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NEW TREATMENT PERSPECTIVES IN AUTISM SPECTRUM DISORDERS

Topic Editors
Roberto Canitano and
Yuri Bozzi

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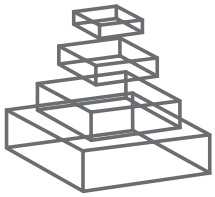

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NEW TREATMENT PERSPECTIVES IN AUTISM SPECTRUM DISORDERS

Topic Editors:
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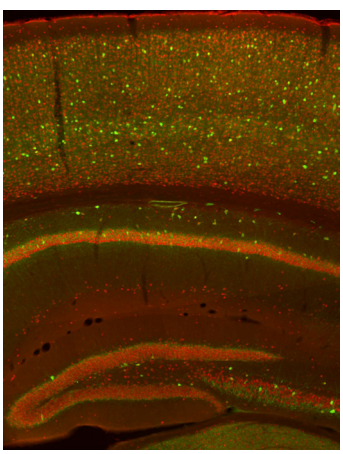

Image shows mouse brain inhibitory interneurons of the cerebral cortex and hippocampus, labelled in green. Recent studies from mouse models suggest that dysfunctions of inhibitory interneurons are associated to the pathogenesis of autism spectrum disorders. Copyright: Marika Maggia and Yuri Bozzi, Laboratory of Molecular Neuropathology, Centre for Integrative Biology (CIBIO), University of Trento, Italy.

Developing novel and more effective treatments that improve quality of life for individuals with autism spectrum disorders is urgently needed. To date a wide range of behavioral interventions have been shown to be safe and effective for improving language and cognition and adaptive behavior in children and adolescents with ASD. However many people with ASD can receive additional benefit from targeted pharmacological interventions. One of the major drawback in setting up therapeutics intervention is the remarkable individual differences found across individuals with ASD. As a matter of fact the medications that are currently available address only symptoms associated with ASD and not the core domains of social and communication dysfunction. The pathogenesis paradigm shift of ASD towards synaptic abnormalities moved the research to pathway to disease that involve multiple systems and that are becoming the forefront of ASD treatment and are pointing toward the development of new targeted treatments. Some new therapeutics have been tested and others are being studied. In this context single gene disorders frequently associated with ASD such as Rett Syndrome, Fragile X and Tuberous Sclerosis have been of significant aid as neurobiology of these disorders is more clear and has a potential to shed light on the altered signaling in ASD. However much research is needed to further understand the basic mechanisms of disease and the relationship to idiopathic ASD. Clinical trials in children are underway with agents directed to core symptoms and to the associated disorders in the search of new therapeutics and progress are expected with possible new option for

therapeutics in ASD in the upcoming future. Children and adolescents with ASD and their families can provide important information about their experience with new treatments and this should be a priority for future research. In addition, research performed on genetic mouse models of ASD will keep on providing useful information on the molecular pathways disrupted in the disease, thus contributing to identify novel drug targets.

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EDITORIAL
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New treatment perspectives in autism spectrum disorders

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Keywords: autism spectrum disorders, autism, autism spectrum disorders and treatment, diagnostic markers, pharmacology

In the past 10 years, research on autism spectrum disorders (ASD) has made a considerable progress, leading to the identification of a number of genes and signaling pathways associated to ASD pathogenesis. Nevertheless, our understanding of the complexity of these disorders remains elusive, and effective treatments are still lacking (1). In this e-book, we present 16 articles addressing several different aspects of both clinical and basic research on ASD. A particular emphasis is put on the efforts that are currently made to identify reliable diagnostic markers and novel therapeutic strategies, as well as on the progress of ongoing clinical trials.

Though ASD are recognized as cross-cultural disorders, discrepancies in early diagnosis and interventions are present in western countries. Genetic testing procedures in Europe and USA are still discordant; these issues are addressed by Amiet and coworkers (2). New ASD treatments are emerging and deserve to be mentioned. A number of novel medications have been used off-label in various studies, including drugs approved for Alzheimer's disease, as reviewed by Rossignol and Frye (3). Among these, it has to be outlined that in a large multisite controlled study memantine was not shown to improve core and associated symptoms in ASD and the open phase of the trial was in fact suspended. Similarly, cholinesterase inhibitors did not show substantial modifications in ASD core symptoms and their use is still not warranted. Drugs commonly used to treat mitochondrial diseases such as L-carnitine, complex B vitamins, antioxidants etc. have been found to improve ASD symptoms in some studies, but results are still conflicting and more research is needed. As a whole, the field is active and in progress but reports are discordant and do not yet allow us to draw firm conclusions regarding safety and efficacy. Further, research is needed to define subgroups of children with ASD in which these treatments may be most effective (4). ASD children treated with a ketogenic diet (KGD) showed decreased seizure frequencies and improved learning abilities and social skills, as proposed by Napoli and coworkers (5); however, replications of this investigation are urgently awaited to have of a clearer picture of KGD role in ASD. Excitation and inhibition (E/I) imbalance in ASD has been demonstrated in preclinical models, and targeted treatments directed either to reduce excessive glutamatergic transmission or increase inhibition through stimulation of GABAergic signaling have been introduced with promising preliminary results. The implication of oxytocin in social development and affiliative behaviors has been ascertained and findings from clinical trials in children with ASD showed encouraging results especially in social cognition. Stimulation of excitatory synapses and neuronal density has been achieved with insulin-like growth factor 1 (IGF-1) administration and it has been positively tested in two single gene disorders associated with ASD, Rett syndrome, and Phelan-McDermid syndrome. These preliminary clinical trials point to additional research in larger samples. Notably, modifications of neural pathways of ASD have been observed after behavioral developmental interventions through the evaluation by functional neuroimaging and electroencephalography, providing evidence of a dynamic neural substrate susceptible of functional changes. This new conceptualization paves the way to a modern treatment approach to this group of disorders once thought as hard wired and not amenable of changing, as discussed in the papers by Pini and colleagues (6) and Canitano (7).

The role of melatonin in the establishment of circadian rhythms and the synchronization of peripheral oscillators is probably linked to the synchrony of motor, emotional, and social rhythms that are altered in ASD. Potential therapeutic benefits of melatonin in the recovery of circadian rhythms have been demonstrated in a growing number of studies in ASD. Developmental behavioral interventions that emphasize synchrony (in some cases combined with melatonin) seem to provide substantial improvement in ASD, as reviewed by Tordjman (8) and reported by Fulton (9). Research on outcome of interventions is currently an active field of investigation, though data available do not allow to answer the question of "what works for whom" in ASD. This is critical to delineate the guidelines for behavioral interventions, as reviewed by Vivanti and colleagues (10). Among the bothersome ASD symptoms that need to be addressed, auditory hypersensitivities may be tackled by means of novel techniques such as the listening protocol projects, as proposed by Porges (11). Poor reciprocal social communication is a hallmark characteristic of ASD and is under intense investigation for treatment; Corbett and colleagues report a novel theatre intervention that combines interaction of trained peers with patients to facilitate the performance-based theatrical treatment (12).

The last part of the book is dedicated to basic studies highlighting the importance of investigating mouse models to unravel the molecular mechanisms underlying ASD pathogenesis. Two review articles, respectively by Cellot and Cherubini (13) and Giovedí and coworkers (14), address the role of GABAergic neurotransmission and synaptic vesicle proteins in the pathogenesis of ASD, strengthening the notion of ASD as a "synaptic neuropathology". Finally, three original research articles investigate novel pathological aspects in three different ASD mouse models. Provenzano and

colleagues show growth hormone and IGF-1 signaling deficits in Engrailed-2 mouse mutants (15); Gigliucci et al. demonstrate oxytocin signaling alterations in mice lacking opioid receptors (16), and Michetti and coworkers identify a series of pathological features (including defects in glutamatergic neurotransmission) in reeler mice (17). Taken together, these mouse studies confirm the importance of IGF-1, oxytocin, and glutamate signaling in the pathogenesis of ASD, as extensively described in several clinical studies presented in this e-book.

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ORIGINAL RESEARCH ARTICLE
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Are there cultural differences in parental interest in early diagnosis and genetic risk assessment for autism spectrum disorder?

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Background: There are many societal and cultural differences between healthcare systems and the use of genetic testing in the US and France. These differences may affect the diagnostic process for autism spectrum disorder (ASD) in each country and influence parental opinions regarding the use of genetic screening tools for ASD.

Methods: Using an internet-based tool, a survey of parents with at least one child with ASD was conducted. A total of 162 participants from the US completed an English version of the survey and 469 participants from France completed a French version of the survey. Respondents were mainly females (90%) and biological parents (94.3% in the US and 97.2% in France).

Results: The mean age of ASD diagnosis reported was not significantly different between France (57.5 ± 38.4 months) and the US (56.5 ± 52.7 months) ($p = 0.82$) despite significant difference in the average age at which a difference in development was first suspected [29.7 months (±28.4) vs. 21.4 months (±18.1), respectively, $p = 7 \times 10^{-4}$]. Only 27.8% of US participants indicated that their child diagnosed with ASD had undergone diagnostic genetic testing, whereas 61.7% of the French participants indicated this was the case ($p = 2.7 \times 10^{-12}$). In both countries, the majority of respondents (69.3% and 80% from France and the US, respectively) indicated high interest in the use of a genetic screening test for autism.

Conclusion: Parents from France and the US report a persistent delay between the initial suspicion of a difference in development and the diagnosis of ASD. Significantly fewer US participants underwent genetic testing although this result should be regarded as exploratory given the limitations. The significance of these between country differences will be discussed.

Keywords: autism spectrum disorders, survey, parents' opinion, genetics, France, US

BACKGROUND

Autism spectrum disorders (ASDs) are a group of highly heritable developmental disorders characterized by early impairments in communication and social interaction, and restricted interests, and repetitive behaviors (1, 2). Recent reports estimate the median prevalence rate to be 62/10,000 (3) but there is clear evidence that rates have increased over time. In the US, the CDC estimates that the prevalence of ASD is approximately 1 in 88 children (4). In France, the most recent epidemiological survey confirmed this increase and found a prevalence rate among 8 year olds of 33.5/10,000 (5).

In terms of treatment, a combination of developmental and behavioral approaches is now recommended focusing on early, intensive intervention and parental collaboration (6). Recently, several meta-analyses were published suggesting that comprehensive early intensive behavioral interventions programs lead to positive effects regarding intellectual functioning, language skill, and adaptive behavior outcome (7–10). However, there were many differences between meta-analyses and potential confounds and limitations that might have lead to discrepant findings across these studies (11). The only randomized controlled trial of an early intensive behavioral intervention [the early start Denver model (ESDM)] demonstrated significant gains in visual processing and improvements in language abilities, with subsequent gains in intellectual quotient and adaptive behaviors, among children receiving the ESDM (6). Moreover, a secondary outcome measurement from this trial suggested that ESDM is associated with normalized brain activity patterns related to social attention and engagement, and that these normalized brain activity patterns are correlated with improvements in social behavior (12).

Despite an increase in ASD research in recent years, there continues to be much debate on autism leading to extensive media coverage (13). For example, between psychiatric care and educational and behavioral methods (14), between parent's advocacy groups and associations of individuals with autism (15), and between media presentation of research advances and evidence-based data *per se* (16). Among the many issues debated, much focus has been given to early diagnosis and the contribution of genetic factors to the cause of autism. Although the importance of early diagnosis and treatment of autism is well accepted, the methods used to achieve early diagnosis are debated (17). To date, specific clinical screening instruments do not show high sensitivity despite acceptable specificity (18). Delays in diagnosis are not only due to a shortage of accessible specialists, but also due to clinical and developmental limitations regarding infant/toddler assessment tools (19). In many instances, parents' initial concerns regarding their child's development are not specific to autism and may be associated with many other conditions in the differential diagnosis (20). Moreover, some clinicians consider that sharing a possible diagnosis of ASD with parents may alter the way these parents will interact with their child as shown in siblings at-risk of ASD (21). Finally, the cost of general population screening is also a concern in countries that do not have free access to healthcare. The use of family history (one or more older siblings with ASD) (22) or perinatal factors (e.g., prematurity) (23) has been suggested as an alternative method to general population screening in identifying children at-risk for ASD.

In regard to genetic risk for ASD, the majority of research has consistently shown that ASDs have a strong genetic component with heritability rates ranging from 60 to 80% (24, 25) and close to 40% in the largest recent twin study (26). Therefore, there is little doubt of the importance of genetic factors in autism (27–29). However, most authors agree that the number of ASD cases explained by a single genetic abnormality is limited to approximately 15–20%. These include single gene mutations (e.g., Fragile X syndrome) or copy number variants (CNVs) (e.g., at 15q11–q13 locus). Although other causal factors (e.g., pre-, peri-, post-natal factors) have been studied in autism (20, 30), interest in the media and in high-impact scientific journals has been limited (31). Yet, they may account for some part in the recent increase in prevalence rate (32). As a result, the majority of cases of autism are often described as a combination of (1) rare genetic variants with high effect size, (2) common genetic variants with low effect size, and (3) environmental (or non-genetic) risk factors (29, 31, 33). A recent study addressed the challenges of early diagnosis and use of genetic risk factors by reviewing the research related to biomarkers associated with ASD (16). The authors recognized the widespread hope that the discovery of valid biomarkers for autism will enable earlier diagnosis and treatment. However, careful review of the literature shows major scientific challenges and ethical concerns related to the development of biomarkers and their clinical application (16).

In addition, societal and cultural attitudes and practices may also contribute to differing opinions regarding ASD as seen with other neurodevelopmental conditions. For example, there is considerable controversy regarding the use of medications in children (34) or the diagnosis of pediatric bipolar disorder (35) despite similar national recommendations in the US and Europe. Healthcare systems are also very different in Europe and the US, which may influence both expectations and access to care. Finally, regulations related to genetic testing and societal acceptance of genetic contributions to ASD may differ drastically influencing both standard of care and patients' willingness to undergo genetic testing.

To investigate the impact of societal and cultural differences in the approach to health care and autism on the diagnostic experience and parental attitudes regarding genetic risk assessment testing for autism, we conducted two parallel web surveys in the US (36) and France. These countries were chosen due to their cultural and social contrasts regarding both healthcare systems and genetic testing regulations (**Table 1**). The French healthcare system combines universal coverage with a public–private mix of hospital and ambulatory care, whereas the US healthcare system depends on private, for-profit health insurance. Although France has a higher volume of service provisions than the US (37), millions of Americans have inadequate or limited access to care because they have inadequate or no health insurance. Data from the 2011 National Health Interview Survey (NHIS) show that the percentage of person uninsured at the time of interview was 15.1% (46.3 million) for persons of all ages and 7% (5.2 million) for children below 8 years (38). Regarding early developmental risk, both the US and French pediatric associations recommend careful early follow-up and systematic examinations during the first 2 years of life to screen for early-onset condition and disabilities. However, only a few states in the US support specific programs for low-income families, whereas in France, these examinations are supported by two parallel regulations: (1) seven free medical examinations during pregnancy[1] and 20 pediatric free examinations during the first 6 years (Code of Public Health – article L.2132-2); (2) free access "dispensaries" funded by the *Protection Materno-Infantile* program located in all areas that offer medico-social action to promote maternal and child health (Code of Public Health – article R2112-1 to R.2112-8). When a diagnosis of autism is suspected, the child can be referred to local mental health ambulatory services and/or to the Regional Center de Resources for Autism where the diagnosis is confirmed (39). Again, within the French healthcare system, these services are free. In the US, first-line primary care services and specialty clinics for ASD are also available, but access to the services is variable as explained above. In terms of diagnosis classification, the DSM-IV is the standard in the US, whereas in France, clinicians use DSM-IV, ICD-10, or CFTMEA. Correspondence algorithms are available (40).

Similarly, regulations for genetic testing differ in the two countries. Genetic tests for over 2,000 diseases are currently available for use in clinical settings in the US. In addition, a growing number of tests are being developed to look at multiple genes that may increase or decrease a person's risk of common diseases. In 2008, the former Secretary's Advisory Committee on Genetics, Health and Society of the US Department of Health and Human Services released a report identifying gaps in the regulation, oversight, and usefulness of genetic testing. However, few recommendations have been addressed[2]. In France, regulations are more stringent.

[1] http://www.ameli.fr/professionnels-de-sante/sages-femmes/exercer-au-quotidien/formalites/la-maternite.php

[2] http://www.cdc.gov/genomics/gtesting/index.htm

Table 1 | A brief overview of the use of genetic testing in the US and France.

	U.S.	France
GENERAL CONTEXT		
Health care system insurance	No universal coverage Mainly private services, for-profit health insurance	Universal coverage Mix of private and public services
Access to genetic testing	Few recommendations Variable insurance coverage	Governed by law Fully covered
SPECIFIC CONTEXT OF ASD		
Classification for ASD	**DSM-IV**	**DSM-IV, ICD-10, CFTMEA**
Diagnosis	Occurs mainly in private settings	Occurs mainly through free and prepaid public services for child early development (pediatrician) and mental health including public clinics for autism
Treatment setting	Mainly private, and through special education	Mix of public health, private non-profit care and special education
Recommended genetic testing	High-resolution karyotype study Fragile X	High-resolution karyotype study Fragile X
Genetic diagnostic test	Numerous chromosomal microarray tests Numerous sequencing tests for ASD	No genetic diagnostic test available
Genetic screening tests for ASD	One genetic screening test for siblings	No genetic screening test available

Only DNA analyses for medical, legal, or scientific purposes are authorized by law, and the numerous predictive genetic analyses being developed and available online are raising ethical and legal questions (41).

Regarding ASD, the clinical genetic tests currently available are limited to diagnostic use and serve to identify the underlying genetic etiology of a child already diagnosed with an ASD. They include chromosomal microarray, Fragile X analysis, and tests for known genetic syndromes associated with ASD. In the US and France, it is recommended that a child diagnosed with ASD undergo high-resolution karyotype studies and Fragile X testing (42, 43). Clinical chromosome microarray tests are commonly available in the US through medical prescription, but insurance reimbursement for such testing is variable. In France, chromosomal microarray analyses are also commonly available. Reimbursement is systematic after medical prescription. Recently, based on studies showing the combined effect of common single nucleotide polymorphisms (SNPs) on ASD susceptibility (44), an SNP-based DNA test has become available in the U.S which assesses the risk of ASD in children who have an older sibling diagnosed with ASD (ARISk Test; IntegraGen, Cambridge, MA, USA). This test is also only available through a medical prescription by a qualified, licensed, medical professional.

In summary, the aim of this study was to compare the diagnostic experience with ASD, access to genetic testing and parental attitudes regarding the use of a genetic risk assessment tool to aid in the earlier diagnosis of ASD between France and US families considering the differences in healthcare models, genetic testing regulations and early screening and diagnostic practices for ASD between the two countries. To our knowledge, no similar study comparing France and US population samples has been published previously.

