BDNF expression in PD, also, the role of other NTFs has been widely investigated including the GDNF and its family member NRTN.

Different from BDNF, whose distribution is diffused in frontal cortex, hippocampus, basal ganglia, cerebellum, SN, and other

brainstem regions (He et al., 2013), GDNF is only expressed in the dorsal and ventral striatum, anteroventral nucleus of the thalamus, septum, and subcommissural organ (Pascual et al., 2011). Curiously, there are no GDNF receptors mRNAs in the striatum, but they are highly expressed in the nigral cells (Trupp et al., 1997), suggesting a specific action on nigral dopaminergic neurons.

GDNF resulted to be more potent than BDNF on survival of SN pars compacta (SNpc) dopaminergic neurons in the brain of lesioned animal models of PD (Lu and Hagg, 1997; Rosenblad et al., 2000; Sun et al., 2005); GDNF is also able to stimulate axonal sprouting of lesioned SNpc neurons, but less effective in promoting striatal reinnervation or functional recovery in 6-OHDA lesion model (Rosenblad et al., 2000).

The therapeutic benefits of this NTF have been demonstrated to be effective in different neurotoxin-induced [6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] models of PD both in rodents (Choi-Lundberg et al., 1997; Tenenbaum and Humbert-Claude, 2017) and in non-human primates (Gash et al., 1996). In addition, the GDNF-family ligand NRTN has a neuroprotective effect, similar to GDNF, both on damaged nigral dopaminergic neurons in an animal model of PD and on ventral mesencephalic dopaminergic neurons *in vitro* (Horger et al., 1998; Fjord-Larsen et al., 2005). Nevertheless, NRTN diffuses less efficiently than GDNF in the parenchyma, due to its higher heparin-binding properties (Hadaczek et al., 2010; Runeberg-Roos et al., 2016). Recently, NRTN variants with lower affinity to heparin were showed to have increased brain biodistribution, to have increased chemical stability, and to be more efficient than GDNF in 6-OHDA rat model (Runeberg-Roos et al., 2016).

The inability of NTFs to cross the blood–brain barrier (BBB), the poor diffusion into brain tissues due to their large molecular size, together with a short half-life, led to the development of different strategies to improve the delivery of NTFs in a cell-specific and inducible manner, including cell grafts and viral vectors.

The implantation of genetically engineered human fibroblasts, protected by a semipermeable polymer capsule, enabled the direct production of GDNF in the striatum of a bilateral 6-OHDA lesion rat model, resulting in a significant improvement of movement performance associated with striatal reinnervation of TH-positive fibers (Sajadi et al., 2006). Recently, a novel strategy used genetically modified hematopoietic stem cell transplantation, capitalizing the propensity of derived macrophages to home to sites of neurodegeneration: macrophage-mediated GDNF delivery protected against dopaminergic neurodegeneration and lead to significant reversal of both motor and non-motor dysfunction in non-neurotoxin mice model of PD (Chen C. et al., 2019).

Delivery *via* viral vectors has the clear advantage that the expression can be elicited only in selected cells, which might mimic a more natural distribution of the ligand inside the tissue. In the last decades, different kinds of virus vectors were engineered to sustained long-term expression of NTFs in the treatment of neurodegenerative diseases (Kordower and Bjorklund, 2013). The viral strategies have been improved to obtain a temporal and quantity control of GDNF expression with the aim to avoid compensatory mechanisms on DA homeostasis, such as downregulation of TH transcription (Georgievska et al., 2002). Neuroprotective effects in the absence of TH downregulation have been obtained by applying low-GDNF doses either by injecting a low amount of viral vector (Eslamboli et al., 2005) or by controlling the level of transgene expression through a inducible viral vector (Tereshchenko et al., 2014; Chtarto et al., 2016).

More recently, cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) formed a new, evolutionarily conserved family of NTFs due to their unique structures and potent protection of embryonic DA neurons (Parkash et al., 2009). In mammals, CDNF expression occurs broadly in the central nervous system and in peripheral tissues, including the cerebral cortex, hippocampus, cerebellum, thalamus, SN, and striatum, showing a partial overlap with MANF expression (Lindholm et al., 2007, 2008). Different from other NTFs, CDNF and MANF are partially retained in the lumen of endoplasmic reticulum (ER) and their primary function appears to be the modulation of the unfolded protein response (UPR) pathway that is activated in response to ER stress (Jäntti and Harvey, 2020). Prolonged ER stress is associated with the onset and progression of chronic neurodegenerative diseases, including PD and Alzheimer's disease.

CDNF and MANF diffuse slightly better than GDNF in the brain tissue (Voutilainen et al., 2011) and protect nigrostriatal DA neurons from 6-OHDA–sinduced degeneration if injected in the striatum both before and after the induction of the lesion (Lindholm et al., 2007; Voutilainen et al., 2009). However, data from severe rat 6-OHDA neurorestoration models of PD suggest that CDNF could be more efficient than MANF (Voutilainen et al., 2011).

The neuroprotective and the neurorestorative effects of CDNF have also be demonstrated in the mouse MPTP model of PD (Airavaara et al., 2012) and through gene therapy strategies using adenovirus-associated virus (Bäck et al., 2013; Ren et al., 2013). However, contrary to previous results, the intrastriatal delivery of CDNF and MANF by lentiviral vector showed no beneficial effect on 6-OHDA rat model of PD; only the combined intranigral viral delivery of both factors was able to improve motor deficit and to lead to greater increase in striatal TH-positive fiber and significant protection of DA neurons in the SN (Cordero-Llana et al., 2015).

In addition to neurotrophic effects, recent studies on primary glial cell cultures and in 6-OHDA rat model of PD suggest that CDNF has an anti-inflammatory effect alleviating ER stress and reducing nitrosative stress and pro-inflammatory cytokines (Cheng et al., 2013; Nadella et al., 2014).

Frequently, pre-clinical studies on NTFs showed controversial results mainly due to different experimental settings and animal models. The dose of NTFs and the duration of the administration are important variables to take into account, and it was one of the main pitfalls for GDNF. Indeed, the sustained striatal overexpression of GDNF in lesioned animal has been associated with aberrant sprouting of the regenerating dopaminergic fibers and reduced DA synthesis, resulting in undesirable behavioral

effects (Georgievska et al., 2002; Sajadi et al., 2005). Similarly, also, the long-term effects of elevated BDNF levels should be considered: an earlier study documented hypodopaminergic phenotype with increased rotatory behavior and decreased TH expression after chronic intranigral delivery of BDNF (Lapchak et al., 1993).

Another important question is that, currently, there is no preclinical animal model of PD that fully demonstrates all of the features of the human disease and this justifies the variability of NTFs results. The neuroprotective and restorative capacity demonstrated by GDNF in neurotoxin-based models is flanked by the failure of GDNF application in α-syn rodent models. Intriguingly, GDNF had no effect on dopaminergic neuron survival and motor symptoms both in a wild-type and A30P mutant α-syn overexpression model of PD (Lo Bianco et al., 2004; Decressac et al., 2011). This failure has been attributed to the decreased level of transcription factor Nurr1, upstream regulator of GDNF receptor Ret whose downregulation block the response to this neurptrophic factor (Decressac et al., 2012). However, the direct correlation between accumulation of α-syn and Ret/Nurr1 downregulation has not yet been totally confirmed (Su et al., 2017).

THE HARD TRANSITION FROM PRE-CLINICAL STUDIES TO HUMAN TRIALS

Previous chapters have highlighted that multiple lines of evidence, derived from preclinical investigations, indicated anomalous expression of various CNS NTFs in neurodegenerative disease involving basal ganglia (Howells et al., 2000; Chauhan et al., 2001).

Theoretically, NFTs may take part to PD pathogenesis. On one hand, it has been hypothesized that the α-syn accumulation may influence the expression of GDNF and BDNF inducing the down-regulation of BDNF transcription and worsening of BDNF trafficking in neurons (Kawamoto et al., 1999, 2000; Yuan et al., 2010; Chu et al., 2012; Pramanik et al., 2017). On the other hand, and aside from the specific interplay between α-syn accumulation and endogenous NTFs, CDNF, and MANF are localized mainly to the lumen of ER and, hence, might play a role in ER stress, *via* the UPR signaling pathways, in neurodegenerative diseases. Both routes (α-syn and mitochondrial energy failure) indicate NFTs as important putative target for therapeutic modulation.

More specifically, since the late 1990s, different post-mortem studies demonstrated the reduction of BDNF protein in SNpc and striatal nuclei (caudate and putamen) of patients with PD (Mogi et al., 1999; Parain et al., 1999), as more recently confirmed by the results in the work of Nagatsu and Sawada (2007). Moreover, *in situ* hybridization data documented the significant reduction of BDNF mRNA in SNpc surviving dopaminergic neurons of patients with PD than their healthy subject counterparts, which express high BDNF mRNA levels (Howells et al., 2000).