MATERIALS AND METHODS

POPULATION

Two self-administered internet-based surveys were administered consecutively using software available from SurveyMonkey[3]. The initial survey was conducted in English only and limited to US residents who were parents or guardians of one or more children with ASD. Requests for participation were e-mailed to potential participants by representatives from local and regional autism advocacy groups [for more details see Ref. (36)]. Responses to the survey were collected from February 13, 2012, until March 23, 2012. The second survey was conducted in French only and was administered to parents or guardians from France or other French-speaking countries with one or more children with ASD. Participants were recruited over a web link posted on the blog "autisme infantile,"[4] the Facebook group "handicap et éducation spécialisée,"[5] and the association "Ecolalie." The survey was conducted from June 27, 2012, until July 9, 2012.

SURVEY DESIGN

The survey was designed to assess parental opinions regarding the ASD diagnostic process for their child and genetic testing related to ASD. It consisted of four sections: (1) parental perceptions regarding the ASD diagnostic process related to the respondent's most recently diagnosed child. This included questions regarding the timing of initial suspicion of a difference in development, the referral process, and age at diagnosis; (2) parental experiences with genetic testing for their child diagnosed with ASD. This sections also included questions focused on the perceived role of genetics

[3]http://www.surveymonkey.com
[4]http://autismeinfantile.com/
[5]http://www.facebook.com/groups/chapellier/

in ASD; (3) for parents who reported having a younger, undiagnosed child younger than 48 months, parental opinion regarding whether they would want to have their younger child receive a genetic test which assessed the child's risk of ASD, even if it could not confirm a diagnosis; (4) demographic data of the respondent including age, gender, and education level. Participants who did not have a child with ASD were excluded from the survey. Survey questions were yes/no, multiple choice answers or 4-point Likert scale (1 – highly likely; 2 – somewhat likely; 3 – somewhat unlikely; 4 – not likely). All questions were presented in a fixed order. Respondents were allowed to change their responses to any question prior to submitting as "final" the survey, after which no further changes could be made.

The survey was reviewed and pilot tested with select parents of children with ASD and with specialists who provide clinical care and diagnostic services for children with developmental delays and/or ASD. All surveys were completed anonymously and no information was collected that permitted the identification of any individuals completing the survey.

Descriptive statistics included means, medians, ranges, and/or percentages. Responses from French and US participants were compared using the χ^2 test.

RESULTS

CHARACTERISTICS OF THE SAMPLES

A total of 162 participants completed the US survey and 554 participants (469 from France, 40 from Canada, and 45 from other countries) completed the French survey. For the purpose of this study, only participants from the US and France were included. Their characteristics are detailed in **Table 2**. In both samples, most of the participants were females (90%). The majority of responders (97.2 and 94.3% in France and the US, respectively) indicated that they were biological parents, the remaining were adoptive parents, stepfathers, grandparents, aunts/uncles, or guardians. French respondents were significantly younger, although more than 40% of respondents in both groups were between the ages of 36 and 45 years. Approximately 76% of the US respondents and only 55% of the French respondents had at least a college degree, meaning that socioeconomic status was on average lower in the French sample.

AGE OF FIRST CONCERN AND AGE OF DIAGNOSIS

We identified a significant difference in the average age at which a difference in development was first suspected between the two populations with a mean age of 29.7 months (±28.4) reported in the French sample and 21.4 months (±18.1) reported in the US sample ($p = 7 \times 10^{-4}$). However, the mean reported age when ASD was diagnosed was 57.5 months (±38.4) in the French sample compared to 56.5 months (±52.7) in the US sample ($p = 0.82$), reflecting no statistical difference in the age at diagnosis between the US and France.

ACCESS TO GENETIC TESTING

Most participants declared that they believed genetic factors contributed to the cause of ASD. Eighty-two percent of the US participants and 59% of the French participants indicated that ASD was a combination of genetic and environmental factors. Twelve percent of US participants and 24% of French participants indicated that ASD was almost entirely a result of genetic factors ($p = 0.0005$). However, only 27.8% of US participants indicated that their child diagnosed with ASD had undergone genetic testing, whereas 61.7% of French participants indicated so ($p = 2.7 \times 10^{-12}$) (**Table 3**).

Table 2 | Sample characteristics.

	France, *N* (%)	US, *N* (%)	*p*
GENDER OF THE RESPONDERS			
M	42 (9.3)	16 (11.0)	0.555
F	410 (90.7)	130 (89.0)	
AGE OF THE RESPONDERS			
<36	178 (37.0)	33 (23.6)	3×10^{-8}
36–45	202 (42)	58 (41.4)	
46–55	45 (9.4)	38 (27.1)	
>55	56 (6.9)	11 (7.9)	
EDUCATION LEVEL OF THE RESPONDERS			
Less than high school	88 (20.0)	0 (0)	0.004
High school/GED	110 (25.1)	35 (23.5)	0.98
College degree	159 (36.2)	59 (39.6)	0.97
Graduate degree	82 (18.7)	55 (36.9)	0.01
RESPONDERS INVOLVED IN A PARENTS ASSOCIATION			
Yes	242 (54.5)	74 (50)	0.34
No	202 (45.5)	74 (50)	
REPORTED DIAGNOSIS			
Autistic disorder	185 (42.6)	86 (54.9)	0.15
PDD-NOS	209 (48.2)	31 (19.1)	0.71
Asperger syndrome	40 (9.2)	35 (21.6)	0.04
Unknown	35 (7.5)	4 (4.3)	

Table 3 | Reported rates of genetic testing performed in affected individuals.

	France, *N* (%)	US, *N* (%)	*p*
Yes	284 (61.7)	42 (27.8)	3.5×10^{-13}
Genetic testing recommended but declined	17 (3.7)	12 (7.9)	0.03
No genetic testing recommended	150 (32.6)	93 (61.6)	1.3×10^{-10}
Don't know	9 (2.0)	4 (2.7)	

RISK OF AUTISM IN THE SIBLING

Twenty-five participants from the US survey and 101 participants from the French survey reported that their family included a sibling, as of yet not diagnosed with an ASD and under the age of 48 months. Of these, 13 (52%) respondents from the U.S and 48 (47%) respondents from the French survey felt that the younger sibling was somewhat or highly likely to develop ASD. However, 15 respondents (60%) from the US survey and 58 respondents (57%) from the French survey indicated that they had a somewhat or very low level of anxiety regarding the younger sibling's risk of ASD. Seventy respondents from the French survey (69.3%) and 20 respondents from the US sample (80%) with a younger undiagnosed child, <48 months old, indicated that they would want their

child tested if a genetic test were available that could identify risk for ASD, even if it could not confirm or rule out the diagnosis.

DISCUSSION

France and the US differ in many sociocultural aspects such as healthcare systems and the regulation of genetic testing (**Table 1**). These differences may have consequences on early autism diagnosis and parental opinions about genetic testing and access to it. In our study, the mean age of first concern was significantly later in the French sample than in the US sample. However, our results are not strictly consistent with previous French studies (45, 46). In the largest study conducted to date ($N = 424$), the mean age when French parents first reported early signs of ASD in their children was 19 months (±11.7), and 27 months (±17.5) when their children were first evaluated by a medical professional (20). However, in both the US and French samples, the mean reported age of ASD diagnosis was approximately 57 months. These results coincide with reports in the literature suggesting that children may not receive a formal diagnosis until the age of 4 (4, 46, 47). Recently, French parents' advocacy organizations have claimed that a delay in diagnosis is still ongoing in France. However, the current results contradict these claims and are consistent with Chamak et al.'s (46) recent study, showing that the age of diagnosis of autism has decreased over the years in France since the beginning of the 1990s (**Figure 1**).

With a majority of the participants from France (59%) and the US (82%) indicating that ASD was a result of the genetics and non-genetic factors, most of participants' knowledge in both countries is in accordance with the state of the art. Significantly more participants from France (24%) than from the US (12%) indicated that ASD was almost entirely a result of genetic factors. This result may be surprising considering the numerous genetic tests currently available for clinical use in the US. However, this may reflect the influence in French popular opinion regarding the role of genetic factors in the origin of autism (13, 15).

A significantly higher number of children diagnosed with ASD were reported to have undergone clinical genetic testing in the French sample (60%) compared to the US sample (28%). Despite the American Academy of Pediatrics suggestion that primary care physicians should obtain high-resolution chromosome studies and Fragile X testing when the diagnosis of an ASD is made (48) and the American College of Medical Genetics recommendation that a genetic consultation should be offered to all persons and families with ASD (43), only 28% of the US participants indicated that their child diagnosed with ASD had undergone genetic testing. This result may be consistent with Selkirk et al.'s study performed in the US reporting that only 24% of 255 parents whose children were diagnosed with an ASD reported seeing a genetic counselor (49). Similarly, in a recent qualitative study of parents' experience, Chen et al. reported that only 28% of 42 parents with at least one child diagnosed with ASD had reported that their child had undergone genetic testing. Moreover, 63% of the parents whose children had not undergone genetic testing for ASD reported that they had never heard of such testing before the interview (50). Sixty percent of French respondents indicated that their child had undergone genetic testing, as recommended since 2005 by the French *Haute Autorité de Santé* (HAS). This is in accordance with Chamak et al. who reported that 51.5% of the individuals with ASD in a French sample of 200 families had undergone clinical genetic testing (personal communication).

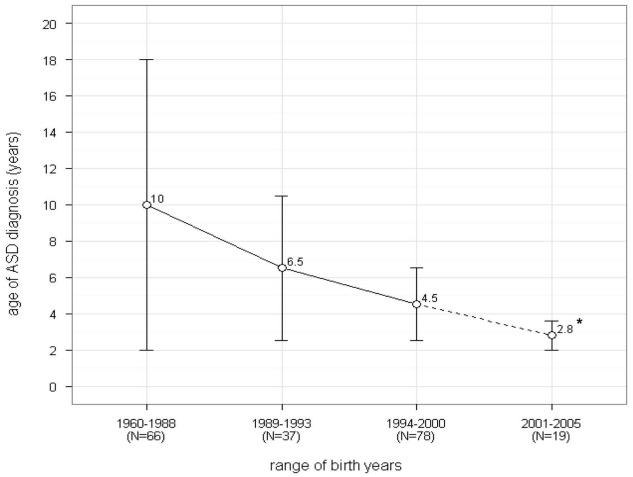

FIGURE 1 | Changes in age of ASD diagnosis in France during the last 40 years [extracted from Ref. (46); courtesy of Claude Bursztejn]. *The dot line indicates that the 2001–2005 result should be regarded with caution as based on only $N = 19$ cases.

With 60% of the parents from the US reporting that no genetic testing was recommended, the low rate of genetic testing reported for the children from the US (28%) may be mainly attributed to a lack of healthcare provider's referral. This observation is consistent with previous findings showing the importance of physicians referral in accessing genetics services for children with ASD (51); ASD, Down syndrome and/or mental retardation (52); or hearing loss (53). However, little information is available in the literature about healthcare provider's attitudes toward genetic testing. Recently, a literature review of genetic testing in psychiatry found that not all psychiatrists felt competent about their genetic knowledge (9–70%) or ability to offer and interpret genetic test (54). More recently, a large e-mailed survey explored psychiatrists' and neurologists' (mostly with an adult practice) practices and knowledge concerning genetic testing and found that only 33% of respondents felt confident about how to order and where to send genetic tests (55). Almost half of neurologists and over 75% of psychiatrists were not aware of a genetic counselor or geneticist to whom to refer patients (55). One may also be questioning physician knowledge about the inheritance of autism: in 2008, nearly half of a small group of randomly selected psychiatrists were found to rate that genetics has a "weak influence" or "no influence" on the heritability of autism (56). Moreover, because evidence-based interventions for ASD are primarily behavioral, the healthcare provider may not see the need for genetic testing.

The impact of free access to care may also be a key factor in determining the frequency with which clinical genetic testing is used in autism. Considering that (1) higher education, as seen in the US sample, is related to better genetic knowledge and increased access to genetic testing (50); and (2) regulations regarding genetic testing are more stringent in France, a higher rate of genetic

testing would have been expected in the US sample rather than the French sample. It is likely that free access to care in France may in part explain the better accordance with genetic testing recommendations in ASD found in this country.

This study should be interpreted in the context of its numerous limitations. First, the comparative survey relied on retrospective self-reporting data collection from parents with numerous biases due to recollection. Second, the parents who responded to the survey may not be representative of the population of families with ASD in France and in the US since most of the parents were contacted via advocacy organizations indicating a higher degree of involvement, and all participants had to have access to a computer. It is probable that the rates reported here are overestimated. Moreover, it is not possible to determine the response rate for an internet survey and it is unknown whether respondents differed in salient ways from non-respondents. For example, families who do not have regular access to a computer or time to respond a survey may not be reflected in the survey. Third, internet-based survey is considered less reliable than direct or e-mail interviews and does not permit clinical diagnosis confirmation of included patients. Also, the collection of ethnicity data was not allowed by the French National Informatics and Liberty Commission (*Commission nationale de l'informatique et des libertés*, CNIL), therefore no comparison could be done with the US data. Fourth, survey participants were not asked to indicate the gender and the age of the affected child. Considering recent changes in the US and French guidelines regarding ASD screening and diagnostic practices, it is unclear whether our results reflect these changes or not, and as a consequence whether older patients could have skewed the results. Indeed, most of what we know about genetics and autism has been learned in the past 20 years. We cannot exclude that the results from the US sample reflect unavailability of genetic testing for older patients. However, the percentage of US families reporting genetic testing in our survey is similar to that found in two recent studies based on a direct interview (50) and an anonymous survey (49). Finally, possible biases due to local variation of accessibility to genetic testing cannot be adjusted in such an internet survey as far as anonymity prevented collection of participant's addresses. This may be an issue for the US sample given that France is no more than a regular US state in terms of surface.

CONCLUSION

Parents from France and the US report a persistent delay between the initial suspicion of a difference in development and the diagnosis of ASD. Age of diagnosis was about 4.5 years in both countries. Additionally, most parents from both countries with a younger undiagnosed child reported that they would pursue genetic risk assessment testing that could identify risk for ASD in a younger sibling even if it could not confirm or rule out a diagnosis. However, significantly fewer US participants underwent genetic testing. We hypothesize that this is the result of economic issues.

AUTHOR CONTRIBUTIONS

Claire Amiet participated in the design of the internet survey, the acquisition and the interpretation of data, and to draft the manuscript. Elizabeth Couchon participated in the design of the internet survey, the acquisition and the interpretation of data, and to draft the manuscript. Kelly Carr participated in the design of the internet survey and the acquisition of data. Jerôme Carayol performed the statistical analysis and participated to the interpretation of data. David Cohen participated in the interpretation of data and to draft the manuscript. All authors read and approved the final manuscript.

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The use of medications approved for Alzheimer's disease in autism spectrum disorder: a systematic review

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects 1 in 68 children in the United States. Even though it is a common disorder, only two medications (risperidone and aripiprazole) are approved by the U.S. Food and Drug Administration (FDA) to treat symptoms associated with ASD. However, these medications are approved to treat irritability, which is not a core symptom of ASD. A number of novel medications, which have not been approved by the FDA to treat ASD have been used off-label in some studies to treat ASD symptoms, including medications approved for Alzheimer's disease. Interestingly, some of these studies are high-quality, double-blind, placebo-controlled (DBPC) studies. This article systematically reviews studies published through April, 2014, which examined the use of Alzheimer's medications in ASD, including donepezil (seven studies, two were DBPC, five out of seven reported improvements), galantamine (four studies, two were DBPC, all reported improvements), rivastigmine (one study reporting improvements), tacrine (one study reporting improvements), and memantine (nine studies, one was DBPC, eight reported improvements). An evidence-based scale was used to rank each medication. Collectively, these studies reported improvements in expressive language and communication, receptive language, social interaction, irritability, hyperactivity, attention, eye contact, emotional lability, repetitive or self-stimulatory behaviors, motor planning, disruptive behaviors, obsessive–compulsive symptoms, lethargy, overall ASD behaviors, and increased REM sleep. Reported side effects are reviewed and include irritability, gastrointestinal problems, verbal or behavioral regression, headaches, irritability, rash, tremor, sedation, vomiting, and speech problems. Both galantamine and memantine had sufficient evidence ranking for improving both core and associated symptoms of ASD. Given the lack of medications approved to treat ASD, further studies on novel medications, including Alzheimer's disease medications, are needed.

Keywords: autism, Alzheimer's disease, acetylcholinesterase inhibitors, NMDA antagonist, medications

BACKGROUND

Autism spectrum disorder (ASD) is a heterogenous neurodevelopmental disorder that is defined by behavioral observations and characterized by developmental delays in communication and social interaction and by repetitive behaviors and/or restricted interests. The most recent prevalence of ASD in the United States (U.S.) is now 1 in 68 children, including 1 in 42 boys (1). Only two medications have been approved by the U.S. Food and Drug Administration (FDA) for ASD and these medications, risperidone and aripiprazole, are indicated to treat irritability, an associated but not core symptom of ASD (2, 3). Since irritability is not a core feature of ASD, there are currently no U.S. FDA approved medications for treating the core symptoms of ASD.

A number of novel medications have been used to treat the symptoms of ASD (4, 5). Some of these medications are approved for the treatment of Alzheimer's disease. A connection between Alzheimer's disease and autism has been proposed by some investigators (6) and will be reviewed below. Because of the evidence of this connection, several medications approved for Alzheimer's disease have been investigated for use in ASD. To date, five medications have been approved by the U.S. FDA for the treatment of Alzheimer's disease: Tacrine (Cognex®, 1993), Donepezil (Aricept®, 1996), Rivastigmine (Exelon®, 2000), Galantamine (Razadyne®, 2001), and Memantine (Namenda®, 2003). Donepezil, galantamine, rivastigmine, and tacrine are cholinesterase inhibitors, and work by preventing the breakdown of acetylcholine. Galantamine also stimulates nicotinic cholinergic receptors and therefore can increase the release of acetylcholine (7). Memantine is distinct from these other medications and modulates glutamate neurotransmission.