The connection between decreased levels of BDNF and PD development has been further outlined *in vivo* by a (123) I-PE2I single-photon emission computer tomography study showing a positive correlation between serum BDNF levels and striatal DAT availability in patients with PD (Ziebell et al., 2012). In addition, GDNF was linked to the dopaminergic system efficiency in PD development. In fact, both striatal and nigral GDNF expression may be markedly decreased in PD. Chauhan et al. (2001) reported a significant reduction of GDNF in the SNpc of patients with PD, which was up to eight times greater than the reduction of other NTFs, thus suggesting that GDNF may be considered as the most vulnerable and earliest NTF to decrease in SNpc neurons that survived to neurodegeneration. Conflicting reports are also available; a lack of difference between healthy subjects and patients with PD, for instance, was documented in a post-mortem study (Mogi et al., 2001).

The translation of those evidence into clinical facts has been hampered by huge limitations. As pointed by Chmielarz and Saarma (2020) "Neurotrophic factor (NTF)–based therapies for PD hold great promise, yet they have so far failed to enter the clinic." Technical limitations, such as incomplete delivery protocols, and difficulties in selection of the optimal NTF regimen might explain unsatisfactory results with NTFs in PD clinical trials. Here, we summarize some results and pitfalls.

A Brief Recap on Recent Human Trials Follows

Clinical trials with GDNF date back to over two decades ago. Previously, experience with GDNF did provide inconclusive or, somehow, disappointing results. In particular, the intraventricular GDNF administration (monthly, for 8 months) did not reach any significant benefit, and side effects prevailed (Nutt et al., 2003). On the contrary, the direct delivery (intra-caudate, reminiscent of preclinical studies) appeared to either induce some modest clinical amelioration or PET-confirmed increased functioning of DA neurons (Gill et al., 2003; Love et al., 2005; Slevin et al., 2005, 2007). However, the latter were small open-label studies and the subsequent placebo-controlled one raised more uncertainties (including the development of neutralizing antibodies to GDNF, see Lang et al., 2006; Patel et al., 2013).

Recently, a randomized, double-blind, placebo-controlled trial focused on the intermittent intraputaminal GDNF administration in patients with motor fluctuating PD was conceived (Whone A. et al., 2019; Whone A. L. et al., 2019). This study reached intriguing but still inconclusive results, suggesting some large improvement in OFF state in a sub-cohort of patients with PD. Actually, an open-label phase 1 single-center trial is ongoing (NCT01621581), with safety and tolerability ambitions. It utilizes an adeno-associated virus serotype 2 vector (AAV2) containing human GDNF complementary DNA. In this study, bilateral catheters are placed surgically through the skull into the brain and the vector being delivered by convection-enhanced delivery (CED) to both putamina (450 microliters per hemisphere) of 24 patients with advanced PD. AAV2-GDNF vector represents, indeed, a rather ambitious effort, supposed to run for 5 years in patients with advanced PD, who are currently candidates to surgical DBS therapy; yet, it is not recruiting at

the moment, as far as we know. Hopefully, results will be by NINDS along 2022.

Neurturin and Cerebral Dopamine Neurotrophic Factor

Among the different NTFs tested in clinical trials for PD, three studies using the NRTN as gene therapy stand out. The first study, an open-label trial with intraputamental infusion of CERE-120 (adeno-associated virus serotype 2-neurturin), although it has not shown changing in PET markers, reported patients' motor improvement in unified Parkinson's disease rating scale (UPDRS) (Marks et al., 2008). However, these encouraging clinical results were not duplicated in the two subsequent double-blinded studies, where AAV2-NRTN was delivered into putamen (Marks et al., 2010) or simultaneously into both putamen and to SN (Bartus et al., 2013). Nonetheless, NRTN studies, which stratified patients by time from disease diagnosis, indicated some benefits related to in the earliest stage of PD (Marks et al., 2010; Olanow et al., 2015).

As for the studies on CDNF therapy for PD, a double-blind clinical phase I/II trial was started in autumn 2017 (Huttunen and Saarma, 2019). Of note, "CDNF is delivered by a convection-enhanced delivery system once a month for 6 months, which will be followed by an open-label extension period where all patients will be given CDNF." Results were expected in autumn 2020, but, to our knowledge, they are still missing. However, preliminary data indicate CDNF as a safe and hopeful tool to improve biological activity in PD with possible disease-modifying potentials.

An interesting lesson arises from the abovementioned AAV-NRTN injection studies, as far as post- mortem investigations became possible in two patients, who had received either injections to both putamen and SN or to putamen only, 8 or 10 years after the virus injection (Chu et al., 2020). In these patients, the lack of significant clinical benefit was related to the fact that NRTN expression was limited to 3–12% in the putamen and 9–40% in SN. However, this study proved a large increase of dopaminergic innervation in those putaminal and SN areas where NRTN was express, suggesting a long-term benefits of NRTN in PD. This datum highlights "the capability of NRTN to protect and restore the function of dopamine neurons over a span of almost a decade." Nevertheless, "saving dopamine neurons in the SN have not improved innervation of the putamen," as if the already occurred axonal degeneration or perturbed axonal trafficking did impede efficient rescue.

PUTATIVE MODERN STRATEGIES: A ROLE FOR FOCUSED ULTRASOUND?

It is extensively known that Magnetic Resonance–guided Focused Ultrasound (MRgFUS) is a versatile tool for clear-cut clinical indications. These clinical applications utilize the high-intensity focused ultrasound (HIFU) protocols. In terms of action mechanism, HIFU causes significant increase in temperature in a defined focal plane of the ultrasound beam, inducing protein denaturation, DNA breakdown, and eventually thermal coagulative necrosis, the latter representing the desired mechanism of action of FUS. In human tissues, therapeutic thermal ablation is achieved at temperatures above 54–56°C. Advances in skull aberration corrective techniques and phased-array transducers have led to the development of fully non-invasive therapeutic transcranial sonication. Combined with MR guidance for targeting, real-time monitoring, and thermal feedback, FUS can be applied through the intact skull and deliver energy to a few millimeters *anywhere* from the cortex to deep lying structure, at selected sites, to produce discrete therapeutic results.

We are collaborating with the dedicated team in L'Aquila, which has been applying the procedure, so far, to 67 essential tremor (ET) and 72 patients with PD with significant short-term and long-term reduction in tremor (**Figure 1**; Bruno et al., 2020a, 2021a).

As several other teams worldwide, they are acquiring experience around potential limitations (i.e., skull density ratio and skull volume in the treatment area, which limit the energy transfer throughout the skull and influence the temperature increase in the lesioning area; the number and maximal average temperature—Tmax-Avg—of sonication during treatment; and the accuracy of targeting, the optimal lesion volume), rare adverse events, and the degree of tremor re-occurrence during follow-up (Bruno et al., 2020b, 2021b; Tommasino et al., 2021; **Figure 2**).

Aside from the standard indication, which was to neutralize ventral intermediate nucleus and/or the dentatorubral tract and/or pallidothalamic tract to relieve drug-resistant ET, clinical lesioning FUS is currently examined in a variety of other potential indications, from exploring bilateral applications for ET, to revealing possible effectiveness in patients with extremely asymmetric PD, up to the chance, in rather patients with advanced PD, to promoting strategic micro-lesions of the GPi to contain dyskinesias that survived both pharmacological optimization and traditional DBS in STN.

Central to our commentary is the following question: well above the HIFU approach, might MRgFUS provide a tool capable to overcome the limitations so far experienced in terms of delivery of NFTs to CNS?

One of the pitfalls experienced so far, and briefly estimated in the previous chapter, concerns the poor penetration of the administered molecules. The majority of NTFs do not easily cross the BBB (i.e., consider the large size of the GDNF and NTFs homodimer structure). Besides, although different compounds are theoretically able to cross the BBB, the current routes of administration failed to achieve a consistent increase of neuronal BDNF expression or oligodendroglial GDNF in humans, thus hampering the translatable of experimental results into putative disease-modifying therapeutics. Experimental approaches included, i.e., GDNF encapsulated in microspheres composed of biodegradable polymer materials (Garbayo et al., 2009, 2011), DNA nanoparticle gene transfer to achieve long term GDNF expression (Fletcher et al., 2011), encapsulated fibroblasts transfected to produce GDNF and confer behavioral improvements in the 6-OHDA rat model of Parkinson's disease (Grandoso et al., 2007).

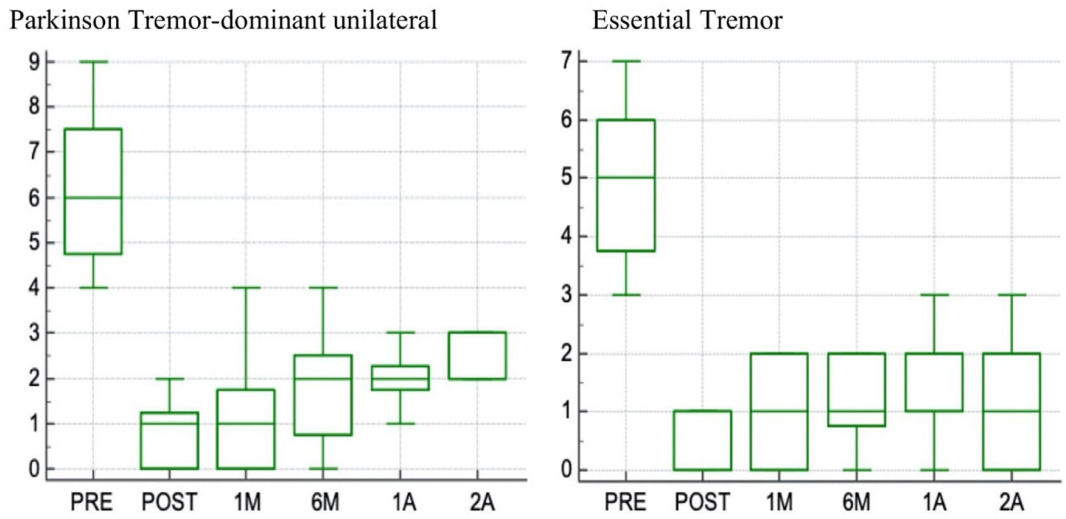

FIGURE 1 | Box plot of changes in scores at the Fahn-Tolosa-Marin (FTM) part A side treated (range 0–9) with 2-years follow up in patients with Parkinson tremor dominant unilateral and in patients with Essential tremor.

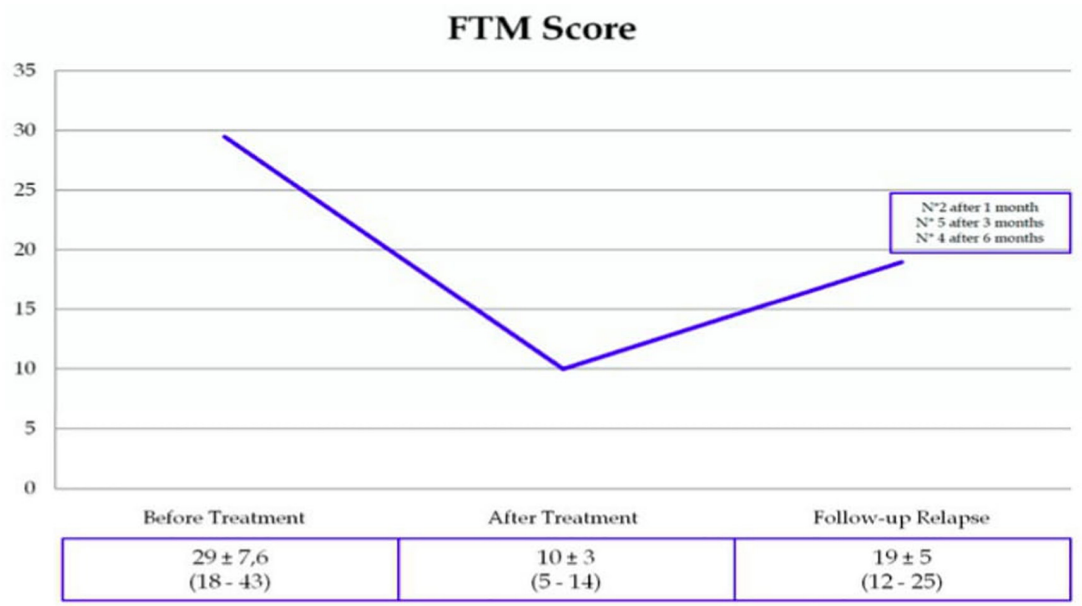

FIGURE 2 | Tremor Recurrence (13%), as estimated in a cohort of MRgFUS-treated ET patients (Sucapane, 1'Aquila).

Actually, the *chronic* reduced integrity of BBB might play a detrimental role in neurodegenerative disorders. Previous works from our department indeed emphasized to what extent an altered BBB may correlate with cognitive impairment in patients with advanced PD (Liguori et al., 2017). The issue, here, is instead dealing with what already acclaimed by experimental studies, showing consistently that the temporary and reversible opening through LIFU in conjunction with intravenously administered preformed microbubbles may be strategic.

Among the applications of the MRgFUS, the ability of this technique to transiently increase the BBB permeability, through the temporary and focalized (localized) opening of BBB tight junctions, should be emphasized. Further, the transient and reversible approach with MRgFUS, different from other pioneering methods proposed to improve CNS-drug delivery, such as receptor modifying nanoparticles, intranasal (IN) injections, and chemo-agent wafers (Gabathuler, 2010; Lochhead and Thorne, 2012; Bregy et al., 2013; Torres-Ortega et al., 2019) should not imply (or render negligible) the risk of iatrogenic injury or irreversibly toxicity. There are several experimental studies of opening the BBB on patients with brain tumors that show the safety and feasibility of the procedure (Lee et al., 2019;

Chen K.T. et al., 2021). In recent years, FUS has been applied for BBB opening for therapeutic purposes also in AD and other neurological diseases.

Actually, mechanisms underlying LIFU are quite complex, given that modulatory functions on neurons and glial cells, either neuroexcitatory (i.e., membrane depolarization) or neuroinhibitory depend upon on the ultrasound parameters, whose evaluation is well above the scopes of this manuscript. However, for our purposes (delivery of NTFs), it is, of note, that pulsed LIFU *at controlled energy* is important, since being capable to produce stable oscillation of microbubbles, which transiently opens the BBB by separating the endothelial tight junction, enhancing the localized delivery of therapeutic agents including antibodies, growth factors, nanoparticles, nucleic acid, viral vectors, and cells to the brain (Sheikov et al., 2004; Fishman and Frenkel, 2017; Lee et al., 2019). On the other hand, high energy might induce inertial cavitation and bubbles collapsing. Several studies focus on the enhancement of the acoustic pressure below a certain threshold to render the delivery procedure safe. A recent review clarifies the modalities of application at both end of the acoustic spectrum (Conti et al., 2021). In humans, MR guidance is the routine, although recent experience on two non-human primates have been designing "the feasibility of BBB opening, through a portable, robotic-assisted clinical system with a therapeutic transducer able to promote real-time cavitation monitoring (hence, provided the availability of neuronavigation system, eliminating the constant need of MRI)" (Pouliopoulos et al., 2020).

First applications, quite surprisingly, date back to the early 2000s when Hynynen et al. (2001) applied LIFU, instead of HIFU, in conjunction with intravenously administered preformed microbubbles for BBB opening.

At present, the big question here is to what extent FUS, effectively experienced in disease models, can definitively help the implanting of microparticles or transfected cells in the human brain. A brilliant review (Lapin et al., 2020) was recently confirming that "a method that overcomes those limitations is FUS in the presence of systemically circulating microbubbles" (Sheikov et al., 2004; Meijering et al., 2009; Chopra et al., 2010). Indeed, FUS has been conceived as a therapy-delivering approach for malignancies, neurodegenerative diseases, and movement disorders (Lin et al., 2015, 2016; Szablowski et al., 2018; Chen K.T. et al., 2019). Albeit the transition from laboratory to daily clinical practice remains hard, pioneering investigations are emerging. In Alzheimer's rodent's models, it was shown that FUS-induced BBB permeability represents a critical requirement to deliver a significant amount of intravenous immunoglobulin (489 ng/mg) to the targeted hippocampus of TgCRND8 mice (Dubey et al., 2020); see also the MRgFUS-mediated neurogenesis and improved cognition, promoted by the allowed delivery of a TrKA agonist in the TgCRND8 model of AD (Xhima et al., 2021). In humans, an ongoing protocol is fascinating. Rezai et al. (2020) lately presented the "initial clinical trial results evaluating the safety, feasibility, and reversibility of BBB opening with FUS treatment of the hippocampus and entorhinal cortex (EC) in patients with early AD." Post-FUS in contrast to MRI revealed immediate and sizable hippocampal parenchymal enhancement indicating BBB opening, followed by BBB closure within 24 h, hence favoring FUS as a safe, non-invasive, transient, reproducible, and focal mediator of BBB opening in the hippocampus/EC in humans."

Next paragraph will focus on the current evidence for the efficacy of FUS approach in experimental PD models (as premise for translational applications).

FOCUSED ULTRASOUND-ASSISTED APPROACH FOR DELIVERING NTFS IN PARKINSON'S DISEASE MODELS

As previously reported, FUS in conjunction with intravenously injected microbubbles induce a safe, non-invasive, and reversible enhancement of BBB permeability. Since 2012, the key parameters in determining drug delivery across the BBB (namely, time window and molecular size) were estimated with mathematical models (Marty et al., 2012). The therapeutic application of BBB opening by FUS for the delivery of NTFs has proved, indeed, efficacious in different experimental PD models (Karakatsani et al., 2019a).