Several lines of evidence have implicated abnormalities in the cholinergic system in ASD (8). First, studies examining postmortem brain samples from individuals with ASD have reported abnormalities in the cholinergic system (9–11). Early studies compared the cholinergic system in frontal, parietal, hippocampus, and cerebellar tissue from typically developing adults to similarly aged autistic adults with intellectual disability. One of these studies found decreases in muscarinic M_1 receptors in the parietal cortex and nicotinic receptors in the frontal and parietal cortexes, with a decrease in α_4 and β_2 nicotinic subtypes confirmed

by immunochemistry in the parietal cortex (9). Another study found significant changes in nicotinic receptors but no significant changes in muscarinic receptors or in the presynaptic cholinergic enzyme choline acetyltransferase in the cerebellum. Consistent nicotinic receptor changes included decreases in the α_4 receptor subtype in several types of cells including granule and Purkinje cells, as well as increases in the α_7 receptor subtype in the granule cell layer (11). In contrast, using quantitative receptor autoradiographic studies, no changes in cholinergic receptor binding were found in the hippocampus (12). Later, a study comparing typically developing and autistic adults showed a decrease in α_7 and β_2 but not α_4 nicotinic acetylcholine receptor subunits in the thalamus (10). Secondly, a positron emission tomography study reported a decrease in acetylcholinesterase activity in the bilateral fusiform gyri in ASD adults, as compared to typically developing adults, with this decrease correlated with objective scales of individual participant social disability (13). Thirdly, functional analysis of gene networks altered in individuals with ASD implicate synaptic cholinergic receptor families of genes (14) and epigenetic changes in both ASD and Rett syndrome have been linked to decreased expression of the CHRNA7 gene encoding the nicotinic receptor subtype α_7 (15). Lastly, some studies have implicated cholinergic abnormalities in an animal model of ASD. The BTBR ASD mouse model demonstrates lower basal levels of extracellular acetylcholine in the prefrontal cortex (16) and injection of the acetylcholinesterase inhibitor donepezil into the BTBR mouse (systemically or directly) in the dorsomedial striatum, the rodent homolog of the caudate nucleus, ameliorated many core ASD behaviors (17). Thus, there is substantial support for the idea that treatments that modulate the cholinergic system might be helpful in ASD (18).

Several lines of scientific evidence have also pointed to abnormalities in glutamate metabolism in individuals with ASD as identified by imaging, genetic, and post-mortem studies. Proton magnetic resonance spectroscopy has demonstrated abnormalities in glutamate metabolism in individuals with ASD. For example, the glutamate + glutamine peak has been found to be increased in individuals with ASD as compared to controls in the auditory cortex (19), anterior cingulate cortex (20, 21), and basal ganglia (22). Another study found that the glutamate/glutamine ratio in the amygdala–hippocampal region was increased in ASD individuals as compared to controls (23), while one study found an increase in glutamate/creatine in the putamen in individuals with ASD (24). Interestingly, the magnetic resonance spectroscopy glutamate + glutamine peak in the basal ganglia of ASD individuals was correlated with a measure of impairment in social communication (22) and the glutamate/creatine ratio in the putamen was correlated with ASD symptoms (social interaction) (24). However, studies examining glutamate in frontal brain regions have demonstrated inconsistent results. For example, as compared to controls, individuals with ASD demonstrated a decrease in the GABA/glutamate ratio in the frontal cortex in one study (25) while the glutamate + glutamine peak was not different between ASD and control individuals in the prefrontal cortex in another study (22). The glutamate/creatine ratio was decreased in ASD individuals in the frontal lobes, as compared to controls, in another study (26).

Genetic studies have implicated abnormalities in ionotropic glutamate receptors in ASD. Genetic studies have associated ASD with abnormalities in subunits of *N*-methyl-D-aspartate (NMDA) (27–30), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (31), and kainate (32, 33) receptors and the mitochondrial aspartate/glutamate carrier (34–36). Genetic syndromes that have a high prevalence of ASD features demonstrate abnormalities in glutamate neurotransmission. For example, haploinsufficiency of SHANK3, a gene that encodes postsynaptic scaffolding proteins for glutamate receptors, is characteristic of many patients with Phelan–McDermid syndrome, a genetic disorder with a high prevalence of ASD (37). Interestingly, genetic animal models of ASD have also demonstrated involvement of glutamate receptors. For example, one of the main areas of research is the Fragile X mouse model where abnormalities in metabotropic glutamate receptor subtype 5 (mGluR5) have been linked to behavioral and cognitive abnormalities that overlap symptoms associated with ASD (38).

Lastly, post-mortem studies have also implicated glutamate abnormalities. Cerebellum samples from individuals with ASD have demonstrated an increase in mRNA for AMPA 1, 2, and 3 receptors and glutamate/aspartate transporter 1 and 2, an increase in protein levels of glutamate/aspartate transporter 1 and 2 and AMPA 1 and NMDA 1 receptors, but a decrease in AMPA receptor density (39). In addition, other studies have demonstrated an increase in mGluR5 protein in the vermis (40) and superior frontal cortex (41) of children with ASD. One study reported decreased levels of kidney-type glutaminase in the anterior cingulate cortex of individuals with ASD (42), while another study demonstrated an increase in the mitochondrial aspartate/glutamate carrier in the prefrontal cortex but not in the cerebellum of individuals with ASD (43). Thus, there is substantial evidence for glutamate abnormalities in individuals with ASD, suggesting the treatments that modulate glutamate may be helpful in ASD.

Memantine is an NMDA receptor antagonist and regulates the activity of glutamate, a neurotransmitter involved in memory and learning. Normally glutamate attaches to NMDA receptors allowing calcium to then enter freely into cells. Memantine prevents this by partially blocking NMDA receptors. Memantine has been reported to help obsessive–compulsive disorder (OCD) as well as impulsive behaviors in open-label (44–48), single blind (49), and placebo-controlled studies (50, 51). In animal models, memantine has also been shown to decrease evidence of neuroinflammation (52).

Given this background, this article reviews studies that have been published to date, which have reported on the use of these Alzheimer's medications in ASD individuals. Overall, this review demonstrates that there is significant scientific support for some of these medications in the treatment of core ASD symptoms, suggesting that further clinical trials may be helpful to help define the role for some of these medications for the treatment of individuals with ASD.

METHODS

SEARCH STRATEGY

A computer-aided search of PUBMED [website (http://www.ncbi.nlm.nih.gov/entrez)] and Google Scholar databases from

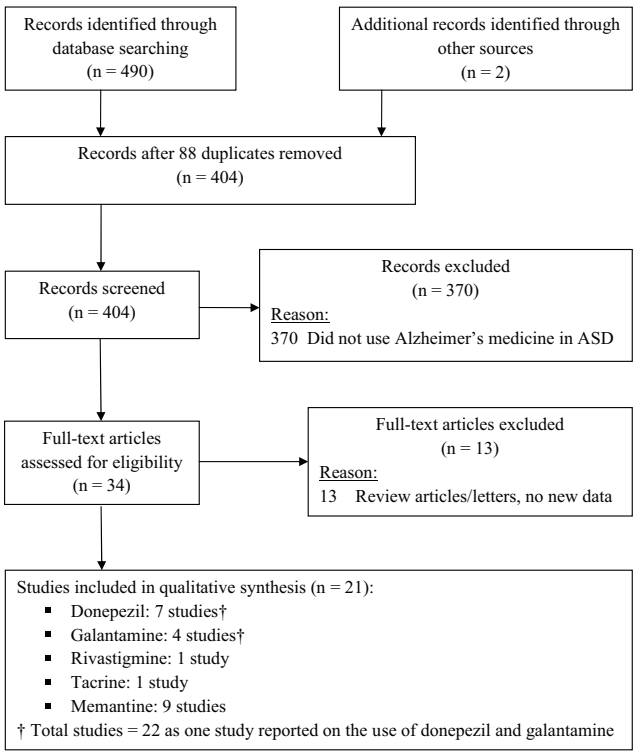

FIGURE 1 | Study flow chart.

inception through the end of April, 2014 was conducted to identify pertinent articles using the search terms "autism," "autistic," "ASD," "pervasive developmental disorder," "PDD," and "Asperger" in all combinations with the terms: "donepezil," "galantamine," "memantine," "rivastigmine," "tacrine," "Alzheimer," "cholinesterase inhibitor," and "NMDA antagonist." The references cited in identified articles were also searched to locate additional studies (two studies total). **Figure 1** depicts the studies identified during the search process.

STUDY SELECTION

Studies were included if they: (1) involved individuals with ASD, and (2) administered a medication approved for Alzheimer's disease to at least one individual with ASD. Articles that did not present new or unique data (such as review articles or letters to the editor) and animal studies were excluded. Studies on Rett syndrome and Childhood Disintegrative Disorder were also excluded. One reviewer screened titles and abstracts of all potentially relevant studies. After screening all records, 34 publications were identified and independently reviewed by both reviewers; 21 studies met inclusion criteria (see **Figure 1**). Within each section of this review, strengths and limitations of studies are discussed and recommendations for additional research are offered.

LEVEL OF EVIDENCE RATINGS

Although we considered conducting a meta-analysis on identified treatments, the lack of standard outcomes and the limitations in study design prevented a meta-analysis of any identified treatment.

Table 1 | Levels of evidence.

Level	Description
1a	SR or meta-analysis of RCTs with homogeneity or Cochrane review with favorable findings
1b	Prospective high-quality RCT
2a	SR of cohort (prospective, non-randomized) studies with homogeneity
2b	Individual cohort (prospective, non-randomized) study or low-quality RCT
3a	SR of case–control (retrospective) studies with homogeneity
3b	Individual case–control (retrospective) study
4	Case-series or reports
5	Expert opinion without critical appraisal or based on physiology or bench research

RCT, randomized controlled trial; SR, systematic review.

Table 2 | Grade of recommendation.

Grade	Description
A	At least one level 1a study or two level 1b studies
B	At least one level 1b, 2a, or 3a study, or two level 2b or 3b studies
C	At least one level 2b or 3b study, or two level 4 studies
D	Level 5 evidence, or troublingly inconsistent or inconclusive studies of any level, or studies reporting no improvements
N	No studies identified

As an alternative, we provide a grade of recommendation (GOR) for each treatment based on the level of evidence (LOE). Using a well-established scale (53), each study was individually assessed to determine the LOE, ranging from level 1 to 5 (**Table 1**). After assessing all identified studies for each treatment, a GOR ranging from A (solid evidence) to D (limited, inconsistent, or inconclusive evidence) was assigned (**Table 2**). Since a treatment could be a GOR of D for several reasons, we specified if the treatment received this rating because the evidence was a single case report or series (SC), was only based on bench research (BR), demonstrated an overall neutral effect (NE), or was found to be possibly detrimental (DE). If no studies were identified for a treatment, a GOR of N (no studies) was assigned. The overall ratings of Alzheimer disease medications used for ASD are found in **Table 3**. **Table 4** lists the types of symptoms improved with each particular medication, while **Table 5** compiles the side effects by medication.

RESULTS

DONEPEZIL

Seven studies reported on the use of donepezil in individuals with ASD, with five studies (71%) reporting improvements. Five studies were open-label or retrospective case-series. The first identified study was an uncontrolled, retrospective, open-label study of eight children (LOE 4) with autistic disorder (mean age 11.0 ± 4.1 years) and reported improvements in irritability and hyperactivity with donepezil (up to 10 mg/day) after at least 2 months of use; side effects included mild irritability (one patient) and gastrointestinal problems (nausea and vomiting in 1 patient) (54). Another

uncontrolled, retrospective case-series (LOE 4) of eight children with PDD (ages 10–17 years) used donepezil (2.5–30 mg/day; mean 18 weeks of treatment) for ADHD-type symptoms and reported improvements in ADHD symptoms, communication, and socialization; one patient had to stop the medication due to side effects (tremor, irritability, and distractibility) (55). An uncontrolled, open-label study (LOE 4) of five children with ASD (ages 2.5–6.9 years) with deficits in REM sleep administered donepezil, which increased the percentage of REM sleep as measured by polysomnography after 1 month of treatment (56). In a case report (LOE 4) of a 5-year-old child with ASD, treatment with donepezil (5 mg at bedtime) over 6 weeks led to significant improvements in communication, eye contact, and hyperactivity (57). Finally, one case report (LOE 4) of three adults with autism treated with galantamine reported that the use of donepezil (dose not reported) led to verbal and behavioral regression in one adult (58).

Two studies were double-blind, placebo-controlled (DBPC) studies. The first DBPC study contained 43 children (LOE 1b) with autism (mean age 6.8 years) and administered donepezil or placebo over 6 weeks and reported improvements in expressive and receptive speech and a decrease in overall autistic behaviors in the treatment group; side effects included diarrhea and stomach

Table 3 | Overall ratings of Alzheimer disease medications used for autism spectrum disorder.

Medication	Uncontrolled studies positive (positive/total)	Controlled studies positive (positive/total)	Grade of recommendation
Donepezil	80% (4/5)	50% (1/2)	D – NE
Galantamine	100% (2/2)	100% (2/2)	B
Rivastigmine	100% (1/1)		D – SC
Tacrine	100% (1/1)		D – SC
Memantine	88% (7/8)	100% (1/1)	B

Table 4 | Improvements reported in studies of Alzheimer disease medications in ASD.

Symptom	Donepezil	Galantamine	Rivastigmine	Tacrine	Memantine
Expressive language	X	X	X	X	X
Receptive language	X				X
Social interaction	X	X			X
Irritability	X	X		X	X
Hyperactivity	X	X		X	X
Attention	X	X			X
Eye contact	X	X		X	X
Emotional lability		X			
Repetitive or self-stimulatory behaviors					X
Motor planning					X
Disruptive behaviors					X
Obsessive–compulsive behaviors					X
Lethargy		X			
Overall ASD symptoms	X		X		
Increased REM sleep	X				

Table 5 | Reported side effects of Alzheimer disease medications in ASD.

Side effect	Donepezil	Galantamine	Rivastigmine	Tacrine	Memantine
Irritability	X				X
Gastrointestinal problems	X				X
Verbal or behavioral regression	X	X			
Headaches		X			
Worsened behaviors					X
Irritability					X
Rash					X
Excessive sedation					X
Vomiting					X
Speech problems					X
Tremor	X				
Distractibility	X				
Increased seizures					X

cramps (59). In the second DBPC study of 34 children (LOE 1b) with ASD (age range 8–17 years), donepezil (dose 5–10 mg/day), or placebo was administered over 10 weeks, with a 10-week open-label trial of the medication for children in the placebo group who did not respond. No significant improvements were found in the donepezil group compared to the placebo group; no serious adverse events were observed (60).

Overall, the evidence for donepezil is inconsistent for improvements in ASD symptoms as one of the two DBPC studies was negative and one of the five case-series reported detrimental effects rather than beneficial effects of donepezil. As two case-series reported improvements in ADHD symptoms and one case-series demonstrated improvements in REM sleep, donepezil may have favorable effects on subsets of children with ASD who have these specific symptoms. Clearly, several studies have demonstrated favorable effects of donepezil, suggesting that further studies focused on specific symptoms may be warranted in the future.

GALANTAMINE

Four studies reported on the use of galantamine in individuals with ASD, with all four studies reporting improvements. Two of the studies were uncontrolled, open-label, or case-series (LOE 4). In the first case-series (LOE 4) of three adults with autism (21 to 42 years old), galantamine 4–16 mg/day was reported to increase expressive language and communication; however; one individual had a regression when put on donepezil (58). The second study was a 12-week, uncontrolled, open-label study (LOE 4) of galantamine in 13 children with autism (mean age 8.8 ± 3.5 years), which reported improvements in parent-rated social withdrawal and irritability on the Aberrant Behavior Checklist (ABC) and improvements in attention and emotional lability on the Conner's Parent Rating Scale – Revised; eight patients were rated as improved on the Clinical Global Impression Scale (CGI); no significant adverse effect were found except for headaches in one child (61).

Two studies were DBPC. The first DBPC study of 20 children (LOE 2b) with autism (mean age 7.4 ± 3.2 years) reported a significant improvement with galantamine (dose not noted) compared to placebo on the ABC in irritability, eye contact, hyperactivity, and inappropriate speech; side effects were minimal (62). The second DBPC study of 40 children (LOE 1b) with autism (ages 4–12 years) reported that galantamine (up to 24 mg/day) for 10 weeks led to significant improvements in lethargy/social withdrawal and irritability on the ABC compared to placebo; side effects were similar in both groups (18).

Given that galantamine has been shown to improve both core and associated ASD symptoms in both open-label and DBPC studies (one high-quality and one of lower quality), it is given a GOR of B. Given this preliminary positive evidence, it is clear that large multicenter high-quality controlled trials should be conducted to provide efficacy data to further define the role of galantamine in the treatment of ASD.

RIVASTIGMINE

An open-label study of 32 children (LOE 4) with autism used rivastigmine (0.4–0.8 mg twice a day) for 12 weeks and reported improvements in expressive speech and overall autism symptoms (63). Given the limited number of studies on rivastigmine for the treatment of ASD, this treatment is given a GOR of D – SC. Because of the positive preliminary results and the fact that it was well tolerated, further larger open-label or blinded studies may be warranted for this treatment in ASD.

TACRINE

An open-label study of three individuals (LOE 4) with ASD (mean age 17.4 ± 33.2 years) administered 20 mg of tacrine daily and reported mild improvements in irritability, hyperactivity, eye contact, and inappropriate speech as rated by combined parent and teacher scales (ABC); no significant side effects were reported (64). Given the limited number of studies on tacrine for the treatment of ASD, this treatment is given a GOR of D – SC. Because of the positive preliminary results and the fact that it was well tolerated, further studies may be warranted for this treatment in ASD to see if a wider number of children with ASD respond to this treatment.

MEMANTINE

Nine studies reported on the use of memantine in individuals with ASD, with eight studies (89%) reporting improvements. Eight studies were open-label or retrospective case-series. In the first study, Chez et al. (63) administered open-label (LOE 4) memantine (mean dose 8.1 mg/day, range 2.5–10 mg/day) in 30 children (mean age 8.92 years) with ASD and reported that for those treated more than 8 weeks (mean duration 18 weeks, range 8–40 weeks), 16 (53%) demonstrated significant improvements and 10 (33%) showed more mild improvements in attention, eye contact, language (expressive and receptive), repetitive behaviors, and motor planning; no significant side effects were observed (65). One case report (LOE 4) described the effects of memantine on a 23-year-old man with autistic disorder and observed improvements in disruptive behavior with memantine 10 mg/day over an 8-month period (66). An uncontrolled, open-label (LOE 4) study of 151 individuals with ASD (ages 2.58–26.33 years old) used memantine at a dose ranging from 2.5 to 30 mg and reported improvements in language, self-stimulatory behaviors, and social behavior; 22 patients (15%) had worsened behavior as a side effect (67).

An uncontrolled, open-label, and retrospective study (LOE 4) examined the effects of memantine (maximum dose 20 mg/day; duration of use 1.5–56 weeks) in 18 children with ASD (age 6–19 years) and reported improvements in social withdrawal and inattention; 7 patients (39%) had side effects including irritability (4 patients), rash (1 patient), excessive sedation and vomiting (1 patient), and an increase in seizures (1 patient); 4 patients had to discontinue memantine (68). An uncontrolled, open-label (LOE 4) study of four patients with ASD (mean age 17.4 ± 33.2 years) administered memantine 20 mg daily for 4 weeks and reported significant improvements on combined parent and teacher ratings on the ABC in irritability, hyperactivity, and inappropriate speech ($p < 0.05$ for all three); no side effects were reported (69). An uncontrolled, open-label study (LOE 4) used memantine (starting at 5 mg, increasing every 2 weeks up to 20 mg/day; mean final dose 18.3 ± 2.6 mg/day; mean use 34.7 ± 36.5 weeks, range 8–104 weeks) in six individuals with Fragile X and concomitant PDD (mean age 18.3 ± 3.8 years, range 13–22 years; four had autistic disorder and two had PDD). Four of the six patients were rated

as "much improved" or "very much improved" on the CGI; non-significant improvements were observed on the ABC and SRS; two individuals experienced irritability, which led to drug discontinuation (70). A case report (LOE 4) of a 15-year-old boy with Asperger disorder, OCD, and Tourette disorder described the use of memantine (2.5 mg increasing to 10 mg/day) to treat the OCD symptoms and observed a significant reduction in OCD symptoms, including rituals and intrusive thoughts as well as improvements in social interaction; no significant adverse events were observed (71). One case study (LOE 4) reported stuttering and speech loss in two children with ASD who were taking memantine; in one child the speech improved with stopping the medication, while in the other child it improved while continuing the medication (72).

Only one study was DBPC and was a 10-week study of 40 children (LOE 1b) with ASD (ages 4–2 years). This study administered memantine (up to 15 mg/day if 10–40 kg; 20 mg/day if over 40 kg) compared to placebo and reported significant improvements on the ABC in irritability ($p < 0.001$), stereotypy ($p < 0.01$), and hyperactivity/non-compliance ($p < 0.01$); side effects were similar in both groups (73).

Given that the majority of clinical studies, including a DPBC study, provide positive evidence of improvements in both core and associated ASD symptoms, a GOR of B is provided for memantine for the treatment of ASD. Several studies have outlined adverse effects in certain patients with memantine treatment. Interestingly, memantine has been reported to both improve and worsen irritability. This suggests that there might be specific subgroups of children with ASD that respond optimally to memantine. Clearly larger, well-designed, and blinded studies are needed to further evaluate the efficacy of memantine in children with ASD as well as define the subgroups that might optimally respond to this medication.

DISCUSSION

This manuscript reviews the evidence for the use of medications which are FDA approved for Alzheimer's disease in individuals with ASD. These medications target two neurotransmitter systems, acetylcholine and glutamate, which are both neurotransmitter systems with abnormalities associated with ASD. Overall five medications, four which target acetylcholine neurotransmission and one that targets glutamate neurotransmission, which are FDA indicated for Alzheimer's disease have been used in individuals with ASD. To provide recommendations on the evidence for the potential usefulness of these medications for the treatment of ASD, we used an objective scale to rate the evidence for the utility of these medications for treating core and associated symptoms of ASD. Overall, we found that one medication that targets acetylcholine neurotransmission, galantamine, and one medication that targets glutamate neurotransmission, memantine, have reasonable evidence for the treatment of core and associated symptoms of ASD, although both require larger controlled studies to provide further efficacy data and define subgroups of individuals with ASD who may best respond to these treatments with limited adverse effects. Two medications, rivastigmine and tacrine, both of which target the acetylcholine neurotransmitter system, have only preliminary uncontrolled studies to support their use, so further studies need to be performed before recommendations can be made. One medication that targets acetylcholine, donepezil, has several studies investigating its use in individuals with ASD but the results of some of the studies, particularly the DBPC studies, are inconsistent, making recommendations difficult at this time, although in certain subgroups (ADHD symptoms or REM sleep problems) it might be of use.

Given the fact that there is no FDA approved medication for the core symptoms of ASD and considering the limited proven effective treatments for ASD, studies are needed to identify novel treatments. Because several of the medications reviewed here show promising evidence for effectiveness for treating core and associated ASD symptoms, such medications should undergo further study in clinical trials to confirm their effectiveness for treating individuals with ASD.

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REVIEW ARTICLE
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Treatments for biomedical abnormalities associated with autism spectrum disorder

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Recent studies point to the effectiveness of novel treatments that address physiological abnormalities associated with autism spectrum disorder (ASD). This is significant because safe and effective treatments for ASD remain limited. These physiological abnormalities as well as studies addressing treatments of these abnormalities are reviewed in this article. Treatments commonly used to treat mitochondrial disease have been found to improve both core and associated ASD symptoms. Double-blind, placebo-controlled (DBPC) studies have investigated L-carnitine and a multivitamin containing B vitamins, antioxidants, vitamin E, and co-enzyme Q10 while non-blinded studies have investigated ubiquinol. Controlled and uncontrolled studies using folinic acid, a reduced form of folate, have reported marked improvements in core and associated ASD symptoms in some children with ASD and folate related pathway abnormities. Treatments that could address redox metabolism abnormalities include methylcobalamin with and without folinic acid in open-label studies and vitamin C and *N*-acetyl-L-cysteine in DBPC studies. These studies have reported improved core and associated ASD symptoms with these treatments. Lastly, both open-label and DBPC studies have reported improvements in core and associated ASD symptoms with tetrahydrobiopterin. Overall, these treatments were generally well-tolerated without significant adverse effects for most children, although we review the reported adverse effects in detail. This review provides evidence for potentially safe and effective treatments for core and associated symptoms of ASD that target underlying known physiological abnormalities associated with ASD. Further research is needed to define subgroups of children with ASD in which these treatments may be most effective as well as confirm their efficacy in DBPC, large-scale multicenter studies.

Keywords: autism spectrum disorders, mitochondria, folate receptor alpha, folinic acid, folate metabolism, redox regulation, oxidative stress, tetrahydrobiopterin

BACKGROUND

The autism spectrum disorders (ASD) are a group of behaviorally defined neurodevelopmental disorders with lifelong consequences. They are defined by impairments in communication and social interaction along with restrictive and repetitive behaviors (1). The definition of ASD has recently undergone revision. Previously, the Diagnostic Statistical Manual (DSM) Version IV Text Revision divided ASD into several diagnoses including autistic disorder, Asperger syndrome, and pervasive developmental disorder-not otherwise specified. The new revision of the DSM now does not differentiate between these ASD subtypes and considers communication and social impairments together in one symptom class (2). Complicating this change is the fact that over the past several decades, most research has used a framework from the former DSM versions.

Autism spectrum disorder has been recently estimated to affect 1 out of 68 individuals in the United States (3) with four times more males than females being affected (4). Over the past two decades, the prevalence of the ASDs has grown dramatically, although the reasons for this increase are continually debated. Despite decades of research on ASD, identification of the causes of and treatments for ASD remain limited. The standard-of-care treatment for ASD is behavioral therapy that requires full-time engagement of a one-on-one therapist typically requiring many years of treatment, and recent reviews have pointed out that controlled studies on commonly used behavior therapies are generally lacking (5). The only medical treatments approved by the United States of America Food and Drug Administration for ASD are antipsychotic medications. However, these medications only treat a symptom associated with ASD, irritability, but not any core ASD symptom. In children, these medications can be associated with significant adverse effects, including detrimental changes in body weight as well as triglyceride, cholesterol, and blood glucose concentrations within a short time (6) and they also increase the risk of type 2 diabetes (7). In some studies, the percentage of children experiencing these side effects is quite high. For example, one recent study reported that 87% of ASD children had side effects with risperidone, including drowsiness, weight gain, and rhinorrhea (8).

A great majority of ASD research has concentrated on genetic causes of ASD (9) despite the fact that inherited single gene and chromosomal defects are only found in the minority of cases

(10). In fact, several recent studies that have conducted genome wide searches for common genetic defects across large samples of ASD children have only identified rare *de novo* mutations, thereby pointing to acquired mutations and/or mutations secondary to errors in DNA maintenance rather than inherited genetic syndromes (11, 12). As research in the field of ASD continues, it is becoming clear that the etiology of most ASD cases involves complicated interactions between genetic predisposition and environmental exposures or triggers. Indeed, a recent study of dizygotic twins estimated that the environment contributes a greater percentage of the risk of developing autistic disorder as compared to genetic factors (13). Another study of over two million children reported that environmental risk factors accounted for approximately 50% of ASD risk (14). Recent reviews have outlined the many environmental factors that are associated with ASD and have described how polymorphisms in specific genes can combine with the environment to cause neurodevelopmental problems (15).

Recent studies have suggested that ASD is associated with impairments in basic physiological processes such as redox (16) and mitochondrial (9) metabolism as well as abnormalities in regulating essential metabolites such as folate (17), tetrahydrobiopterin (18–20), glutathione (21–23), cholesterol (24), carnitine (25–28), and branch chain amino acids (29). Although many of these studies have based their findings on peripheral markers of abnormal metabolism, many studies have documented some of these same abnormalities in the brain of individuals with ASD, including mitochondrial dysfunction and oxidative stress (30) and one study has demonstrated a link between oxidative stress, inflammation, and mitochondrial dysfunction in the brain of individuals with ASD (23). Interestingly, several of these physiological abnormalities are also observed in genetic syndromes associated with ASD. For example, mitochondrial dysfunction is prevalent in both idiopathic ASD (31) and is associated with Rett syndrome (32–34), PTEN mutations (35), Phelan-McDermid syndrome (36), 15q11-q13 duplication syndrome (37, 38), Angelman syndrome (39), Septo-optic dysplasia (40), and Down syndrome (41, 42).

Identifying the metabolic or physiological abnormalities associated with ASD is important, as treatments for such abnormalities may be possible. Thus, a better understanding of these abnormalities may allow for the development of novel treatments for children with ASD. Below the evidence for metabolic abnormalities related to ASD that may be amenable to treatment are discussed along with the evidence of potential treatments for these disorders. **Figure 1** provides a summary of the pathways and demonstrates which pathways are targeted by the better studied treatments. In addition, a section on the common adverse effects of these treatments follows the discussion of treatments.

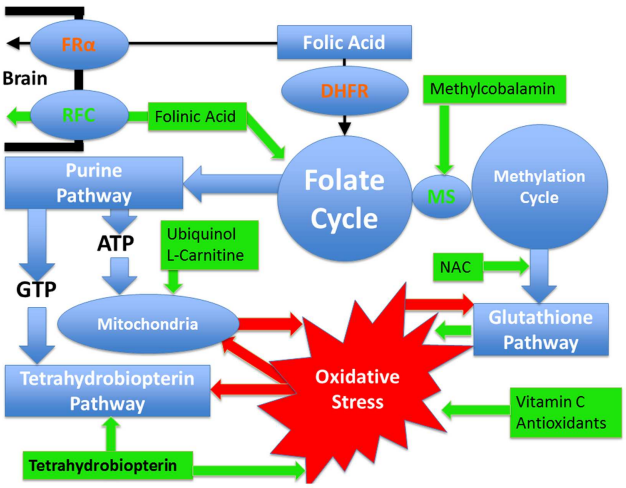

FIGURE 1 | Pathways affected in autism spectrum disorder that are discussed in this article as well as the treatments discussed with their points of action. Pathways are outlined in blue while treatments are outlined in green. Oxidative stress is outlined in red and the red arrows demonstrate how it can negatively influence metabolic pathways. Certain pathways such as glutathione and tetrahydrobiopterin pathways have an antioxidant effect and a reciprocal relationship with oxidative stress such that they can improve oxidative stress but at the same time oxidative stress has a direct detrimental effect on them. Mitochondrial dysfunction and oxidative stress have mutually negative effects on each other such that oxidative stress causes mitochondrial dysfunction while mitochondrial dysfunction worsens oxidative stress. Dihydrofolate reductase (DHFR) is colored in red since polymorphisms in this gene, that are commonly seen in individuals with autism, have a detrimental effect on the reduction of folic acid such that the entry of folic acid into the folate cycle is decreased. Folinic acid enters the folate cycle without requiring this enzyme. Similarly the folate receptor alpha can be impaired in individuals with autism by autoantibodies and by mitochondrial dysfunction. In such cases, folinic acid can cross the blood–brain barrier by the reduced folate carrier. Methionine synthase (MS) connects the folate and methylation cycles and requires methylcobalamin as a cofactor.

REVIEW OF TREATABLE CONDITIONS AND THEIR POTENTIAL TREATMENTS

MITOCHONDRIAL DYSFUNCTION

Recent studies suggested that 30–50% of children with ASD possess biomarkers consistent with mitochondrial dysfunction (31, 43) and that the prevalence of abnormal mitochondrial function in immune cells derived from children with ASD is exceedingly high (44, 45). Mitochondrial dysfunction has been demonstrated in the postmortem ASD brain (23, 30, 46–49) and in animal models of ASD (50). Novel types of mitochondrial dysfunction have been described in children with ASD (28, 51, 52) and in cell lines derived from children with ASD (53, 54). Several studies suggest that children with ASD and mitochondrial dysfunction have more severe behavioral and cognitive disabilities compared with children who have ASD but without mitochondrial dysfunction (55–57). Interestingly, a recent review of all of the known published cases of mitochondrial disease and ASD demonstrated that only about 25% had a known genetic mutation that could account for their mitochondrial disease (31).

Treatments that are typically used for patients with mitochondrial disease have been shown to improve functioning in some children with ASD (31). Several studies, including two double-blind, placebo-controlled (DBPC) studies (58, 59) and case reports (25, 37, 60–63) have reported improvements in core and associated ASD behaviors with L-carnitine treatment. Two DBPC studies using a multivitamin containing B vitamins, antioxidants, vitamin E, and co-enzyme Q10 reported various

improvements in ASD symptoms compared to placebo (64, 65). Several other antioxidants (66), including vitamin C (67), methylcobalamin (68–70), *N*-acetyl-L-cysteine (71–73), ubiquinol (74), and carnosine (75), have also reported to demonstrate significant improvements in ASD behaviors and may function to improve mitochondrial function.

Thus, many treatments that are believed to improve mitochondrial function have been shown to be helpful for some children with ASD. However, none of these studies have specifically selected children with mitochondrial dysfunction or disease to study, so it is difficult to know if individuals with ASD and mitochondrial dysfunction would benefit the most from these treatments or whether these treatments are effective for a wider group of children with ASD. One study did demonstrate that the multivitamin used for treatment resulted in improvements in biomarkers of energy metabolism (as well as oxidative stress) suggesting that the effect of the multivitamin may have been at least partially related to improvements in mitochondrial function (65). Clearly, this is a fertile area for research but there remain several complications that could impede moving forward in a systematic way. For example, given the inconsistency in the prevalence estimates of mitochondrial disease and dysfunction across studies (ranging from about 5–80%), the notion that mitochondrial abnormalities are even associated with ASD is somewhat controversial. This may be, in part, due to the unclear distinction between mitochondrial disease and dysfunction. However, even the lower bound of the prevalence estimate of 5% is significant, as mitochondrial disease is only believed to affect <0.1% of individuals in the general population and given the current high prevalence of ASD, a disorder that affects even 5% of individuals with ASD would add up to millions of individuals who have the potential to have a treatable metabolic abnormality. Other complicating factors include the fact that there are many treatments for mitochondrial disease and these treatments have not been well-studied (76). Hopefully, the increased interest in treatments for mitochondrial disease will help improve our knowledge of how to best treat mitochondrial disease so that such information can be applied to children who have mitochondrial disease and dysfunction with ASD. Other recent approaches include the *in vitro* assessment of compounds that may improve mitochondrial function in individuals with ASD (53).

FOLATE METABOLISM

Several lines of evidence point to abnormalities in folate metabolism in ASD. Several genetic polymorphisms in key enzymes in the folate pathway have been associated with ASD. These abnormalities can cause decreased production of 5-methyltetrahydrofolate, impair the production of folate cycle metabolites and decrease folate transport across the blood–brain barrier and into neurons. Indeed, genetic polymorphisms in methylenetetrahydrofolate reductase (22, 77–85), dihydrofolate reductase (86) and the reduced folate carrier (22) have been associated with ASD.

Perhaps the most significant abnormalities in folate metabolism associated with ASD are autoantibodies to the folate receptor alpha (FRα). Folate is transported across the blood–brain barrier by an energy-dependent receptor-mediated system that utilizes the FRα (87). Autoantibodies can bind to the FRα and greatly impair its function. These autoantibodies have been linked to cerebral folate deficiency (CFD). Many cases of CFD carry a diagnosis of ASD (88–94) and other individuals with CFD are diagnosed with Rett syndrome, a disorder closely related to ASD within the pervasive developmental disorder spectrum (95–97). Given that the FRα folate transport system is energy-dependent and consumes ATP, it is not surprising that a wide variety of mitochondrial diseases (91, 94, 97–102) and novel forms of mitochondrial dysfunction related to ASD (52) have been associated with CFD. Recently, Frye et al. (17) reported that 60% and 44% of 93 children with ASD were positive for the blocking and binding FRα autoantibody, respectively. This high rate of FRα autoantibody positivity was confirmed by Ramaekers et al. (103) who compared 75 ASD children to 30 non-autistic controls with developmental delay. The blocking FRα autoantibody was positive in 47% of children with ASD but in only 3% of the control children.

Many children with ASD and CFD have marked improvements in clinical status when treated with folinic acid – a reduced form of folate that can cross the blood–brain barrier using the reduced folate carrier rather than the FRα transport system. Several case reports (89) and case series (90, 91) have described neurological, behavioral, and cognitive improvements in children with documented CFD and ASD. One case series of five children with CFD and low-functioning autism with neurological deficits found complete recovery from ASD symptoms with the use of folinic acid in one child and substantial improvements in communication in two other children (90). In another study of 23 children with low-functioning regressive ASD and CFD, 2 younger children demonstrated full recovery from ASD and neurological symptoms, 3 older children demonstrated improvements in neurological deficits but not in ASD symptoms, and the remainder demonstrated improvements in neurological symptoms and partial improvements in some ASD symptoms with folinic acid; the most prominent improvement was in communication (91). Recently, in a controlled open-label study, Frye et al. (17) demonstrated that ASD children who were positive for at least one of the FRα autoantibodies experienced significant improvements in verbal communication, receptive and expressive language, attention, and stereotypical behavior with high-dose (2 mg/kg/day in two divided doses; maximum 50 mg/day) folinic acid treatment with very few adverse effects reported.

Thus, there are several lines of converging evidence suggesting that abnormalities in folate metabolism are associated with ASD. Evidence for treatment of these disorders is somewhat limited but it is growing. For example, treatment studies have mostly concentrated on the subset of children with ASD who also possess the FRα autoantibodies. These studies have only examined one form of reduced folate, folinic acid, and have only examined treatment response in limited studies. Thus, large DBPC studies would be very helpful for documenting efficacy of this potentially safe and effective treatment. In addition, the role of other abnormalities in the folate pathway beside FRα autoantibodies, such as genetic polymorphisms, in treatment response needs to be investigated. It might also be important to investigate the role of treatment with other forms of folate besides folinic acid, but it might also be wise to concentrate research on one particular form of folate for the time being so as to optimize the generalizability of research studies in order to have a more solid understanding of the role of folate

metabolism in ASD. Given the ubiquitous role of folate in many metabolic pathways and the fact that it has a role in preventing ASD during the preconception and prenatal periods (104), this line of research has significant potential for being a novel treatment for many children with ASD.