The potential of FUS method to deliver neuroprotective agents had received a seminal support by Samiotaki et al. (2015), whose experiments showed an efficient NRTN bioavailability in both the SN and CP through the optimization of acoustic parameters. Moreover, the authors demonstrated the activation of NRTN signaling pathway, i.e., intracytoplasmic increase of Erk1/2 phosphorilation and the downstream transcription factor cAMP response element-binding protein (CREB), playing a well-known role inside the nigrostriatal circuitry.

Another report that addressed BBB opening and neurotrophin delivery in a PD model combined FUS and transvascular non-viral gene delivery system (microbubbles conjugated with GDNF-plasmid) (Fan et al., 2016). This strategy showed a meaningful recovery of DA concentration and restoration of behavioral function in a standard 6-OHDA–lesioned PD model (Fan et al., 2016). Interestingly, Karakatsani et al. (2019b) investigated a "more discrete" PD model in mice, obtained following subacute MPTP injections (leading to an apoptotic-like, partially reversible degeneration, reminiscent of an "early" PD). In this model, it was shown that only the combination of FUS and intravenous NFT delivery (NRTN) increased unilateral TH staining in SN; further, only repeated FUS/NRTN exposures were able to recover the TH fibers density in caudate putamen (CPu), hence affecting DA release, as quantified by HPLC-determined levels of major metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in midbrain and striatum (Karakatsani et al., 2019b).

A clever synergistic use of FUS and gene-carrying liposome to perform non-invasive GDNF delivery was proposed in two different PD models, respectively, MPTP-treated mice (Lin et al., 2016) and 6-OHDA–lesioned rats (Yue et al., 2018a,b). In this case, MRI-guided singleFUS treatment of 6-OHDAlesioned striatum, combined with brain-penetrating polymeric nanoparticles, induced relevant GDNF level increase in the target region, lasting up to 10 weeks after

treatment, and a prolonged improvement in locomotor function (Mead et al., 2017).

Last but not least, the IN route has opened new avenues: the first demonstration dates back to 2016, when fluorescence imaging of brain slices found that IN administration of BDNF followed by FUS sonication achieved a significant enhancement of BDNF distribution in FUS-treated CPu compared with the corresponding controls (Chen et al., 2016). These preliminary data were extensively applied in Ji et al. (2019) to an early stage PD mouse model in which the up-regulation in TH expression and the corresponding behavioral changes indicated that applied FUS-mediated-BBB opening improved the delivery of IN BDNF into the target regions.

So far, a rapid translational road is still uncertain. Whether analogous approach would be efficacious for patients with advanced PD is matter of present research (Foffani et al., 2019; Fishman and Fischell, 2021). It will be critical to minimize possible adverse events and concerns, as brilliantly summarized by Todd et al. (2020).

CONCLUSION

- Bioavailability of NTFs in the target tissue remains a major challenge for NTF-based therapies; yet, contradictory results should not discourage our efforts.
- The difficulty to solve NTFs delivery (Marty et al., 2012; Conti et al., 2019) was partially overcome by revisiting FUS approach through multimodal protocols.
- MRgFUS, through dedicated approach designed to transiently modify the BBB permeability, may indeed be considered a safe method of relatively simple implementation, and, likely, effective when compared to the surgical procedures, which administered NTFs in specific intracerebral target nuclei by stereotactic delivery, either directly (Patel et al., 2005; Lang et al., 2006) or *via* viral vectors (Bartus et al., 2013; Olanow et al., 2015). Consistent experience with FUS-facilitated delivery of neurotrophic agents, either assisted by MR guidance or not, accumulated in experimental PD models and has paved the road for ongoing and up-coming clinical trials.
- However, in many respects, combined strategies should not be excluded. Functional neurosurgery is undergoing a fascinating renovation (Stefani et al., 2017; Priori et al., 2021), and multi-disciplinary teams are welcome. In this content, some experimental studies utilize synergistic approach: a Finnish group, for instance (Huotarinen et al., 2018), detected that "CDNF delivery improved the effect of acute STN DBS on front limb use asymmetry at 2 and 3 weeks after CDNF injection."

The ambition of promoting neuro-rescue, in PD and other movement disorders, so far unmet need, will require an abundant dose of experimental courage and clinical wisdom.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

This manuscript received contribution from Bric 2019 to AS.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC declared a shared affiliation, with several of the authors AS, MP, LS, RC, MC, and SC to the handling editor at the time of the review.

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frontiers | Frontiers in Pharmacology

PERSPECTIVE
published: 14 April 2022
doi: 10.3389/fphar.2022.786475

Exploiting Focused Ultrasound to Aid Intranasal Drug Delivery for Brain Therapy

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OPEN ACCESS

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 30 September 2021
Accepted: 07 March 2022
Published: 14 April 2022

Citation:
Barbato G, Nisticò R and Triaca V (2022) Exploiting Focused Ultrasound to Aid Intranasal Drug Delivery for Brain Therapy. Front. Pharmacol. 13:786475. doi: 10.3389/fphar.2022.786475

Novel effective therapeutic strategies are needed to treat brain neurodegenerative diseases and to improve the quality of life of patients affected by Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic Lateral sclerosis (ALS) as well as other brain conditions. At present no effective treatment options are available; current therapeutics for neurodegenerative diseases (NDs) improve cognitive symptoms only transiently and in a minor number of patients. Further, most of the amyloid-based phase III clinical trials recently failed in AD, in spite of promising preclinical and phase I-II clinical trials, further pinpointing the need for a better knowledge of the early mechanisms of disease as well as of more effective routes of drug administration. In fact, beyond common pathological events and molecular substrates, each of these diseases preferentially affect defined subpopulations of neurons in specific neuronal circuits (selective neuronal vulnerability), leading to the typical age-related clinical profile. In this perspective, key to successful drug discovery is a robust and reproducible biological validation of potential new molecular targets together with a concomitant set up of protocols/tools for efficient and targeted brain delivery to a specific area of interest. Here we propose and discuss Focused UltraSound aided drug administration as a specific and novel technical approach to achieve optimal concentration of the drug at the target area of interest. We will focus on drug delivery to the brain through the nasal route coupled to FUS as a promising approach to achieve neuroprotection and rescue of cognitive decline in several NDs.

Keywords: neurodegenerative diseases, brain circuit vulnerability, drug delivery, focused ultrasound, clinical trials

Abbreviations: AD, Alzheimer's Disease; ALS, Amyotrophic lateral sclerosis; BBB, Blood brain barrier; BDNF, Brain-derived neurotrophic factor; CPu, caudate putamen; CT, computer tomography; ET, essential tremor; EMA, european medicine agency; FDA, Food and Drug Administration; FUS, focused ultrasound; HP, hippocampus; HD, Huntington Disease; MB, microbubbles; ND, neurodegenerative diseases; MCI, Mild Cognitive Impairment; PD, Parkinson's Disease; NGF, Nerve growth factor; SN, substantia nigra.

INTRODUCTION

Alzheimer's disease (AD) and other neurodegenerative diseases (NDs) affect over 50 million people worldwide according to World Health Organization (WHO), with nearly 10 million new cases per year, projected to reach 82 million in 2030 and 152 in 2050 because of a rise in life expectancy to 60 years aged population (World Health Organization, 2017). A number of initiatives to prioritise dementia in the European policy agenda have been established by EU (https://www.alzheimer-europe.org/policy/eu-action) and WHO to facilitate the discovery of disease-modifying treatments through the "*Global Action Plan on the Public Health Response to Dementia 2017–2025*" (World Health Organization, 2017) and its recent implementations, including the recently published Report on Global status (WHO, ISBN: 978-92-4-003324-5).

At present, available treatment options have limited efficacy. Most current therapeutics for AD only transiently improve cognitive symptoms in a minor number of patients (Mauricio et al., 2019). At best, they provide limited cognitive benefit in approximately 40% of people living with dementia, and they have no impact on the underlying disease process or the rate of cognitive decline. While development of symptomatic treatments has slowed, the search for dementia-preventing or dementia-modifying treatments has increased significantly. Very recently, the Food and Drug Administration (FDA) granted accelerated approval for Aduhelm (aducanumab), a human monoclonal antibody that selectively targets aggregated amyloid beta (Aβ). Being a disease modifying therapy, aducanumab holds a great potential for clinical benefit over current symptomatic therapies, however its approval -largely criticized- has been based on the reduction of a surrogate marker (amyloid beta) with questionable data on clinical efficacy (Nisticò and Borg, 2021). Moreover, it is under scrutiny for side effects, including amyloid related imaging abnormalities (ARIA) and brain haemorrhage. In line with this, the European Medicines Agency (EMA) has recently recommended the refusal of the marketing authorisation for Aduhelm (https://www.ema.europa.eu/en/medicines/human/summaries-opinion/aduhelm; EMA 750220/2021).