REDOX METABOLISM

Several lines of evidence support the notion that some children with ASD have abnormal redox metabolism. Two case-control studies have reported that redox metabolism in children with ASD is abnormal compared to unaffected control children (22, 105). This includes a significant decrease in reduced glutathione (GSH), the major intracellular antioxidant, and mechanism for detoxification, as well as a significant increase in the oxidized disulfide form of glutathione (GSSG). The notion that abnormal glutathione metabolism could lead to oxidative damage is consistent with studies which demonstrate oxidative damage to proteins and DNA in peripheral blood mononuclear cells and postmortem brain from ASD individuals (23, 30, 106), particularly in cortical regions associated with speech, emotion, and social behavior (30, 107).

Treatments for oxidative stress have been shown to be of benefit for children with ASD. In children with ASD, studies have demonstrated that glutathione metabolism can be improved with subcutaneously injected methylcobalamin and oral folinic acid (69, 105), a vitamin and mineral supplement that includes antioxidants, co-enzyme Q10, and B vitamins (65) and tetrahydrobiopterin (20). Interestingly, recent DBPC studies have demonstrated that *N*-acetyl-L-cysteine, a supplement that provides a precursor to glutathione, was effective in improving symptoms and behaviors associated with ASD (72, 73). However, glutathione was not measured in these two studies.

Small (64, 67), medium (72, 73), and large (108) sized DPBC trials and small and medium-sized open-label clinical trials (68, 70) demonstrate that novel treatments for children with ASD, which can address oxidative stress are associated with improvements in core ASD symptoms (68, 70, 72), sleep and gastrointestinal symptoms (64), hyperactivity, tantruming, and parental impression of general functioning (108), sensory-motor symptoms (67), and irritability (72, 73). These novel treatments include *N*-acetyl-L-cysteine (72, 73), methylcobalamin with (69, 70) and without (68) oral folinic acid, vitamin C (67), and a vitamin and mineral supplement that includes antioxidants, co-enzyme Q10, and B vitamins (64, 65).

Several other treatments that have antioxidant properties (66), including carnosine (75), have also been reported to significantly improve ASD behaviors, suggesting that treatment of oxidative stress could be beneficial for children with ASD. Many antioxidants can also help improve mitochondrial function (31), suggesting that clinical improvements with antioxidants may occur through a reduction of oxidative stress and/or an improvement in mitochondrial function.

These studies suggest that treatments that address oxidative stress may improve core and associated symptoms of ASD. Furthermore, these treatments are generally regarded as safe with a low prevalence of adverse effects. Unfortunately many studies that have looked at antioxidants and treatments that potentially support the redox pathway did not use biomarkers to measure redox metabolism status in the participants or the effect of treatment on redox pathways. Including biomarkers in future studies could provide important information regarding which patients may respond to treatments that address redox metabolism and can help identify the most effective treatments. Since there are many treatments used to address oxidative stress and redox metabolism abnormalities in clinical practice and in research studies, the most effective treatments need to be carefully studied in DBPC studies to document their efficacy and effectiveness. Overall, the treatments discussed above have shown some promising results and deserve further study.

TETRAHYDROBIOPTERIN METABOLISM

Tetrahydrobiopterin (BH_4) is a naturally occurring molecule that is an essential cofactor for several critical metabolic pathways, including those responsible for the production of monoamine neurotransmitters, the breakdown of phenylalanine, and the production of nitric oxide (19). BH_4 is readily oxidized by reactive species, leading it to be destroyed in the disorders where oxidative stress is prominent such as ASD (18). Abnormalities in several BH_4 related metabolic pathways or in the products of these pathways have been noted in some individuals with ASD, and the cerebrospinal fluid concentration of BH_4 has been reported to be depressed in some individuals with ASD (19). Clinical trials conducted over the past 25 years have reported encouraging results using sapropterin, a synthetic form of BH_4, to treat children with ASD (19). Three controlled (109–111) and several open-label trials have documented improvements in communication, cognitive ability, adaptability, social abilities, and verbal expression with sapropterin treatment in ASD, especially in children younger than 5 years of age and in those who are relatively higher functioning at the beginning of the trial (19).

Frye has shown that the ratio of serum citrulline-to-methionine is related to the BH_4 concentration in the cerebrospinal fluid, suggesting that abnormalities in both oxidative stress and nitric oxide metabolism may be related to central BH_4 deficiency (18). More recently, Frye et al. demonstrated, in an open-label study, that sapropterin treatment improves redox metabolism and fundamentally alters BH_4 metabolism in children with ASD. Interestingly, serum biomarkers of nitric oxide metabolism were found to predict response to sapropterin treatment in children with ASD (20), thereby suggesting that the therapeutic effect of BH_4 supplementation may be specific to its effect on nitric oxide metabolism.

The potential positive effects on nitric oxide metabolism by BH_4 supplementation could be significant for several reasons. The literature supports an association between ASD and abnormalities in nitric oxide metabolism. Indeed studies have documented alterations in nitric oxide synthase genes in children with ASD (112, 113). In the context of low BH_4 concentrations, nitric oxide synthase produces peroxynitrite, an unstable reactive nitrogen species that can result in oxidative cellular damage. Indeed, nitrotyrosine, a biomarker of reactive nitrogen species, has been shown to be increased in multiple tissues in children with ASD, including the brain (22, 23, 107, 114, 115). Thus, BH_4 supplementation could help stabilize nitric oxide synthase as well as act as an antioxidant and improve monoamine neurotransmitter production. Further

DBPC studies using biomarkers of metabolic pathways related to BH_4 metabolism will be needed to determine which children with ASD will most benefit from formulations of BH_4 supplementation like sapropterin.

POTENTIAL ADVERSE EFFECTS

Although many of the treatments discussed within this manuscript are considered safe and are generally well-tolerated, it is important to understand that these treatments are not without potential adverse effects. In general, these treatments are without serious adverse effects but some children may not tolerate all treatments well. Systematic and controlled studies are best at providing data on adverse effects, so the true adverse effects of the supplements discussed will only be based on the limited treatments that have been studied in such a fashion. It is also important to understand that because of the complicated nature of the effects of these treatments, they should only be used under the care of a medical professional with appropriate expertise and experience.

Controlled studies for treatments that address mitochondrial disorders include L-carnitine and a multivitamin with various mitochondrial supplements. In one small DBPC study, there were no significant adverse events reported in the 16 children treated with L-carnitine (59) while a second small DBPC trial reported no differences between the adverse effects reported by the treatment and placebo groups; notably, more patients in the placebo group withdrew from the study because of adverse effects (58). Thus, there is no data to suggest that L-carnitine has any significant adverse effects. In the large DBPC multivitamin study, about equal numbers of children in the treatment and placebo groups withdrew from the study because of behavior or gastrointestinal issues (65). In another small DBPC study, the investigators noted that two children began to have nausea and emesis when they started receiving the treatment at nighttime on an empty stomach (64). This adverse effect resolved when the timing of the treatment was adjusted. Thus, with proper dosing of this multivitamin, it appears rather safe and well-tolerated.

Controlled studies for folate pathway abnormalities only include folinic acid. In a medium-sized, open-label controlled study, 44 children with ASD and the FRα autoantibody were treated with high-dose folinic acid (2 mg/kg/day in two divided doses; maximum 50 mg/day) and four children discontinued the treatment because of an adverse effect (17). Of the four children who discontinued the treatment, three children, all being concurrently treated with risperidone, demonstrated increased irritability soon after starting the high-dose folinic acid while the other child experienced increased insomnia and gastroesophageal reflux after 6 weeks of treatment. Since there was no placebo in this study, the significance of these adverse effects is difficult to determine. For example, it is not clear whether this was related to concurrent risperidone treatment or was related to a baseline high irritability resulting in the needed for risperidone. All other participants completed the trial without significant adverse effects. Due to the timing of the adverse events in the children on risperidone in this trial, to be safe, the authors suggested caution when using folinic acid in children already on antipsychotic medications.

Clinical studies for treatments that could address redox metabolism include *N*-acetyl-L-cysteine, methylcobalamin, methylcobalamin combined with oral folinic acid and a multivitamin (as previous mentioned). One small open-label study that provided 25–30 μg/kg/day (1500 μg/day maximum) of methylcobalamin to 13 patients found no adverse effects (68) while a medium-sized, open-label trial that provided 75 μg/kg subcutaneously injected methylcobalamin given every 3 days along with twice daily oral low-dose (800 μg/day) folinic acid to 44 children noted some mild adverse effects (69, 70). Four children discontinued the treatment, two because their parents were uncomfortable given injections and two because of hyperactivity and reduced sleep. The most common adverse effect in the participants that remained in the study was hyperactivity, which resolved with a decrease in the folinic acid to 400 μg/day. Lastly, two medium sized, DBPC studies examined *N*-acetyl-L-cysteine, one as a primary treatment and another as an add-on to risperidone. The trial that used *N*-acetyl-L-cysteine as a primary treatment noted no significant differences in adverse events between the treatment and placebo groups, although both groups demonstrated a high rate of gastrointestinal symptoms and one participant in the active treatment phase required termination due to increased agitation (72). In the add-on study, one patient in the active treatment group withdrew due to severe sedation (73). In this latter study, adverse effects were not compared statistically between groups, but most adverse effects were mild and had a low prevalence. Such adverse effects included constipation, increased appetite, fatigue, nervousness, and daytime drowsiness. Lastly, a small DPBC study using vitamin C did not report any adverse effects from the treatment (67). Thus, there are several relatively safe and well-tolerated treatments for addressing abnormal redox metabolism, but there does appear to be a low rate of adverse effects, reinforcing the notion that a medical professional should guide treatment.

Three DBPC studies, one small (110), one medium (111), and one medium-to-large (109) sized, were conducted using sapropterin as a treatment for ASD. None of these studies have reported a higher prevalence of adverse effects in the treatment group as compared to the placebo group and none of these studies attributed any dropouts to the treatment. Thus, sapropterin appears to be a well-tolerated treatment.

DISCUSSION

One advantage of the treatments outlined above is that the physiological mechanisms that they address are known and biomarkers are available to identify children who may respond to these treatments. Preliminary studies suggest that there are a substantial number of ASD children with these metabolic abnormalities. For example, mitochondrial abnormalities may be seen in 5–80% of children with ASD (31, 43–45, 53, 54) and FRα autoantibodies may be found in 47% (103) to 75% (17) of children with ASD. Clearly, further studies will be required to clarify the percentage of these subgroups.

Further large-scale, multicenter DBPC clinical trials are needed for these promising treatments in order to document the efficacy and define the subgroups that best respond to these treatments. As more treatable disorders are documented and as data accumulates to demonstrate the efficacy of treatments for these disorders, clinical algorithms to approach the work-up for a child with ASD need to be developed by a consensus of experts. Indeed, developing

guidelines will be the next step for applying many of these scientific findings. Clearly many children with ASD may be able to benefit from such treatments, which are focused on improving dysfunctional physiology. Given the fact that no approved medical treatment exists which addresses the underlying pathophysiology or core symptoms of ASD, these treatments could make a substantial difference in the lives of children with ASD and their families. With the high prevalence of ASD, treatments that successfully treat even only a fraction of children affected with ASD would translate into substantial benefits for millions of individuals with ASD and their families. In summary, it appears that many of these treatments may provide benefit for a substantial proportion of children with ASD.

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Potential therapeutic use of the ketogenic diet in autism spectrum disorders

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The ketogenic diet (KGD) has been recognized as an effective treatment for individuals with glucose transporter 1 (GLUT1) and pyruvate dehydrogenase (PDH) deficiencies as well as with epilepsy. More recently, its use has been advocated in a number of neurological disorders prompting a newfound interest in its possible therapeutic use in autism spectrum disorders (ASD). One study and one case report indicated that children with ASD treated with a KGD showed decreased seizure frequencies and exhibited behavioral improvements (i.e., improved learning abilities and social skills). The KGD could benefit individuals with ASD affected with epileptic episodes as well as those with either PDH or mild respiratory chain (RC) complex deficiencies. Given that the mechanism of action of the KGD is not fully understood, caution should be exercised in ASD cases lacking a careful biochemical and metabolic characterization to avoid deleterious side effects or refractory outcomes.

Keywords: epilepsy, autism spectrum disorders, dietary intervention, mitochondria, bioenergetics, ketogenic diet, oxidative stress

BIOCHEMISTRY OF THE KGD

The ketogenic diet (KGD) is a nutritional approach constituted by high-fat content with adequate protein amount for growth but insufficient levels of carbohydrates for metabolic needs (1), thus forcing the body to primarily use fat as a fuel source. The original KGD was designed as 4:1 lipid:non-lipid (carbohydrate plus protein) ratio with 80% fat, 15% protein, and 5% carbohydrate. Most of the fat is provided as long-chain triglycerides, composing ~80% of the estimated caloric dietary requirement (2). To date, several modifications to the original KGD have been introduced such as lowering the lipid:non-lipid ratio (3) and decreasing the caloric intake from fat (~60–70%) with either no restriction in calorie amount with unlimited protein and fat intake (modified Atkins diet) (4, 5), or with fat provided as triglycerides esterified with medium-chain fatty acids (FA) (to overcome deficits in carnitine metabolism; medium-chain triglyceride diet) (6).

The hormonal changes associated with a KGD include changes in circulating insulin (due to insulin reduction in response to decreasing plasma glucose) and/or leptin (7–9), thus limiting glucose utilization. Under normal conditions, FA mobilized from adipose tissue are catabolized to acetyl coenzyme A (CoA) via β-oxidation, and then oxidized to CO_2 and H_2O in the Krebs' cycle. However, when an imbalance is created between the rate of FA mobilization and the capacity of the Krebs' cycle to process acetylCoA (e.g., low-carbohydrate and/or protein diet), the liver converts the excess of acetylCoA into ketone bodies (KB), namely acetoacetate (ACA) and D-β-hydroxybutyrate (BHB). A significant fraction of acetone (~30%), the product of the spontaneous decarboxylation of ACA, is found in urine, sweat, and breath (10, 11). KB are utilized as fuel by peripheral tissues sparing glucose and muscle wasting. They generate a comparable amount of energy to protein or carbohydrates (2.7 vs. 4 kcal/g) and, unlike FA, KB can cross the blood–brain barrier (12) constituting the main fuel sources for the brain during fasting periods (13). Most ATP from BHB is via Complex I (70–80%), with the rest via Complex II (14). The low-carbohydrate intake forces the body to sustain systemic glycemia by hepatic gluconeogenesis from non-carbohydrate precursors (e.g., lactate, glucogenic amino acids, and glycerol).

At the center of intermediary metabolism reside mitochondria. These dynamic organelles whose morphology, composition, and function adapt to changes in response to pathological and physiological signals respond to nutritional variations such as those introduced by KGD. Several reports in the literature document changes in mitochondrial number or function in a variety of biological systems, from *in vitro* to *in vivo*, when exposed to KGD or KGD-mimetics (**Table 1**).

THERAPEUTIC USE OF THE KETOGENIC DIET IN HUMAN DISEASES

By providing alternative sources of acetylCoA, KGD is the dietary intervention for inborn genetic disorders in pyruvate dehydrogenase (PDH) and glucose transporter 1 (GLUT1) (**Table 1**), proven effective also in other metabolic conditions, including phosphofructokinase deficiency and glycogenosis type V (McArdle disease) (37). The KGD has also been investigated for the management of neurological disorders such as Alzheimer's and Parkinson's diseases (38).

Ketogenic diet has been utilized for >80 years in epilepsy treatment (39, 40) especially in children and adolescents (1, 41) with reduction in seizure frequencies (2, 42) and improvements in developmental progress (26).

Evidence supporting the use of the KGD for patients with intractable epilepsy and respiratory chain (RC) complex defects has been reported in which the majority of patients responded with decreased seizure frequencies, regardless of the RC complex defect or magnitude of deficit (27). The administration of KGD

Table 1 | Examples extracted from the literature on effects of KGD on mitochondrial function with the potential to benefit ASD symptoms.

Experimental model	Diet/treatment	KGD-dependent effects	Source
OUTCOMES RELATED TO ENERGY RESERVES AND/OR ENERGY-SENSING PATHWAYS			
Rat hippocampus	Young rats fed KGD for 9 weeks	Increased gene expression of mt genes; 46% increase in mitochondria number with no changes in citrate synthase or any other mt enzymatic activity; [PCr]/[Cr] higher (due to lower [Cr])	Bough et al. (15)
Rat hippocampus	Young rats fed KGD for 1 month	Decreased (−30%) body weight than controls; few mt genes overexpressed	Noh et al. (16)
Rat brain	Fed HFD for 3 weeks	[ATP]/[ADP] increased by 12%; lower [Cr] with no changes in [PCr]; lower [cAMP] and [cGMP]	DeVivo et al. (17)
Rat hippocampus	Slices from rat hippocampus (4–7 weeks) with BHB and ACA each at 0.5 or 1 mM	KB prevented rotenone- and 3NP-dependent decrease in ATP and decreased 3NP-dependent ROS production	Kim do et al. (18)
Mouse brain	Mice (8–10 weeks) treated with d-BHB or l-BHB via pumps	BHB restored NADH-supported O_2 consumption inhibited by MPP^+, partly the one inhibited by rotenone; BHB increased mtROS. 70–80% ATP from BHB produced via Complex I, the remaining via Complex II	Tieu et al. (19)
Rats	CR-KGD for 7 days	Body weight loss, increased brain expression of IGFR and GLUT3	Cheng et al. (14)
Neuronal human SH–SY5Y cell line	FA (C8 or C10) treatment for 1–6 days	Increased citrate synthase and Complex I activities	Hughes et al. (20)
Rat hippocampus and liver	Rats fed with a 6:1 lipid:non-lipid KGD	Delayed occurrence of epileptic episodes via mTOR inhibition	McDaniel et al. (21)
OUTCOMES RELATED TO NEUROLOGICAL SYMPTOMS/BEHAVIOR WITH RC COMPLEX AND/OR PDH DEFICIENCIES			
Child with Leigh syndrome	KGD	Improvement of cerebral lesions by brain MRI	Wijburg et al. (22)
Individuals with PDH deficiency (PDHA1 an PDHX mutations)	KGD (lipid:non-lipid 3:1)	KGD improved only paroxysmal dysfunction	Barnerias et al. (23)
Child, idiopathic PDH deficiency	KGD for ~3 years (lipid:non-lipid 3:1 later switched to 2:1)	Seizure free; improvement in hypotonia, motor development, relationship with environment; poor weight gain, high ketonemia	Di Pisa et al. (24)
Children with PDHE1 mutations	KGD (varied degrees of carbohydrate restriction)	Improved longevity and mental development	Wexler et al. (25)
Child with PHDX	KGD (lipid:non-lipid 4:1, later switched to 3:1 plus MCT oil)	Weight gain, decreased seizure episodes, improved sociability and activity	El-Gharbawy et al. (26)
Children with intractable epilepsy with ETC defects	Age (mean) 45 months, KGD (4:1 lipid:non-lipid) for (mean) 18 months	Eleven of 14 patients decreased seizure frequency by 50–90%; 8 ceased or lowered antiepileptic medications; 8 showed improved cognitive and behavioral functions	Kang et al. (27)
OUTCOMES RELATED TO MITOCHONDRIAL ANTIOXIDANT DEFENSES AND ROS			
Mouse hippocampus	Young mice fed a 6:1 lipid:non-lipid KGD for 10–12 d	Decreased mtROS; increases in UCP expression	Sullivan et al. (28)