A plethora of other innovative therapeutic approaches are emerging, with the identification of novel mechanisms as potential drug targets. Indeed, robust and reproducible biological validation of putative new molecular targets is key to successful drug discovery. To date, the success rate for the development of disease-modifying drugs for NDs has been disappointing, like the failure of beta secretase inhibitors or monoclonal antibodies targeting amyloid beta in AD clinical trials (Salloway et al., 2021). This applies also to new drugs directed toward tau including those reducing tau hyper-phosphorylation, tau accumulation or preventing the spread of toxic tau species (Imbimbo et al., 2021).

Here we discuss the main features of neuronal subpopulations and circuits most vulnerable to neurodegenerative insults, and how optimal target engagement is critical for ensuing treatment efficacy. In this frame, we will highlight the importance of the non-invasive intranasal route for brain drug delivery coupled with Focused UltraSound (FUS).

Previous preclinical studies and pilot trials have shown that the intranasal administration of NGF (Tuszynski et al., 2015) and insulin (Craft et al., 2012) to mild cognitive impairment (MCI) and early AD patients was safe and resulted in rapid improvement of cognition, even within 30 min upon nasal sniffing. Noteworthy, and in spite of very promising preclinical data, phase 3 clinical trials investigating NGF and insulin failed, possibly because of the poor target engagement attributed to the implanted device used for continuous delivery of the drugs (Castle et al., 2020; Craft et al., 2020). Comparable promising results in terms of synaptic functions recovery have been obtained by direct BDNF infusion into the enthorinal cortex of animal models of pathology (Nagahara et al., 2013), and this technique has been used also in non-human primates (Nagahara et al., 2018). An open label phase1 clinical trial based on Adeno-Associated Virus (AAV)-Based, Vector-Mediated Delivery of Human Brain Derived Neurotrophic Factor (AAV2-BDNF) in subjects with early AD and MCI has been started by the Tuszynski group last year.

Surely, it is demanding to conceptually reconsider the ND field and take advantage of emerging technical opportunities. Efforts in finding new effective drugs slowing down or halting NDs progression should be coupled with efforts addressed to efficient drug delivery systems. The effectiveness of these methods would be strictly dependent on the administration route and on their intrinsic ability to target organs and tissues in a suitable amount and at the right time. In this perspective, we will describe FUS as a novel non-invasive technical paradigm to allow focused drug delivery to precisely target circuits, with the final aim to reach the optimal drug amount in a specific target area and thus improve the outcome in preclinical and clinical trials.

IMPROVING DRUG DELIVERY AND TARGET ENGAGEMENT IN NDS: THE PROMISE OF FUS

Drug Delivery Approaches

Different strategies of drug delivery to the brain have been reviewed recently (Wang et al., 2019; Lee and Leong, 2020). The last decade has seen an enormous research effort spent to develop BBB penetration methods using biochemical or physical stimuli, that have also aided in effective preclinical screening of brain targeting therapeutics and external stimulation, among those the application of Magnetic Resonance guided Focused Ultrasound (MRgFUS) is gaining momentum (Leinenga et al., 2016).

Technology, in fact, is in place supporting feasibility of such interventions in humans, and devices using Therapeutic Ultrasound (TU, at high frequency - 620 kHz–1.0 MHz- and intensity) in non-invasive brain surgical ultrasound treatment of NDs were approved by regulatory organisms (FDA and CE mark), and are considered emerging treatment in essential tremor, PD, neuropathic pain and ablation of brain tumours (Leinenga et al., 2016). The same technology, albeit operating at a different frequency (low frequency i.e., 220 kHz, and

TABLE 1 | Current status (August 2021) of Clinical Trials on BBB opening. AD: Alzheimer's Disease; ALS: Amyotrophic Lateral Sclerosis; PD: Parkinson's Disease.

	ClinicalTrials.gov IDENTIFIER	Cluster	Objective	Condition	Phase	Patients	Status
1	NCT03119961	1	feasibility, safety of BBB opening in AD patients	AD	I/II	10	completed
2	NCT02986932	1	feasibility, safety of BBB opening with IV administration of US contrast agents in AD patients	AD	I/II	6	completed
3	NCT04118764	1	feasibility, safety of BBB opening with IV administration of US contrast agents in AD patients using US guided neuronavigation guidance	AD	I	6	recruiting
4	NCT03671889	1	feasibility, safety of BBB opening in AD patients	AD	I	20	recruiting
5	NCT04526262	1	feasibility, safety of BBB opening in AD patients	AD	I	6	active
6	NCT03321487	1	feasibility, safety of BBB opening in ALS patients	ALS	I	8	active
7	NCT03626896	1	evaluation of safety and find the tolerated ultrasound dose of transient opening of the blood-brain barrier (BBB)	r-Glioblastoma	I	6	completed
8	NCT03712293	1	evaluation safety and feasibility of BBB disruption along the periphery of tumor resection cavity	Glioblastoma	I	20	recruiting
9	NCT03322813	1	Evaluate the Safety and Feasibility of Temporary Blood-Brain Barrier Disruption (BBBD) in Patients With Suspected Infiltrating Glioma	Glioma	I	15	active
10	NCT03739905	2	feasibility, safety and efficacy of repeated, BBB opening in AD patients	AD	IIa	30	recruiting
11	NCT03608553	2	feasibility, safety and efficacy of repeated, BBB opening in PD patients	PD	I	10	active
12	NCT04370665	3	safety and feasibility of three biweekly delivery of Cerezyme® via BBB opening	PD	I	4	active
13	NCT04528680	3	evaluation of Abraxane® drug crossing of BBB, at increasing doses: dose limitin toxicity and 1-yr survival rate	r-Glioblastoma / Gliosarcoma	I / II	39	recruiting
14	NCT04614493	3	evaluation of Temozolomide drug crossing BBB and efficacy in Glioblastoma patients	Glioblastoma	II	66	recruiting
15	NCT02343991	3	evaluation of Doxorubicin drug crossing BBB and accumulation in brain tumor	brain tumor	I	10	active
16	NCT03616860	3	evaluation of safety of BBB disruption in patients following surgical resection and chemo-radiation with temozolomide (TMZ) protocol	Glioblastoma	I	20	recruiting
17	NCT04998864	3	evaluation of safety and feasibility of BBB disruption in high grade glioma patients under standard of care therapy	Glioma	I	5	recruiting
18	NCT03551249	3	evaluation of safety and feasibility of BBB disruption in high grade glioma patients under standard of care therapy	Glioma	I	20	recruiting
19	NCT03744026	3	evaluate dose limiting toxicity (DLT) of escalating n. of ultrasound beams at constant acoustic pressure and standard escalation (Phase I) safety and efficacy of BBB opening	r-Glioblastoma	IIa	33	active

TABLE 2 | Acoustic pressure (MPa) and Frequency (MHz) dynamic ranges for the focused ultrasound application in current clinical settings for sonothrombolysis, ablative surgery (Essential tremors, PD, and tumour ablation), neurostimulation, Imaging, and obicodilation (BBB opening; bold evidenced).

	Sonothrombolysis	Neuromodulation	Obicodilation (BBB opening)	Imaging	Ablative surgery
Acoustic Pressure (MPa)	0.009–0.300	0.02–0.25	**0.08–4.00**	0.1–10.0	1.5–38.0
Frequency (MHz)	0.25–1.15	0.055–1.000	**0.12–1.00**	0.85–18.00	0.09–1.05

intensity), is used to allow BBB tailored opening, also known as obicodilation (McDannold et al., 2012). While UT surgery has already reached clinical acceptance status as valid alternative treatment in several CNS (ET, PD) as well as other diseases (uterine fibroma, prostatic cancer, osteoid osteoma, bone tumour), the use of MRgFUS BBB disruption to favour alternative drug administration routes is undergoing a very intense clinical trial activity, summarized in **Table 1**, for NDs (AD, PD, ALS), and for brain tumours as well.

FUS Technique

The acoustic pressure delivered locally is the key factor distinguishing the different US-based interventional methodologies: the operative ranges for acoustic pressure and frequencies used in different US therapeutic field applications are schematized in **Table 2** (Jun, 2012).

The use of circulating microbubbles (MB)—i.e., clinically approved contrast agents used for ultrasound imaging - combined with low intensity MRgFUS is an emerging

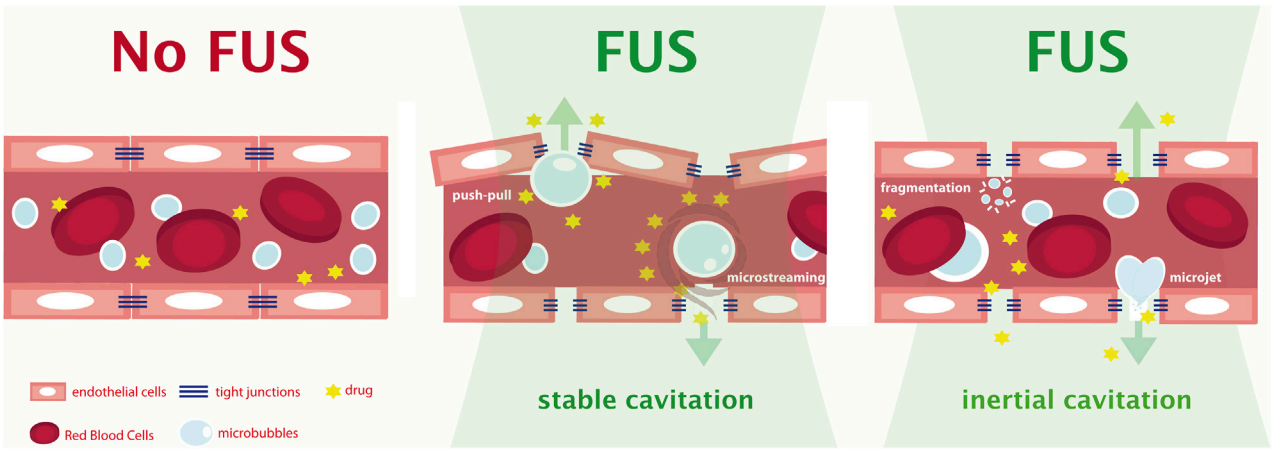

FIGURE 1 | Schematic representation of terminal capillaries targeted by the FUS stream. In the absence of FUS-induced cavitation, an intact BBB of a living animal (No FUS) with flowing red blood cells and peripherally (tail vein) injected microbubbles is depicted. Upon FUS stimulation and depending on the specific combination of acoustic pressure and frequency applied, a controlled stable cavitation with a balanced microbubbles expansion and compression or an inertial cavitation with a wide range of microbubbles diameters may be achieved by the operator at specific brain point locations. Both mechanisms leading to stretching of tight junctions in the endothelial cells of the final capillary allowing a 15′-12 h window of BBB opening and drugs delivery to adjacent brain parenchyma. In case of stable cavitation conditions microbubbles induce the opening of the capillary walls mainly through push-and-pull mechanisms and/or local microstreaming of the blood flow. If inertial cavitation takes place, microbubbles become unstable and collapse emitting high energy microjets or more rarely explode (fragmentation), in both cases facilitating the drug passage through the BBB, however often resulting in local inflammation/oxidative insult.

technology which allows to perform a controllable obicodilation (Konofagou et al., 2012; Leinenga et al., 2016; Wu et al., 2020). The mechanism by which BBB disruption takes place is still not entirely elucidated. Capillary diameter varies in the range of 4–10 μm and commonly used MB sizes are in the range 1–5 μm (SonoVue®, Optison®, Definity®, Sonazoid® etc.), thus cyclic repetition of ultrasound bursts inducing MB expansion and contraction, are thought to generate mechanical stress forces disrupting the integrity of BBB tight junctions (Tung et al., 2010; Hosseinkhah and Hynynen, 2012). The focused US beam may induce two types of cavitation phenomena on MB. Stable cavitation resulting from lower/intermediate acoustic pressures promotes a periodic gas decompression/compression within the microbubble inducing their expansion/contraction cycle regularly (**Figure 1**). In the expansion phase stretching of the vessel may lead to transient opening of the tight-junctions between cells (Dasgupta et al., 2016), while in the contraction phase micro-streams may be produced developing shear stress on the vascular endothelial cells increasing endocytosis (van Bavel, 2007). MB stable cavitation also activates radiation forces pressing and pushing on endothelia and inducing effects leading to increased passive permeability (Dasgupta et al., 2016), **Figure 1**. The mechanism encompasses a series of complex phenomena including the mechanical disruption of the tight junctions (Sheikov et al., 2004), a reduction of the expression levels of ZO-1, claudin 5 and occludin (Sheikov et al., 2008); an increased number of transcytotic vesicles and increased permeability of cell plasma membrane (Sheikov et al., 2006) and a decrease in drug efflux mechanisms (Cho et al., 2011; Aryal et al., 2017; Choi et al., 2019).

Higher acoustic pressures will lead to inertial cavitation, MB collapse, and consequent microjet formation propagating higher energy shock waves which directed upon endothelia would result in local micro-damages, **Figure 1**, increasing its permeability at the expense of enormous increased risk of adverse events, i.e. bleeding. Very recently, inertial cavitation finely tuned control is being explored in pre-clinical applications of histotripsy using high-energy very short US pulses also for brain treatment (Lu et al., 2022).

In a series of pioneering studies, the cavitation was reported to produce obicodilation acting on gas particles dissolved in blood. The high energy high frequency focused ultrasound (HIFU) used, however, produced hemorrhage and tissue damage (Vykhodtseva et al., 1995). The introduction of pre-formed MB of the average size 1–5 μm marked an advancement in the field, lowering the amount of energy needed to induce the cavitation allowing to use low energy low frequency Focused Ultrasound (Hynynen et al., 2001; Burgess et al., 2012). Comparing similar amount of BBB disruption obtained applying different US frequencies (0.26–2.04 MHz) to pre-formed MB, resulted in decreased extravasation at the lower frequencies 0.26 MHz, since at higher frequency the threshold of acoustic pressure needed to induce obicodilation is reaching the range needed to engage MB in the inertial cavitation phenomena (Apfel et al., 1991; McDannold et al., 2008).

The dimension of the openings and the recovery time to reseal depend on several factors, the most relevant being the used FUS parameters (McDannold et al., 2008; Baseri et al., 2010 and, 2012; Chopra et al., 2010; Choi et al., 2011), MB type, size and dose (Choi et al., 2010; Tung et al., 2011; McMahon and Hynyen. 2017; Ohta et al., 2020), effective acoustic pressure (Chopra et al., 2010; Samiotaki et al., 2011). Efficiency of BBB opening is strictly related with acoustic pressure and pulse duration, however these parameters are the sensitive ones that relate also with

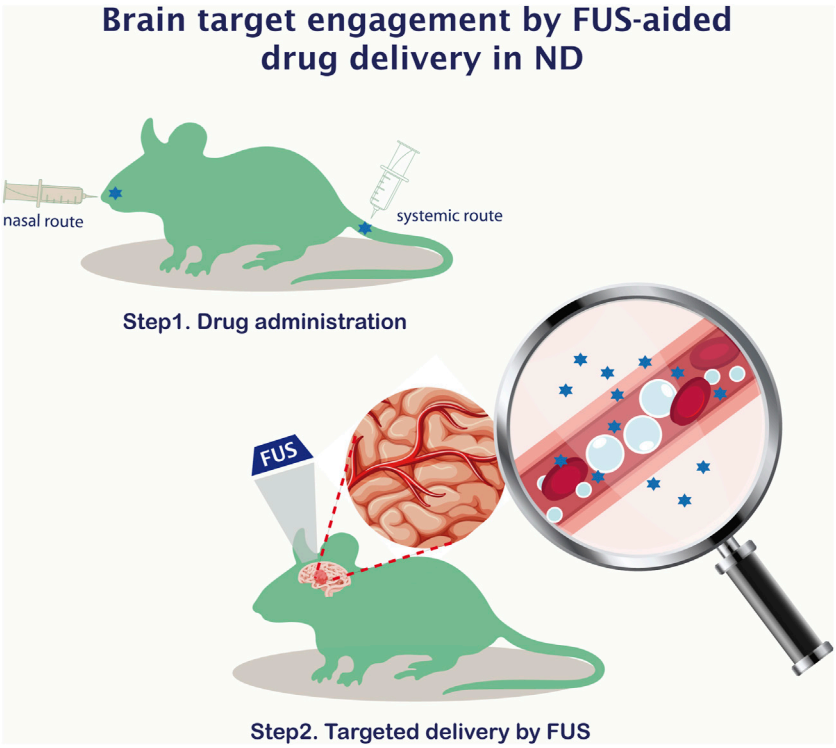

FIGURE 2 | Brain target engagement by FUS-aided delivery of drugs in NDs therapy. A two steps experimental paradigm with the intranasal or systemic administration of the drug of choice, followed by FUS-driven local brain stimulation allowing non-invasive, targeted, and transient opening of the BBB at the region of interest for therapeutic purposes.

tissue damage in the sonicated region, while MB type, size and dose, together with the pulse repetition frequency (PRF) have been shown to impact the BBB opening without relevant tissue damage after sonication (Shin et al., 2018).

A series of preclinical studies on animal models have shed light on the most relevant physical parameters interfering with the transcranial FUS applications, i.e., the influence of the skull interference has been assessed first in non-human primates (macaques) (McDannold et al., 2008; Arvanitis et al., 2012), and recently in humans (Schwartz et al., 2018; Wang et al., 2018).