(Continued)

Table 1 | Continued

Experimental model	Diet/treatment	KGD-dependent effects	Source
Rat hippocampus	Adolescent rats, KGD (78% lipid, 0.76% carbs) for 1, 3 days or 1, 3 weeks	KGD-induced initial mild oxidative stress, activation of Nrf2 pathway	Milder et al. (29)
Rat cortex, cerebellum, and hippocampus	Adolescent rats fed with KGD or BHB for 3 weeks	Increased GPX activity and [GSH]	Ziegler et al. (30), Jarrett et al. (31)
Rat neocortical neurons	Neurons exposed to BHB *in vitro*	Decreased Glu-mediated excitotoxicity mtROS production via increased NADH oxidation	Maalouf et al. (32)
OUTCOMES RELATED TO MITOCHONDRIA-DERIVED NEUROTRANSMITTER METABOLISM			
Mouse forebrain	Ketotic mice fed KGD (50% lipids) for 3 days	Increased GABA and Gln production	Yudkoff et al. (33)
Cerebrospinal fluid	26 children with refractory epilepsy fed KGD for 6 months	Increased [GABA], [taurine], [Ser], and [Gly]. Higher [GABA] (>50–90% seizure reduction)	Dahlin et al. (34)
Zebrafish with PDHE1 mutation, lower acetylcholine in inner retina	Larvae fed a mix of lauric/myristic/palmitic acid, and phosphatidyl choline	KGD rescued vision and prolong survival	Maurer et al. (35)
SSDAH mouse model	At PND 12 were fed KGD for 20–30 days	Increased mitochondrial number and size; increased (ATP), no changes in lifespan or neurological outcomes	Nylen et al. (36)

3-NP, 3-nitropropionic acid; AHA, acetoacetate; BHB, β-hydroxybutyrate; CR-KGD, calorie-restricted ketogenic diet; Cr, creatine; Gln, glutamine; Glu, glutamate; Gly, glycine; GPX, glutathione peroxidase; FA, fatty acids; HFD, high-fat diet; IGFR, insulin-like growth factor receptor; Mt, mitochondrial; MCT, medium-chain triglycerides; Nrf2, Nuclear factor-like 2; PCr, phospho-creatine; PND, post-natal day; Ser, serine.

to epileptic patients (37, 39) has been based on the assumption that KB replace glucose as the major metabolic fuel to the brain, although the precise molecular steps still remain obscure. It has been proposed that KB metabolism is not the primary mechanism of this diet, but rather an outcome of the metabolic shifts that occur with this treatment (43) and that the anticonvulsant effects of the KGD could result from an altered gene expression profile accompanied by cellular adaptation mechanisms (15) needed to modify the brain to utilize KB over glucose over time (39).

THERAPEUTIC USE OF KGD IN ASD

Autism spectrum disorders (ASD) include a complex neurodevelopmental condition characterized by abnormal social interaction, verbal and non-verbal communication, and limited interest in the surrounding environment associated with stereotyped and repetitive behaviors (44). Limited scientific advances have been made regarding the causes of ASD, with general agreement that both genetic and environmental factors contribute to this disorder (44–47). ASD has been associated to metabolic dysfunction (44, 48) and autism is a common trait of epilepsy-associated diseases (49), and syndromes like Landau–Kleffner, Dravet (50, 51), and Rett (52, 53). Thus, given the beneficial effects of KGD on epilepsy and increased mitochondrial function, its use has the potential to ameliorate some of the ASD-associated symptoms.

Beneficial effects of KGD in children with ASD symptoms have been reported in two independent studies (54, 55). The first study evaluated the role of KGD on 30 ASD children (54). The John Radcliffe diet (a modified medium-chain triglyceride diet with a caloric distribution of 30% in medium-chain triglyceride oil, 30% fresh cream, 11% saturated fat, 19% carbohydrates, and 10% proteins) was administered for 6 months, with intervals of 4 weeks interrupted by two diet-free weeks. Of the 30 children, 40% did not comply or did not tolerate the diet. From the rest, the two children with the milder autistic behaviors showed the most improvement (as judged by total Childhood Autism Rating Scale score, concentration and learning abilities, and social behavior and interactions), while the rest displayed mild to moderate improvements. Interestingly, the beneficial effects of KGD persisted even after termination of the trial. Six of the children enrolled in this study had a higher baseline ketonemia with no apparent PDH and/or RC deficiencies; but it is not clear if any of the other patients underwent this screening, before and/or after the administration of the diet in addition to the lack of the inclusion of a control diet before administering the KGD to the ASD group or during the trial.

The other study (55) reports the administration of a gluten-free casein-free modified KGD (1.5:1 lipid:non-lipid ratio; medium-chain and polyunsaturated FA) for 14-months to a 12-year-old child with ASD and seizures with substantial medical comorbidities associated with a family history of metabolic and immune disturbances. Due to the improvements in seizure activity, improved electroencephalogram, cognitive and social skills, language function, and complete resolution of stereotypies, anticonvulsant medication doses were reduced without worsening of seizures. Of note, the administration of the diet was accompanied by a wealth of

medications, a significant weight loss, and transitioning to puberty, so it is difficult to assess the sole role of the diet with this clinical background.

In mouse models of ASD [i.e., Rett syndrome (56), BTBR model (57), and succinate semialdehyde dehydrogenase (SSADH) deficiency (36)], the use of the KGD has improved behavioral abnormalities (increased sociability and decreased self-directed repetitive behavior) and/or decreased the number of seizures, normalized ataxia, and increased lifespan of mutant mice. However, while the KGD was originally designed to be administered under controlled caloric intake (38), most of the mouse studies have been performed under *ad libitum* conditions and/or for a relatively short period [see Ref. (57)]. Moreover, a ketogenic low-carbohydrate diet does not have a significant metabolic advantage over a non-ketogenic low-carbohydrate diet as judged by equal effects in body weight reduction and decreased insulin resistance; however, the former one was associated with higher inflammatory risk and increased perception of fatigue (58).

Although the exact molecular mechanisms underlying the effect of the KGD are still under investigation, several scenarios are reported below to explore the potential therapeutic effects of the KGD in ASD.

KGD IN PDH DEFICIENCY

Peripheral blood mononucleated cell (PBMC) from children with high severity scores for ASD has shown impaired PDH activity (44). The KGD is recommended as an alternative source of the acetylCoA in patients (37) with pathogenic mutations in PDH- or GLUT1-encoding genes (22, 25) leading to amelioration of some symptoms (59, 60) especially in those with milder phenotypes (25, 61). Thus, the use of the KGD in ASD with PDH deficiencies might prove to be beneficial.

KGD IN β-OXIDATION DEFECTS

Some patients with ASD have been reported to have defects in fatty acid β-oxidation evidenced as long-chain acyl dehydrogenase deficiency (62) and high concentrations of short or long acyl-carnitines in plasma (63). Carnitine biosynthesis has been recently identified as a risk factor for ASD (64). Thus in these cases, it is advisable to limit the use of a high-fat diet or improve its safety by switching to short or medium-chain FA, which do not utilize the carnitine system.

KGD IN MITOCHONDRIAL BIOGENESIS

The KGD might improve mitochondrial function by enhancing mitochondrial biogenesis in murine models (15, 65). The medium-chain triglyceride diet (6) has been shown to produce significant increases in citrate synthase and Complex I activity in SH–SY5Y neurons (20). However, the increases in mitochondrial mass would need to result in an OXPHOS outcome of $\geq$30% [30% as the limit for minor diagnostic criteria of mitochondrial RC disorder (66)] for that particular tissue, given that each tissue has a different ATP threshold (67). Otherwise the increases in mass might not be sufficient to rescue the already impaired ATP production in ASD individuals. Moreover, given the presence of mitochondrial DNA (mtDNA) deletions in PBMC from ASD (44, 68, 69), the KGD-driven mitochondrial biogenesis may result in an enrichment of defective mitochondria due to the proliferating advantage of damaged or deleted mtDNA over wild-type (70, 71). Conversely, treatment of cells containing large-scale mtDNA deletions from a patient with Kearns–Sayre syndrome with KB shifted the heteroplasmy between and within cells (72). The observation that KB can distinguish between normal and respiration-compromised cells suggests that the KB may be useful in treating patients with heteroplasmic mtDNA disorders (72).

ROLE OF THE KGD IN RC COMPLEX DEFICITS

Children with ASD display an array of mitochondrial dysfunction (MD) of differing severity (44, 73–75). Electron transport chain (ETC) deficiencies have been reported in ASD, primarily in Complex I and IV, but also affecting others such as Complex II, III, and IV (44, 73, 74, 76). The prevalence of seizures (41%) has been observed to be significantly higher in individuals with ASD and MD than in the general ASD population (11%) (74), raising the possibility that epileptic episodes observed in ASD might have a mitochondrial origin. Indeed, epilepsy is a recurrent feature of many inherited "classic" mitochondrial disorders, like myoclonic epilepsy with ragged red fibers, mitochondrial encephalopathy with lactic acidosis, and stroke-like episodes (77), and Leigh syndrome (78). In a small study on children with ETC defects (**Table 1**), the KGD has been proven to reduce epileptic attacks, with far better prognosis among children with Complex I deficits than Complex IV (27). These results are not surprising given that KGD generates more NADH/$FADH_2$ than glucose (2 vs. 5).

EFFECT OF KGD ON ENERGY-SENSING PATHWAYS ALTERATIONS

Recently, KGD-fed rats showed increased brain expression of insulin-like growth factor receptor (ILGFR) and neuronal GLUT3 (14). The KGD might have a beneficial effect in some ASD cases considering that IGFR is important for brain health throughout life (79–81), and that IGFR and GLUT3 have both been implicated in ASD (82, 83).

Some energy-sensing molecules and metabolism regulators (including the mammalian target of rapamycin, mTOR) have been recently indicated as possible downstream targets of KGD and may be involved in neuroprotective effects associated to the diet (84). Defects in the mTOR pathway have been linked to ASD (85–87). Failure to inhibit mTOR pathway could lead to MD due to decreased mitophagy (88) resulting in an accumulation of dysfunctional mitochondria as observed in a mouse model of ASD with phosphatase and tensin homolog on chromosome ten (*Pten*) gene haploinsuffciency (89). Indeed, inhibition of mTOR has been linked to a delay in the occurrence of the epileptic episodes (90) and KGD-fed rats showed inhibition of the activation of the mTOR pathway in brain (21), thus representing an appropriate treatment to control seizures while enhancing the clearance of defective/damaged mitochondria.

ANTIOXIDANT AND NEUROPROTECTIVE ROLE OF THE KGD

Ketone bodies (without glucose and at concentrations 10-times higher than physiological ones) inhibit mitochondrial reactive oxygen species (ROS) production in rat neurocortical neurons by increasing NADH oxidation following glutamate (Glu) excitotoxicity (32). It has been suggested that the production of NADPH via

oxidation of succinate semialdehyde (SSA) into succinate in the Glu decarboxylase (GAD)/γ-aminobutyric acid (GABA) pathway may buffer the redox changes likely to occur in stressful conditions (91–93). However, other mitochondrial NADPH sources are quantitatively more important than SSADH and fatty acid oxidation produces more mitochondrial ROS than pyruvate oxidation (94).

Thus, the use of KGD could be beneficial in ASD given that higher rates of mitochondrial ROS production and compromised cellular antioxidant status (69, 95, 96) have been reported in peripheral cells from children with ASD (44, 68, 69).

EFFECT OF THE KGD ON GABAergic AND CHOLINERGIC SYSTEMS DISTURBANCES

The GABA shunt bypasses two steps of the tricarboxylic acid cycle – the α-ketoglutarate (KG) dehydrogenase complex and the succinylCoA synthase – for the conversion of KG into succinate (**Figure 1**). It involves three enzymes: a GAD, catalyzing the Glu decarboxylation to GABA, a GABA transaminase, converting GABA to SSA, and an SSADH, catalyzing the oxidation of SSA to succinate (97). This metabolic route (the GAD/GABA pathway) is conserved from bacteria, through yeast and plants, to vertebrates. In higher eukaryotes, SSA can be reduced to γ-hydroxybutyric acid (GHB) by an alternative reaction catalyzed by a GHB dehydrogenase (98–100). It has been proposed that KGD may limit the availability of oxaloacetate to aspartate aminotransferase, an enzyme involved in brain Glu metabolism, resulting in increased Glu or Gln availability to produce GABA (101). The increased conversion of Glu to GABA would be potentially beneficial in ASD (102–105) (**Figure 1**).

Changes in GABA neurotransmission by KGD might explain the decrease in seizure frequencies and improved behavior observed in Rett syndrome (106). Studies in patients with ASD strongly suggest a dysfunction in the GABAergic system (107–109). However, changes in other components (including Gly, taurine, and GABA) cannot be excluded (34). In the case of SSADH deficiency (SSADH), the KGD may work through restitution of GABAergic neurotransmission (36), although the use of KGD in SSADHD has been strongly argued until more research is performed to test its potential detrimental effects in humans (110). Conversely, ketotic rodents fed on KGD showed no changes

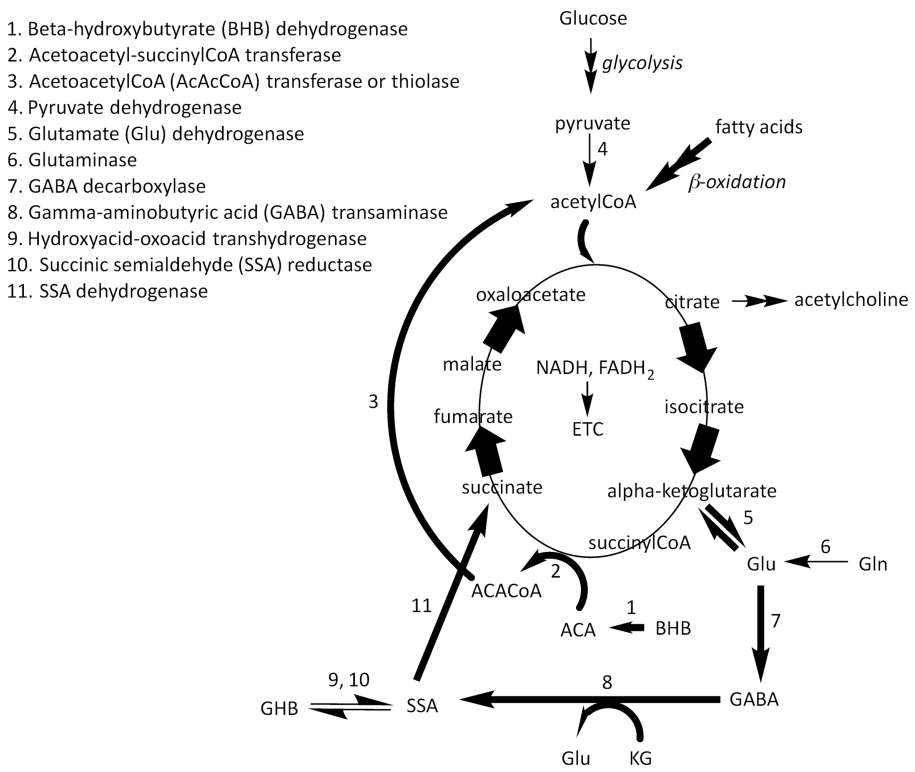

FIGURE 1 | β-hydroxybutyrate and ACA are utilized as fuel molecules in all mitochondria-containing tissues (except liver). BHB is oxidized to ACA by β-hydroxybutyrate dehydrogenase at the inner mitochondrial membrane (arrow 1). ACA acquires the CoA moiety from succinylCoA resulting in succinate and acetoacetylCoA (ACACoA; arrow 2). ACACoA releases acetylCoA catalyzed by ACACoA thiolase (arrow 3). AcetylCoA generated from β-oxidation of fatty acids from the diet and acetylCoA generated by the catabolism of KB is condensed into citrate in the Krebs cycle. The increased flux in the right part of this cycle, increases the concentration of α-ketoglutarate (KG) resulting in increases in the production of Glu via glutamate dehydrogenase (arrow 5) or a transaminase (not shown). Glu from these reactions in addition to that formed from the deamination of glutamine (Gln) via glutaminase (arrow 6) result in the generation of γ-aminobutyric acid (GABA). The GABA shunt bypasses two steps of the Krebs cycle – the KG dehydrogenase complex and the succinyl coenzyme A (CoA) synthase – for the conversion of KG into succinate. It involves three enzymes: a Glu decarboxylase (GAD; arrow 7), which catalyzes the decarboxylation of glutamate to GABA, a GABA transaminase (arrow 8), which converts GABA to succinate semialdehyde (SSA), and an SSA dehydrogenase (arrow 11), which catalyzes the oxidation of SSA to succinate. SSA can be reduced to γ-hydroxybutyric acid (GHB) by an alternative reaction catalyzed by either a hydroxyacid–oxoacid transhydrogenase or SSA reductase (arrows 9, 10).

in whole brain (GABA) [between brackets = concentrations; (33, 111)]; however, regional (GABA) changes cannot be ruled out (112), in addition to species-specific differences in the expression of GABA receptors subtypes (113, 114). Considering that cerebrospinal fluid from children treated with KGD showed higher (GABA) (34), it would be of interest to evaluate GABA and amino acid concentrations in different brain areas in animal models of ASD fed KGD.

Dysfunction in the cholinergic system has been observed when PDH deficits are present (115) because a block in this enzyme decreases (citrate), the precursor of acetylcholine via citrate lyase (116). Studies in humans and animal models of ASD suggested that dysfunction of the cholinergic system underlies ASD-related behavioral symptoms (117–119). Trials conducted on ASD individuals have shown beneficial effects of galantamine (an acetylcholinesterase inhibitor) in the management of aberrant behaviors in children and adolescents with ASD (120–122). Treatment of BTBR mice with the acetylcholinesterase inhibitor donepezil hydrochloride improved social preference, social interaction and decreased cognitive rigidity (123). Thus, a KGD has the potential to exhibit beneficial effects in individuals with both ASD and PDH deficiency because the metabolism of KB overcomes the decrease in (citrate) (124) and that of (acetylcholine).

POTENTIAL SIDE EFFECTS OF KGD IN ASD

Several side effects of KGD have been reported, among them: (a) limitation in protein, carbohydrate, and other nutrients intake can result in a lack of weight gain and growth inhibition (42), which could be detrimental in ASD because of a predisposition for being underweight (125) and the presence of eating disorders (126). Thiamine, lipoic acid, and L-carnitine supplementation have been helpful in selected cases (25). (b) Dyslipidemia from KGD (127, 128) would need to be supervised in ASD patients with β-oxidation deficits, including carnitine deficiency (64, 129) and, for older patients, the additional increased risk in heart disease and atherosclerosis (130). These patients should limit their fat intake or a modified KGD possibly with carnitine and/or coenzyme Q10 supplementation (131), should be used (132). (c) KGD has an increased risk of systemic ketosis, which may result in lower affinity of hemoglobin for oxygen, resulting in severe outcomes (e.g., coma and death) especially in anemic ASD patients (133). (d) Adverse events experienced by patients with RC complex deficits and epilepsy, which could be extrapolated to those with ASD, included symptomatic persistent hypoglycemia, persistent metabolic acidosis, aspiration pneumonia, and pneumonia followed by respiratory failure (27). (e) Initial fasting and prolonged caloric restriction can cause acute metabolic decompensation in ASD patients with metabolic disorders (134). To reduce the adverse effects of fasting, some studies have omitted the initial fasting period and substituted it with a gradual increase in calories (135). (g) Other side effects include constipation, slower growth, kidney stones, and gastroesophageal reflux (136), although most of them are treatable and/or preventable.

CONCLUDING REMARKS

More research is necessary to understand the potential therapeutic use of KGD in ASD as discussed at length for SSADHD (110). More specifically, how this diet may improve mitochondrial function in ASD and how this putative improvement derived from a better energy and/or neurotransmitter management may influence behavioral symptoms. There are concerns about utilizing KGD in patients with metabolic encephalopathies, with specific contraindications in pyruvate carboxylase deficiency, fatty acid oxidation disorders, and Krebs cycle disorders. Thus, given that the mechanism of action of KGD has not been yet fully understood, even in cases of improved behavioral symptoms, KGD in ASD might need to be prescribed on a case-by-case basis, upon careful biochemical characterization and metabolic profiling.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the work and interpretation of the literature, drafted the work, and gave final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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CLINICAL CASE STUDY
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Repeated insulin-like growth factor 1 treatment in a patient with Rett syndrome: a single case study

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Rett syndrome (RTT) is a devastating neurodevelopmental disorder that has no cure. Patients show regression of acquired skills, motor, and speech impairment, cardio-respiratory distress, microcephaly, and stereotyped hand movements. The majority of RTT patients display mutations in the gene that codes for the Methyl-CpG binding protein 2 (MeCP2), which is involved in the development of the central nervous system, especially synaptic and circuit maturation. Thus, agents that promote brain development and synaptic function are good candidates for ameliorating the symptoms of RTT. In particular, insulin-like growth factor 1 (IGF1) and its active peptide (1–3) IGF1 cross the Blood Brain Barrier, and therefore are ideal treatments for RTT Indeed, both (1–3) IGF1 and IGF1 treatment significantly ameliorates RTT symptoms in a mouse model of the disease In a previous study, we established that IGF1 is safe and well tolerated on Rett patients. In this open label clinical case study, we assess the safety and tolerability of IGF1 administration in two cycles of the treatment. Before and after each cycle, we monitored the clinical and blood parameters, autonomic function, and social and cognitive abilities, and we found that IGF1 was well tolerated each time and did not induce any side effect, nor it interfered with the other treatments that the patient was undergoing. We noticed a moderate improvement in the cognitive, social, and autonomic abilities of the patient after each cycle but the benefits were not retained between the two cycles, consistent with the pre-clinical observation that treatments for RTT should be administered through life. We find that repeated IGF1 treatment is safe and well tolerated in Rett patients but observed effects are not retained between cycles. These results have applications to other pathologies considering that IGF1 has been shown to be effective in other disorders of the autism spectrum.

Keywords: Rett syndrome, insulin-like growth factor 1, social cognition, seizures, autonomic functions

INTRODUCTION

The clinical case study performed in this experiment depicts the efficacy and safety of insulin-like growth factor 1 (IGF1) exposure upon two cycles of treatment. The patient is a young girl with a clinical and genetic diagnosis of Rett syndrome (RTT). The study started when the patient was 5 years old. The patient received first round of treatment on the 25th of May 2010 and discontinued on the 11th of November 2010. The second round of treatment started on the 20th of November 2012 and discontinued on the 28th of February 2013.

BACKGROUND

Rett syndrome is taxonomized as a pervasive neurodevelopmental disorder affecting mainly female children (1:10000) due to its X-linked method of inheritance (1). Methy-CpG binding protein 2 (MeCP2) dysfunction is associated with several neurodevelopmental disorders (1), and can cause a wide variety of phenotypes in females. Most males do not live past the first year due to neonatal encephalopathy and death in the classic RTT karyotype (2). The effects of MECP2 mutations lead to a loss of function or overexpression of the MeCP2 protein. If it results in a loss of function state, some examples of effects yield: classic RTT, atypical RTT, autism, and mild mental retardation (2). The majority of RTT patients (85%) have mutations in the MECP2 gene that lead to loss of MECP2 protein function. At this point of time, there is no cure to RTT syndrome, however treatment strategies are starting to emerge.

Rett syndrome is a progressive neurodevelopmental disorder that initially presents in females just past 18 months. Patients with RTT appear to have apparent normal development and milestone achievements up to the first 18 months of age (3). Early indication of neurological disease progression includes: deceleration of head growth, general growth retardation, weight loss, and weak posture via muscle hypotonia (2). Progression of RTT leads to the loss of purposeful use of their hands and the development of stereotypic hand wringing or washing movements: other hand movements are clapping, flapping, and mouthing (2). Withdrawal of social behaviors are noticeable where patients have shown symptoms such as

irritability, self-abusive mannerisms, blank faces, audio hypersensitivity, reduction of eye contact, unresponsiveness to social cues, and indifference to variable environments (2). Eventual losses in motor coordination, ataxia, and gait apraxia are symptoms of motor pathway deterioration (2). Most girls eventually require wheel chair assistance during teenage years. Autonomic perturbations include hyperventilation (and other breathing abnormalities), seizures with variable severity, cardiac abnormalities, constipation, parkinsonian features, and oropharyngeal dysfunction (2). Patients may have a normal appetite but still experience weight loss and osteopenia. Structural abnormalities include scoliosis and rigidity. Behavioral complications involve teeth grinding, temper tantrums, depression, and anxiety. The condition eventually plateaus and the patient may live up to their seventh decade (2).

MeCP2 is an example of a mammalian protein that binds to methylated CpGs. For its ability to bind DNA and influence gene expression, MeCP2 is mostly believed to be a transcription factor (1), however localization of MeCP2 outside the nucleus and in the synaptic compartments (4) suggest that MeCP2 protein may have also functions directly related to synaptic activity. Inheritance of the MECP2 gene follows an X-linked dominant pattern and sporadic mutations are prevalent in 85% of classic RTT cases (2). Mutations that have been noted on the spectrum include missense, nonsense, and frame shift mutations for this protein. Mutations impacting on the nuclear localization sequence of MeCP2 or early truncating mutations have more severe phenotypes in comparison to missense mutations; while C-terminal deletions are thought to cause milder phenotypes. There is an association between the severity of the mutation and the severity of the phenotype (5). The phenotype of the female is highly variable depending on the expression levels of the unaffected copy of MECP2 on the other X chromosome.

The identification of the gene associated with Rett made possible the generation of mutant mice defective in MeCP2 functions. The mice have signs that resemble patients' symptoms and therefore constitute a valid model for the study of neurobiology of Rett and for testing candidate therapeutics. A significant discovery has emerged from two independent groups concluding that in the mouse model, the reactivation of the natural Mecp2 gene in the adult mutant mice partially reestablished normal phenotype (6, 7). This finding implies that when the normal brain has impairment by a dysfunctional MeCP2, control conditions can be restored in the central nervous system. However, Zoghbi's lab has shown that drug treatment for RTT has to be administered during the whole life of the patients (8).

Neurobiological analysis shows that MeCP2 mutations produce defects in central nervous system development, specifically in the maturation of circuits and synapses (4, 9). Therefore, medical treatments focused on brain development and synaptic functioning is promising for the regression of RTT. Indeed, a research study noted that overexpression of brain-derived neurotrophic growth factor (BDNF), which is a neurotrophin that has involvement in brain development and plasticity, resulted in the neutralization of the MeCP2 deficiency (10). This allowed for the consideration of growth factors, which are centralized around their involvement of neuronal and synaptic growth, toward potential treatment options for RTT syndrome. BDNF is incapable of crossing the blood brain barrier, so it is of no use for immediate pharmacological therapy consideration. However, IGF1 and its active peptide (1–3) IGF1, are capable of crossing the blood brain barrier. This quality makes them an ideal drug for treatment in neurodevelopmental disorders like RTT. Research has shown that IGF1 peptide treatment partially restored spine density and synaptic amplitude in the mouse model (9). The treatment was started at 2 weeks of age and was noted to stabilize cortical plasticity to the wild-type level. One of the major effects noted in this study was after the treatment of IGF1; MeCP2 KO with treatment mice improved from an average of 60-day lifespan to an average of 90-day lifespan in comparison with KO without treatment mice. Not only did the KO mice with treatment have increased life span, but also improved respiratory, cardiac, and locomotor function. In the study from Tropea et al. (9) (1–3) IGF1, has already shown to ameliorate the symptoms of RTT in the mouse model of the disease. IGF1 has also been shown to be an effective therapy in MeCP2 KO mice (11). Other studies show that IGF1 is a suitable treatment for human RTT patients and that there are no known risks associated with IGF1 administration (12, 13). In our previous study, six girls with classic RTT, between 4 and 11 years of age, were administered IGF1 subcutaneously twice per day for 6 months. They were evaluated based on the effectiveness of the treatment and it was reported that IGF1 partially restored a significant amount of the symptoms of RTT. From this study, all families and caretakers noticed an improvement in cognitive abilities of the girls and their interactions with the surrounding environment. Considered McGraw's study, lifelong therapy is necessary to combat RTT syndrome, like IGF1 for MeCP2 dysfunction (8).

The aim of this paper is to evaluate if repeated cycles of IGF1 is a suitable method, in respect to safety and efficacy, for a life-long treatment option in the human model. The clinical case study in this report depicts a patient receiving two cycles of IGF1 treatment and clinical parameter assessments. Also, we provide insight on how the patient responds after the treatment with two clinical evaluations after the treatment cycles. The study is an open label study, and the child displays classic RTT syndrome, where the phenotypic onset of the disease took place between the eighth and ninth month. The child started the first IGF1 cycle in May 2010, when the child was in the fifth year of age. IGF1 was administered twice daily (0.1 mg/kg) for 6 months. Two years later, another cycle of treatment was started. The dosage and routes of administration were the same, but the second cycle lasted 4 months. In between each cycle, blood parameters were monitored and autonomic responses evaluated. During both cycles, IGF1 was well tolerated in each cycle and no direct side effect was observed. IGF1 is also shown to cause no interference with the anti-epileptic medications that the patient was undergoing. Based on the conclusion of this experiment, the treatment is believed to promise a strong impact on RTT syndrome.

SUBJECTS AND METHODS

CLINICAL ANALYSIS

We evaluated the RTT patient's overall physical health, RTT-associated phenotypic expressions of the patient, and growth developments. We took notes, recorded in the Section "Results," during various stages of the treatment process during two cycles

of IGF1 exposure. The parents kept a diary with daily annotations of glycemia levels and alterations in behavior (sleep, eating, etc.)

Patient screening

Patient was chosen based on the presentation of classic RTT syndrome and confirmed mutation in the MECP2 gene. The family gave written consent for partaking in this study before any treatment took place. The selection criteria are described in Pini et al. (12).

Treatment administration

Mecasermin–Increlex (IGF1) was subcutaneously administered twice daily (0.1 mg/kg) in two cycles: the first cycle lasted nearly 6 months, and the second cycle lasted 4 months, with a 2 years gap between the two cycles. Possible side effects include hypoglycemia (headache, dizziness, nausea, and perspiration), tonsil hypertrophia, and local irritation at the injection site. The family was thoroughly informed about the treatment process; the caretakers of the patient were trained to perform daily injections, and were provided with a kit to measure glycemic levels. Patients were observed for side effects during every visit and critical events were recorded and are in summarized in the Section "Results."

Post treatment examination

The patient was followed up twice after each cycle to evaluate general health after IGF1 exposure. Patient was physically examined based on cardio-respiratory factors, body growth parameters, and motor abilities. Critical events were recorded along with the corresponding epileptic treatment at that time. Behavioral examination along the parameters of social interaction and mood were also evaluated.

VIDEO ANALYSIS OF COGNITIVE AND SOCIAL ABILITIES

Video analysis utilized approximately 20 h of video recordings of RTT patients and was filmed in Versilia Hospital (Italy). The RTT patient observed in this paper was an analyzed participant in this study. The investigators, analyzing the videos, were not aware that the patient had received IGF1 treatment. Observed preliminary footage from additional patients yielded the design of the scoring criteria. It was decided to score the girls on 20 features, 10 negatives, and 10 positives. The patient was scored in each feature on a scale from 1 to 5, with 1 indicating extreme RTT, 2 indicating severe RTT, 3 indicating moderate RTT, 4 indicating mild RTT, and 5 indicating absent RTT. In this way, a lower score was related to a more prevalent RTT-like behavior. The 10 negative features chosen were: hand wringing, biting, rocking, hitting, indiscriminate moaning, tongue chewing, vacant staring, bruxism, breath-holding/apnea, and Valsalva maneuver. The 10 positive features chosen were: pointing, manipulating, reaching for something, ability to mimic/imitate, indicating yes/no with head gesture, reactivity to a call, reactivity to an object, smiling in response to a stimulus, deliberate vocalization, and attention.

RESULTS

The patient was born from the mother's first pregnancy. The mother suffers from epilepsy and was treated with anti-epileptic drugs (AEDs) since adolescence. The patient was born at week 38 of gestation by cesarean section due to an emergency response. At birth, the patient had the following dimensions: 2600 g in weight, 45.5 cm in length, and 33 cm in head circumference. The newborn received artificial feeding due to maternal agalactia. At the approximate age of 9 months, the child presented with: frequent pyloric stenosis, decreased stimulus to environment, mentally absent, and showed stereotypic hand to mouth and washing movements. The patient's motor development showed independent seat positioning acquired at 8 months with kyphosis of the bust: unimanual walking with support at 18 months with mild postural tremor. At 18 months, the patient's use of hands was discrete and she could grab objects and press buttons. The patient's language development showed production of vocalization and infrequent simple syllabic doublings (bi-bi-, ma-ma, ba-ba). The patient showed bruxism with variable power. The patient had tendency to be constipated and sphincter control was not acquired. The sleep–wake rhythm was characterized by frequent nocturnal awakening and was prescribed Niaprazine (Nopron). On the 2nd of August 2006, genetic testing for RTT indicated a mutation c. 397C > T (R133C) of exon 4 of the MECP2 gene.

The clinical case study performed in this experiment depicts the efficacy and safety of IGF1 exposure upon two cycles of treatment. Careful consideration was executed in monitoring to the effects of IGF1 in both autonomic function and blood parameters. The patient received first round of treatment on the 25th of May 2010 and discontinued on the 11th of November 2010. The second round of treatment started on the 20th of November 2012 and discontinued on the 28th of February 2013. The dosage for IGF1 was the same in both cycles: 0.1 mg/kg maintenance dosages and 0.05 mg/kg first and last weeks were adjustment dosages. Our findings are noted below during each visit with the patient during their cycles of IGF1 treatment.

FIRST CYCLE OF IGF1 TREATMENT

The first experimentation with IGF1 started on 25th of May 2010 (first cycle), when the patients were 5 years and 8 months. We clinically assessed the patient and results were noted as follows: patient has slight ataxic ambulation, which required minimum support; slight hypotonia was noticeable and babinsky positive ROT hyper-excitability; wide-spread bouts of tremor often associated with tachypnea, and possible grasping of objects upon request. Patient demonstrated expressive language, characterized by varied and modulated vocalizations. The patient had a calm demeanor with discreet interest in external environment and surrounding people.

Second visit was on the 31st of August 2010. The patient showed good clinical health and similar ambulation as the first visit. The patient showed coastal navigation with the family. The hypotonia and wide-spread tremor was the same as first visit. The patient showed improvement in grip of objects. The patient continued to demonstrate expressive language, characterized by varied and modulated vocalizations. Communicative intentions were observed and actions were occasionally preceded by anticipatory behaviors. The demeanor remained calm.

Third visit took place on the 23th of November 2010. The patient maintained good general clinical condition and the parents reported a good clinical outcome at home. The patient was

able to ambulate with unimanual support with tendency to externally rotate the march of the lower limbs, and launch of the same. The use of hands to engage in patterns of action with objects have been a new observation, such as pressing a button or lever to get a visual or sound effect. The patient demonstrates good conduct and interaction with unfamiliar people. The food and sleep–wake rhythm is regular, while obstinate constipation persists.

The patient was discontinued from IGF1 on the 27th of November 2010 and clinical assessments were continued.

The fourth visit was on the 15th of February 2011. The clinical observations were almost unchanged from the previous visit. The gait seemed to have improved, even when the child fatigued easily. There was a submission of critical events that took place. On the 8th of February 2011, at 10:40 a.m. the first epileptic crisis lasting 2–3 min was characterized by tremor, hypotonia, eyes wide and staring into space. This was followed by sleep. Secondary crisis showed facial and perioral cyanosis, tremor, and blank stares, followed by sleep. A third crisis at 6:10 p.m. noted guttural verses, staring, stiffening, and blank stares followed by sleep. A fourth crisis occurred at 7:30 p.m. where stiffening, staring, perioral cyanosis, pallor of the face, and strange vocalizations followed by sleep. Vomiting, facial and perioral cyanosis, and guttural sounds characterized the fifth crisis at 9:30 p.m. On the 9th of February 2011, the patient had a fever of 37.3°C. At 12:35 p.m., the first crisis lasted a minute and was characterized by salivation, perioral and facial cyanosis, absent eyes, and potential hypotonia causing the patient to fold on itself.

The second crisis (on February 9th) occurred at 1:38 p.m. and lasted 3 min; characterized by perioral and facial cyanosis and mouth tremors followed by sleep. The third crisis occurred at 5:27 p.m. and lasted for 2 min, characterized by an absent gaze. On the 11th of February 2011 at 10:16 a.m., the patient was noticed to have staring eyes and making guttural sob like sounds. On the 12th of February 2011 at 10:00 a.m., the patient woke up with a frightened look, began trembling with tears, and guttural vocalizations. A second crisis occurred at 3:40 p.m. characterized by neck muscle failure and appearance of sleep while sitting on the toilet, with guttural vocalizations. On the 13th of February 2011, the patient presented with a fever of 38.3°C, pharyngitis, and began Zithromax treatment.

The fifth visit took place on the 24th of May 2011. The clinical examination showed good general condition and parents reported a stable clinical experience. The child had unimanual-supported ambulation with tendency to externally rotate and launch of the lower limbs. The clinician noted a decreased resistance during walking. The patient was observed to have a discreet use of hands when she grabbed objects and pressed buttons to get a visual or sound effect. Social interaction showed observation of the environment and the maintenance of eye contact for a longer duration of time. The patient maintained a serene mood. The food and sleep–wake rhythm were regular and constipation still persisted. Critical incidents occurred almost daily and were characterized by staring, gaze deviation to the left, and clonus of the limbs. On the 13th of April 2011, the child presented a crisis with the semiotics for a 10-min period, which required Micronoan administration. The patient's anti-epileptic therapy dosage was increased: valproate 200 + 200 mg/day.