As the number of PD and ET patients undergoing to MRgFUS ablative application increased, it was noticed that there were cases were the treatments failed to reach the temperature sufficient to cause a lesion. The analysis of these cases led to the formulation of the concept of Skull Density Ratio (SDR) (Chang et al., 2015). Optimal FUS high intensity energy parameters for patients were since determined taking into account predetermined skull local thickness and SDR ratios, and efficacy of the ablative procedures increased. However, obicodilation is conducted using FUS low intensity energy, and there's a difference between the attenuation produced when the US wave crosses through the skull if it is a high or a low energy wave. A more recent study based on clinical data available from the obicodilation procedures on human clinical trials has advanced the hypothesis that the trabecular bone ratio showed a significantly greater correlation with dose/delivered energy than that of thickness and the SDR (Kong et al., 2021).

Currently there are three clinical devices approved for brain use in humans: SonoCloud® (CarThera, France), NaviFus® (NaviFUS Corp., Taiwan) and Exablate 4000 (InSightec Ltd., Israel). The first device avoids skull attenuation by positioning the transducer directly in contact on the dural surface, although at the expense of a small craniotomy (Sonabend and Stupp, 2019). The latter two instruments take advantage of the introduction of the phased array transducer technology which has been recently reviewed in depth (Hynynen and Jones, 2016).

Here we will briefly outline the most relevant advantages of the technology: i) it allows transcranial FUS without need of any skull *tomia*, ii) the beam can be "steered" meaning its focal center moved and axis rotated, to different locations in the field space by adjusting the time at which each array element emits the driving signal (i.e., phase shift), iii) wave front aberrations induced by heterogeneous tissue layers crossed can be minimized, iv) using the amplitude and phase of the signals can modify the focus shape or even generate simultaneous multiple foci in different locations. Phased array transducers allow a finer degree of precision positioning the ultrasound focus since, once fixed the mechanical translational positioning of the focal point, can further fine-adjust its positioning with the single elements phase modulation without further mechanical adjustment. Such a possibility is not available on single elements focused transducers.

Transcranial FUS coupled with the administration of micro-bubbles is proposed as the only non-invasive technique to

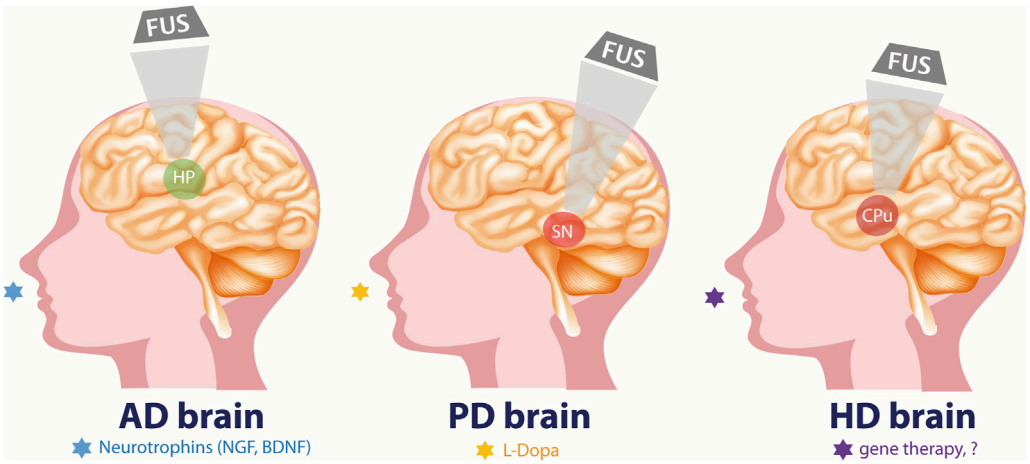

FIGURE 3 | FUS-aided, non-invasive brain delivery of novel or repurposed drugs, as possible therapeutic application in three devastating neurodegenerative diseases of the human central nervous system, namely AD, PD and HD. Specific target regions are identified and selectively reached for FUS-aided drug delivery in neuroprotective/therapeutic approaches: hippocampus (HP) for AD, Substantia nigra (SN) for PD, and Caudate Putamen (CPu) for HD. NGF and BDNF in AD, L-Dopa or dopaminergic drugs in PD, and gene therapy or currently unknown molecules are proposed for intranasal FUS-aided therapy.

transiently, locally, and reversibly disrupt the BBB, allowing a temporal and spatial window for molecules to cross to the brain parenchyma both in preclinical animal set-ups (**Figure 2**) and in patients (Choi et al., 2007; **Figure 3**).

FUS in Brain Delivery

A large body of pre-clinical evidence (recently reviewed by Pandit et al., 2020) has been accumulated in the last decade demonstrating that FUS-mediated BBB opening was able to facilitate delivery of a wide variety of therapeutic agents: conventional chemotherapeutics (doxorubicine, methotrexate, temozolomide), mAbs (trastuzumab, bevacizumab), gene therapy vectors, nanoparticles delivering therapeutics for brain tumour treatments, and even whole immune cells (e.g., natural killer cells).

On the side of NDs, the FUS mediated opening of the BBB has been used to explore innovative therapies for AD (Raymond et al., 2008; Jordão et al., 2010; Leinenga and Gotz, 2015, Nisbet et al., 2017; Janowicz et al., 2019), PD (Kinoshita et al., 2006; Lin et al., 2016; Chen et al., 2016; Mead et al., 2017; Yue et al., 2018a,Yue et al., 2018b; Karakatsani et al., 2019), Huntington (Burgess et al., 2012). In august 2021 a survey of the Clinical Trials Database (https://www.clinicaltrials.gov) resulted in a total of 19 studies (**Table 1**), of which 3 with a completed status (Lipsman et al., 2018; Abrahao et al., 2019; Mainprize et al., 2019), 7 active and nine recruiting. The total number of patients involved so far are 334, for 22 patients the trial has been completed, and for 86 is currently on-going. The above-mentioned completed phase 1 CT has provided the first evidence that this technology is safe in humans under the conditions used. With the increase in the number of clinical studies and recruited patients, the relevance of the secondary effects of the BBB disruption has become a focus of attention. Recent publications have reviewed safety of the transcranial procedure (Pasquinelli et al., 2019) and a first rational overview of the casual effects to brain physiology after BBB disruption (Todd et al., 2020). The former pointed towards a favorable safety profile, while the latter underlined a generalized inflammatory response as the most notable effects, a reduction of both amyloid β plaques and hyper-phosphorylated tau proteins, altered brain transcriptome and proteome profiles, and cerebral blood flow, and finally a transient suppression of neuronal activity. Possible clearance of metabolic waste products through the cerebral spinal fluid (CSF) need further investigations. Notably, the effects reported in the CT are transient with a described time span of few days.

Patients recruited in those studies generally fulfilled the criteria for mild to moderate AD (Lipsman et al., 2018; Meng et al., 2019; Beisteiner and Lozano, 2020), one study targeted early AD patients (Rezai et al., 2020), another patient with PD (Nicodemus et al., 2019) or PD dementia (Gasca-Salas et al., 2021). Although increased BBB permeability was always detected in the targeted regions along with sparse evidence of reduction in Aβ deposition (Park et al., 2021), the clinically meaningful impact of such changes is still questionable (reviewed by Liu et al., 2021) and a sensitive target subpopulation remains elusive.

FOCUSING TARGETS FOR NEURODEGENERATIVE DISEASES

In order to be effective, treatments should target early stages of disease. Currently, we lack conceptual frameworks to identify validated biomarkers relevant to disease progression. A deeper knowledge of genetic and environmental selective neuronal vulnerability/resilience is key to discover novel drug targets, appropriate subjects' selection, and to assess drug-target engagement in clinical trials. Neurodegenerative diseases (NDs) develop over years of progressive metabolic imbalance, synaptic dysfunction and subclinical pathology. Common molecular events include accumulation of a particular misfolded protein, neuronal

dysmetabolism, inflammation, oxidative and mitochondrial stress, ultimately leading to neuronal death (Haass and Selkoe, 2007; Frost and Diamond, 2010) and contributing to functional deficits and loss of cognition (Björkqvist et al., 2009).

Although the above-mentioned similarities, NDs differ in their prevalence, age at onset, and clinical characteristics, and particularly in the vulnerability of the neuronal circuits involved (Fu et al., 2018). For instance, high energy demanding and/or high firing neurons, like hippocampal or basal forebrain cholinergic neurons are well-known to be more susceptible to dysmetabolic events and cell stressors. Further, anatomical properties, like the presence of a long extending axon, confer to neurons more vulnerability to chronic stressors, including excess oxidative conditions (Saxena and Caroni, 2011). Activation of extra-synaptic NMDA receptors increasing circuit excitability as a maladaptive response to early injury, possibly boosting neurodegeneration, represents another mechanism of circuit-driven cognitive demise in NDs (Parsons and Raymond, 2014). Also, genetic and epigenetic heterogeneity introduces further interindividual variation. Moreover, environmental insults like brain trauma are potent triggers of neurodegeneration able to turn the initial pathology into a chronic condition (Mendez, 2017; Jamjoom et al., 2021).

Selectively Vulnerable Brain Circuits

Alzheimer's Disease. AD is the most common neurodegenerative disease, and the most common cause of late-onset dementia (Roussarie et al., 2020). Initial metabolic derangement followed by overt neurodegeneration has been demonstrated to occur in cholinergic circuits innervating frontal cortex and hippocampus, underpinning learning and memory deficits typical of Mild Cognitive Impairment (MCI) and AD (Schliebs and Arendt, 2011; Hampel et al., 2018). Typical AD pathology includes extracellular plaques of amyloid β, and hyper-phosphorylated tau enriched neurofibrillary tangles (Janelidze et al., 2020). Presenilin 1 and 2 mutations characterize early onset familiar AD (Mathews et al., 2000). Amyloid targeting therapy by Adulcanumab has been recently approved by FDA, although controversial for the side effects. No other treatment has been proven to be helpful in halting or delaying the pathology so far.

Parkinson's Disease. PD, the second most common neurodegenerative disease, is a movement disorder, associated to mutations in α-synuclein, LRRK2, parkin, and PINK1, and selectively affecting the substantia nigra dopaminergic (DA) neurons (Surmeier et al., 2017; McGregor and Nelson, 2019). Accordingly, dopaminergic drugs and, in particular, levodopa are current gold standards in PD treatment, although they come with significant side effects. dyskinesia in early onset PD, wearing-off effect, on-off effect, mental symptoms, frozen gait, and last but not least, the irritation and/or other issues at the pump injection site have been reported following chronic levodopa treatment (Vasta et al., 2017).

Huntington's Disease. HD is a fatal genetic disorder affecting muscle coordination and cognition, caused by CAG expansions in the Huntingtin gene and typically involving Huntingtin-enriched inclusion bodies. Striatal medium spiny neurons are selectively vulnerable to HD, resulting in cognitive disabilities early in the disease course, and later progressing to dementia (Rikani et al., 2014; Ruiz-Calvo et al., 2018). No cure is available, and current treatments are mainly symptomatic, including FDA approved tetrabenazine for chorea, antipsychotic drugs and anti-depressant.

Amyotrophic Lateral Sclerosis (ALS, or Lou Gehrig's disease), the most common form of motoneuron disease, is characterized by limb or bulbar initial deficits and leads to progressive paralysis of skeletal muscles.

Spinal alpha-motoneurons, brainstem and upper motor neurons are the specific targets of ALS pathology, which typically presents with deposits enriched in ubiquitin, TDP-43, FUS, and SOD1 (Nijssen et al., 2017). Mutations in SOD1, C9ORF72, TDP-43, FUS, VAPB, and VCP have been described in familial ALS (Taylor et al., 2016; Mejzini et al., 2019). Unfortunately, ALS is an orphan disease, which prognosis is invariably fatal within 3–5 years from diagnosis, with a worldwide incidence of 1.5 individuals per 100.000 yearly worldwide (Xu et al., 2020). ALS manifests as a sporadic disease in 90–95% of ALS affected individuals (Chen et al., 2013). Two stage 3 clinical trials, the first assessing Tofersen called VALOR (Biogen) and based on antisense technologies, and the second with AMX0035 (PHOENIX, Amylix Pharmaceuticals) are currently ongoing (Paganoni et al., 2020).

DISCUSSION

The most challenging issue of recent therapeutic approaches to CNS pathology is safe, efficient and non- invasive target engagement for disease modifying brain drug delivery.

A number of unsuccessful routes have been attempted so far, including systemic administration, and intracerebral injection of stem cells, or cell encapsulated and growth factors releasing devices. Among the major pitfalls of these approaches are the lack of target selectivity, low entry rates, high amount of drug required, and invasiveness of techniques hindering chronic treatments.

Novel experimental paradigms are needed in order to achieve proof of concept more rapidly than traditional approaches, to reduce the risk of negative outcomes and reduce the overall costs for drug development in NDs. The exploitation of a safe efficient tool to achieve target engagement in specific neuronal circuits is a major challenge in the current neuroscience research.

Focused ultrasound (FUS) is a powerful and precision technique allowing multiple, non-invasive targeted delivery of drugs to the brain. In particular, FUS-aided brain drug delivery through the nasal route has been recently proposed as a paradigmatic model to achieve efficient target engagement in brain pathology (Chen et al., 2016). Clearly, both pro and cons should be weighted for exploitation of the FUS technique for brain drug delivery in humans.

Today three main factors make us believe that FUS mediated disruption of the BBB is more than just another promising tool available to neuro-physicians: 1) a large body of pre-clinical evidence accumulated in the last decade and increasing each year is widening its potential applications in several neurological disorders; 2) the exciting results of the first three completed

clinical trials and several others currently being pursued and others in the recruitment phase, with an increasing number of patients that have positively undergone a BBB disruption treatment, including ND patients. At the same time, the current lack of emerging data regarding undesirable side-effects together with the increasing number of studies which are focusing on this issue is definitely pointing towards a realistic wider applicability of the methodology; 3) the technical instrumentation is already available, having gained approval by FDA and CE for specific clinical applications, including medication-refractory essential tremor in 2016, drug-refractory and tremor-dominant PD in 2018, where FUS has indications for non-invasive ablation of the globus pallidus.

Moreover, accumulating evidence pinpoints FUS for both Paclitaxel infusion and ablative surgery in high-grade glioma (Schneider et al., 2020), although some limitations need to be overcome, like skull overheating. Interestingly, regulated BBB disruption by FUS is under investigation for therapy of several NDs, including AD, PD, ALS and BBB opening by FUS has been achieved with success in AD and ALS patients (Elias et al., 2016; Meng et al., 2019; Moosa et al., 2019).

However, more extensive research is warranted regarding possible safety issues. For instance, the prolonged and/or repeated BBB opening might facilitate the brain entry of undesired peripheral immune cells and inflammatory molecules. Also, unexpected mechanical effects, such as focal heating, should be considered in order to reduce the chance of injury along the path. In any case, anatomical and physiological characteristics of each individual should be considered with respect to the capability of BBB opening. Thus exposure parameters should be tailored in order to optimize the amount of acoustic energy delivered while minimizing the potential occurrence of adverse effects.

Noteworthy, despite some minor issues and limitations currently addressed in its clinical use, FUS-aided brain drug delivery is expected to offer significant advantages in clinical settings, like to improve drug pharmacokinetic profile, decrease side-effects, e.g., minimize the risk of haemorrhage and infection compared to more invasive neurosurgical procedures.

In line with its clinical potential, FUS-based brain treatment has been granted by several funding agencies, included NIH, and it is currently under scrutiny for the treatment of ischemic and haemorrhagic stroke, gene therapy and antibody delivery, and neurostimulation, drug-resistant neuropatic pain and trigeminal neuralgia, as recently reviewed (Giammalva et al., 2021).

Nowadays, the application of FUS is gaining particular momentum for brain delivery of current treatments or repurposed drugs. Particularly, FUS application combined with intranasal delivery may be helpful in achieving infusion of neurotrophins, like NGF and BDNF, into specific damaged areas of the brain, of foremost clinical relevance in AD therapy.

NGF or insulin nasal spray have been proposed for human use upon encouraging studies on animal models and humans (Craft et al., 2012; Tuszynski et al., 2015; Manni et al., 2021). However, once at the clinical trial stage, insulin infusion failed to show any effect, supposedly because of the releasing device (Craft et al., 2020). Indeed, NGF based gene therapy has been also attempted in clinical trials, and resulted in failure of cognitive efficacy and/or off-target effects attributed by the authors to the implanted device (Castle et al., 2020).

Noteworthy, levodopa nose-to-brain delivery by nanoparticles (Arisoy et al., 2020) has been interrogated in PD and levodopa inhalation powder (Inbrija, Acorda therapeutics) has been approved by FDA for OFF periods. Thus, FUS-aided intranasal levodopa delivery may be envisaged as a potential FUS application for this devastating brain pathology.

Overall, by allowing targeted delivery of drugs in specific areas of the brain relevant to the different pathologies, the FUS-aided nasal delivery of novel or repositioned drugs may represent a game-changer in treating a wide range of still incurable brain pathological conditions.

AUTHOR CONTRIBUTIONS

GB, RN and VT equally contributed to theoretical conception, writing and final editing of the Perspective. GB conceptualized and realized the tables; VT graphically elaborated the figures.

ACKNOWLEDGMENTS

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