The sixth visit took place on the 21st of October 2011. The clinical examination reported a good general clinical condition. The parents informed that the child was attentive, observational of the environment, interacting with people, and mood was serene. The critical events were reported to occur on awakening and were characterized by wide-eyed, clonus of the limbs, apnea, and cyanosis of the face. The patient then took Valproate: dosage of 250 + 200 mg/day.

Upon collection of the clinical data set for the first cycle, blood test showed no morbidity related to the treatment and a noticeable improvement in bone density. Glycemic values taken during each visit all remained well within reference value (60–110 mg/dl), where the patients' average was 80 mg/dl and the range was between 75 and 86 mg/dl. The starting bone density measurement was taken on the 26th of May 2010 and a second one was taken at the end of first cycle on the 15th of February 2011. This measurement showed a BMD of 0.333 and 0.451, respectively.

Analysis of cognitive and social abilities show mixed results in positive and negative RTT features, in terms of improvement and disease progression (**Figures 1** and **2**). **Figures 1** and **2**, representing video analysis, note that the negative features (9 out of 10) increased more than positive features (5 out of 10). Hitting and breath-holding/apnea were the most improved negative features. Smiling in response to a stimulation, deliberate vocalization, and yes/no with head gesture were the most improved positive features. The patient shows an improvement in height percentile during the course of IGF1 treatment in almost immediate results, however, it returned to previous percentile among the discontinuation of the IGF1 treatment. No other significant correlations to the IGF1 treatment can be made from **Table 1**. In the ISS evaluation, we noted a starting score of 4 in autonomic function, at the first dose of the cycle; and upon the last dose, the patient reduced to a score of 3 in autonomic function. This implies that the autonomic function restored as a result of IGF1 treatment. Another improvement noted by the ISS score was in growth and development from an ISS score of 6, at first treatment, to a score of 5, at last dosage. This also implies that IGF1 treatment improved the patient's growth and development. ISS scores are shown in **Figure 3**.

SUMMARY OF IGF1 EFFECTS AFTER THE FIRST CYCLE OF TREATMENT

We found that IGF1 was well tolerated during the first cycle of treatment and did not show any side affect. The treatment produced an increase in height and BMI of the patient mostly during the first 3 months of treatment, and returned to baseline after the discontinuation of the drug (**Tables 1** and **4**). Significant benefits were noted as reduction of negative stereotypes, while the social and cognitive abilities did not show any significant improvement (**Figures 1** and **2**; **Table 5**). IGF1 did not show any significant effect on ISS, with the exception of the autonomic function, that appear improved also after 6 months from the last IGF1 administration (**Figure 3**; **Table 6**).

SECOND CYCLE OF IGF1 TREATMENT

The next cycle of IGF1 treatment started on the 20th of November 2012, when the patient was 8 years and 2 months. Since the last visit, the patient is continuing the anti-epileptic therapy: Valproate

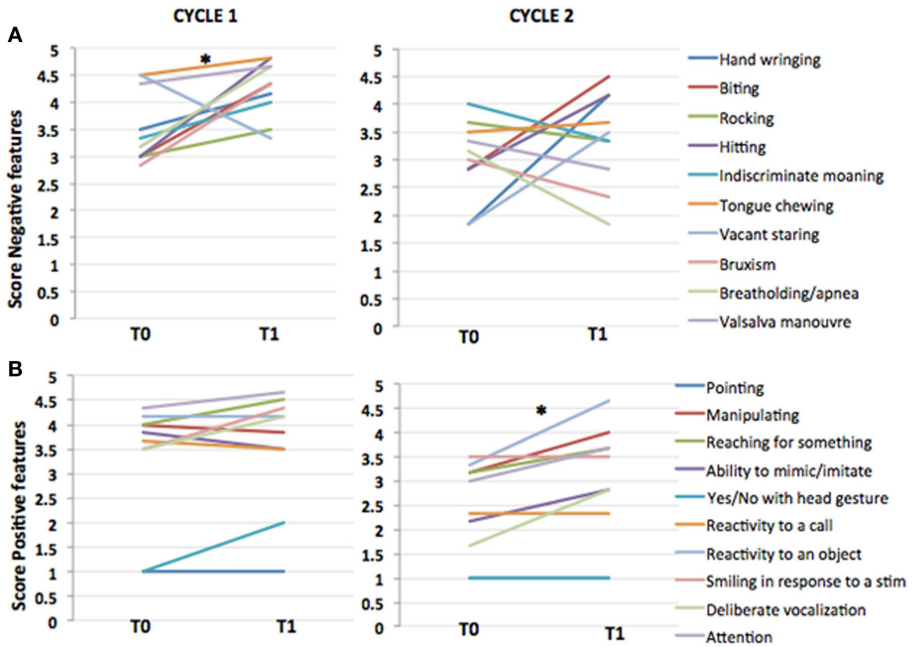

FIGURE 1 | IGF1 treatment affects different features in different cycles. Comparison of the scores of individual features at T0 and T1 for negative **(A)** and positive **(B)** features in cycle 1 (left) and cycle 2 (right). Paired comparison with Wilcoxon test shows that IGF1 treatment improved significantly negative features in cycle 1 (Wilcoxon test, p-value = 0.014), but not in cycle 2 (Wilcoxon test, p-value = 0.07). Viceversa, for positive features the effect was significant in cycle 2 (Wilcoxon test, p-value = 0.016), but not in cycle 1 (0.3).

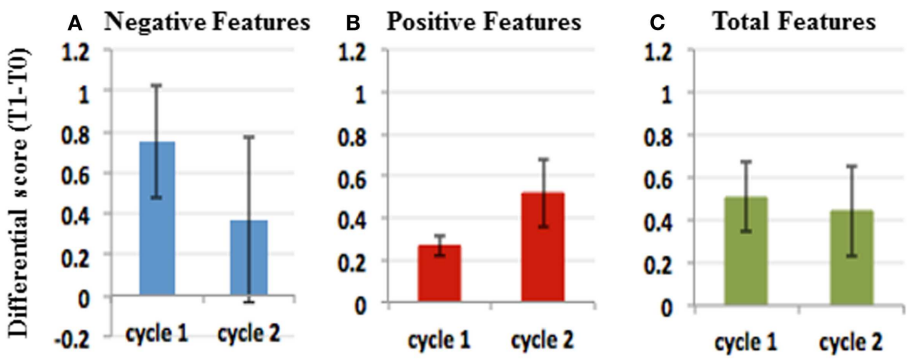

FIGURE 2 | Effects of IGF1 treatment differ in different treatment cycles. Plots of average (T1–T0) and SE in cycle 1 and cycle 2 for Negative features **(A)**, Positive features **(B)**, and Negative plus positive features **(C)**. The data suggest that the negative features improved more in the first cycle than in the second **(A)**. Conversely, the positive features improved more in the second cycle than the first. **(B)** Considering both positive and negative features, the effect of the treatment was comparable in both cycles **(C)**.

(250 + 200 mg/day) and Levetiracetam (300 + 300 mg/day). The clinical examination shows that health is fair and reported: patient has slight ataxic ambulation, which required minimum support; slight hypotonia was noticeable and Babinski positive ROT hyperexcitability; wide-spread bouts of tremor often associated with tachypnea; and possible grasping of objects upon request, but released after a few seconds. The patient has expressive language that is characterized by production of varied and modulated vocalizations. The mood of the patient is serene and there is a discreet interest in the environment and in surrounding people.

The second visit of the second cycle took place on the 29th of January 2013. The patient continues to have seizures three to four times a week characterized by eyes turned in backwards. The patient has an increased in dosage of Levetiracetam (350 + 350 mg/day) and maintained Valproate (250 + 200 mg/day). The parents reported an increase in bruxism with a decrease in stereotypic mannerisms. The patient showed longer attention span, improved eye contact, and smiling. The dietary intake was good and constipation improved. The clinical examination reported: patient has slight ataxic ambulation, which

required minimum support; slight hypotonia was noticeable and Babinski positive ROT hyper-excitability; wide-spread bouts of tremor often associated with tachypnea; and possible grasping of objects upon request, but released after a few seconds. The patient had expressive language characterized by production of varied and modulated vocalizations. The mood of the patient was serene and there was a discreet interest in the environment and in surrounding people. We took extra note in the slight improvement of attention: specifically indicating that the patient holds her gaze for the duration of the smile and she seems more interested in the environment. Motor stereotypes decreased slightly while bruxism increased.

The third visit took place on the 3rd of December 2013. The grandfather informs about an improvement in attention span to external stimuli throughout the visual field laterally. The communication capability is improved and characterized by the improved ability to stare at an object that the patient wants to play with or a place that the patient wants to go. He also noticed an improved grip on objects. The mother reports that the patient eats less and tremors and bruxism have increased. She also states that sleep patterns are altered by presence of numerous episodes of pavor during sleep and wake states. The clinical examination reported: patient has slight ataxic ambulation, which required minimum support; slight hypotonia was noticeable and Babinski positive ROT hyper-excitability; wide-spread bouts of tremor often associated with tachypnea; and possible grasping of objects upon request, but released after a few seconds. The patient has expressive language that is characterized by production of varied and modulated vocalizations. The mood of the patient is serene and there is a discreet interest in the environment and in surrounding people. The clinician took another extra note in the slight improvement of attention: specifically indicating, again, that the patient holds her gaze for the duration of the smile and she seems more interested in the environment. Motor stereotypes decreased slightly while bruxism increased.

Suspension of the second cycle of IGF1 occurred on the 28th of February 2013.

The following clinical observation was recorded on the 21st of May 2013. An anamnestic incident was recorded with a slightly increased frequency of seizures and every crisis has a longer duration of action. There is a constant intense tremor present. Parents observe the patient suffering from pharyngitis and a decrease in appetite. The clinical observation includes the patient being pale and sleepy, while being poorly responsive to environmental stimulation. They informed about two critical episodes in the morning with semiology and in few seconds durations. The gait was possible with minimal support and appeared ataxic. Slight hypotonia was noticeable and Babinski positive ROT hyper-excitability. Wide-spread bouts of tremor were noted in association with tachypnea. There was grasping of objects on presentation, but released after a few seconds. The stereotypes were unchanged.

Clinical data sets provided no insight into the second cycle of IGF1 treatment in blood work or growth parameters (**Table 2**). Glycemic values taken during each visit all remained well within reference value (60–110 mg/dl), where the patients' average was 78.2 mg/dl and the range was between 73 and 84 mg/dl. The starting bone density measurement was taken on the 20th of November 2012 and a second one was taken at the end of the second cycle on the 21st of May 2013. This measurement showed a BMD of 0.426 and 0.465, respectively.

Table 1 | Growth parameters during first cycle of treatment.

Visit	Weight (kg)	Percentile	Height (cm)	Percentile	Head circumference	Percentile	BMI
1	14.2	4	104.5	4	48	2	13
2	15.2	4	108	10	48	2	13.03
3	17	4	108.5	10	48	2	14.5
4	17	4	108.5	10	48.4	2	14.5
5	16.7	4	110.6	4	48.4	2	13.8

This table compares the growth parameters of the RTT patient in the first cycle with the average child of same age. The patient remains in low percentiles, despite improvements in height during IGF1 application.

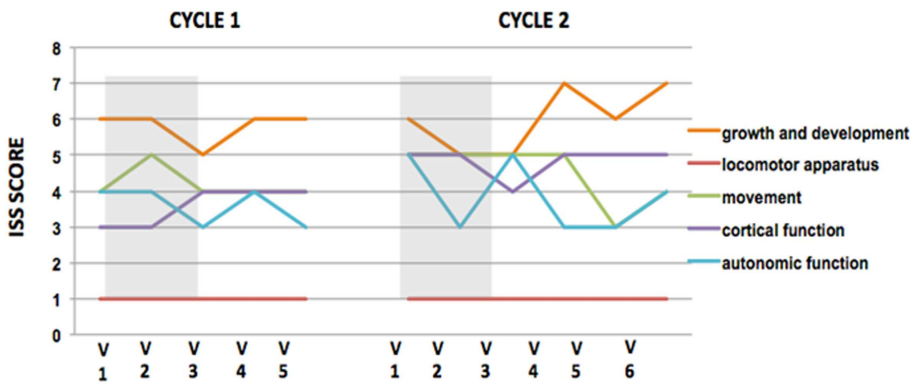

FIGURE 3 | ISS scores plots during IGF1 cycles of treatment. ISS scores reveal that IGF1 improves autonomic function. Plots of International Severity Scale (ISS) score at each visit during cycle 1 (left) and cycle 2 (right) of IGF1 administration. Gray rectangles show the IGF1 Administration. Higher scores represent worst performance. In both cycles, the IGF1 treatment improved the autonomic function, while the other parameters remain unchanged or worsened between the beginning and the ending of each cycle. Note that the improvement is not maintained between cycles.

Table 2 | Growth parameters during second cycle of treatment.

Visit	Weight (kg)	Percentile	Height (cm)	Percentile	Head circumference	Percentile	BMI
1 and 2	15	3	115	4	48	2	11.3
3	16	3	118.5	5	48.2	2	11.5
4	16.1	3	118.5	4	48.2	2	11.5
5	15.4	2	119.4	3	48.2	2	10.8

This table shows the growth parameters during the second cycle of IGF1 treatment. There are no significant contributions to these characteristics given by the treatment. The patient maintains in low percentiles during the second IGF1 treatment cycle.

Table 3 | Cardiovascular reflexes range during cycles.

Cardiovascular reflexes	Examined values	Normal values
Cardiac vagal tone	5.63	6–19
Heart rate	90.1 beats/min	60–140 beats/min
Respiratory rate	20 breaths/min	18–28 breaths/min

This table depicts that the patient falls with in the normal reference range for three out of the four cardiovascular reflexes that we observed. Cardiac vagal tone is just slightly under the reference value.

The patient shows mixed results for positive and negative phenotypes based on the video analysis (**Figures 1** and **2**), however no adverse effects are noted in association with the second cycle of IGF1 treatment. **Figures 1** and **2**, representing video analysis, note that the negative features (4 out of 10) increased less than positive features (6 out of 10). Hand wringing and biting showed most improvement in negative features. Reactivity to an object and deliberate vocalization showed the most improvement in positive features. In the ISS evaluation: we noted a starting score of 6 in autonomic function, at the first dose of the cycle; and upon the last dose, the patient reduced to a score of 5 in autonomic function. This implies that the autonomic function restored, again, as a result of IGF1 treatment. Another consistent improvement noted by the ISS score was in growth and development from an ISS score of 6, at first treatment, to a score of 5, at last dosage. This also implies that IGF1 treatment improved the patient's growth and development. ISS scores are shown in **Figure 3**.

SUMMARY OF IGF1 EFFECTS AFTER THE SECOND CYCLE OF TREATMENT

In the second cycle of treatment, there were no significant effects of IGF1 on the growth parameters of the patient (**Tables 1** and **4**). The scoring of the cognitive and social abilities, as well as the negative features of Rett, shows a general worsening of the conditions between the end of the first cycle and the starting of the second cycle of treatment. The second treatment with IGF1 produces a significant improvement in the scoring for the social and cognitive abilities, but no effects are visible on the negative symptoms of RTT (**Figures 1** and **2**; **Table 6**). The ISS components show a mixed effect of IGF1 on different features, with overall a long term improvement of movement and autonomic functions (**Figure 3**; **Table 5**).

FOLLOW-UP EXAMINATIONS POST CYCLES

On September 24, 2013, the patient was 9 years old with a body mass index of 10.13 (normal values between 19 and 25 kg/m^2). The occipital–frontal cortex circumference was 48.2 cm and her weight was 14.6 kg. The patient's results in this report came from the evaluations of the autonomic nervous system in quiet conditions. **Table 3** depicts the cardiovascular examination that took place on this date.

The results showed normal breathing patterns for 21.2–35.9% of mixed tachypnea breath, 12.2% of deep breaths, 7.4% of breath-holding episodes, 8% of hyperpnea, and 3.8% of apnea episodes. The average heart rate recorded is about 90 beats per minute, while the mean values of CVT in relaxation amounted in about 5.6; where the registered CVT peaks results are of average and significant.

The phenotype of the cardiopulmonary patient was classified as forced (40.2%). The general autonomic framework was that of a basal level vagal tone, which is slightly lower than normal. There were multiple types of respiratory rhythm abnormalities that were indicative of instability within the respiratory centers of the brainstem.

On 11 March 2014, a second follow-up examination was recorded. The parents noted some changes in seizure related events. Since November of 2013, the patient took Carbamazepine (25 mg in the morning and 50 mg in the evening), which was suspended while being treated with Levetiracetam. The latest crisis occurred a week before this examination where the patient had a cluster of six episodes on the same day. The patient grew agitated and had difficulty falling asleep, so Valium was given by the parents.

At this time, the patient was currently attending third grade class with support and rehabilitative physiotherapy continues 2 days a week (partially at ASL and partially privately) with swimming integrated. She was on the following AEDs: depakote (250 mg in morning and 250 mg in the evening), Carbamazepine (5 mg in morning and 50 mg in the evening), and Pepidax.

The general health conditions were satisfactory. The patient was able to autonomously maintain the sitting position with ataxia of the trunk and ambulation requires support. Tremors were noticed and the detection of bruxism. Scoliosis was absent, while there was reduced muscle tropism. The patient made little facial expression, with eye contact being only possible if the child is evoked several times; language was absent. During the visit, there were no manual stereotypes; however, the parents report it occurred when the patient was tired. The patient showed fluctuation in vigilance, and during the visit the child was alert and responsive. During neurological examinations, the child had suddenly fallen asleep for few minutes at a couple of occasions. The physician recommended that it was advisable for the patient to continue the rehabilitation treatment that was already in progress.

COMPARISON OF IGF1 EFFECTS DURING AND AFTER THE TWO CYCLES

In order to compare the effects of IGF1 between the two cycles, we considered the relative values of each parameter (growth parameters, ISS scores, cognitive, and social abilities scores) during and after the termination of the drug administration, grouping

together the effects during IGF1 administration and after the suspension of the drug (**Figure 4**). In particular, for the effects during the two cycles, was considered the differences between the last visit during treatment and the visit before treatment (**Figure 4A**). For the preservation of the effects after the cycles, we considered the difference between the values at the last visit post treatment and the values of the last visit during treatment (**Figure 4B**). We observed that the effects of the drug were comparable between the two cycles, and even with a slight positive effect during the first cycle, there were no significant differences (two tailed t-test, p-value = 0.28).

EPILEPTIC HISTORY DURING OBSERVATION

The patient was enlisted into the study in March 2010, but because of an onset of epilepsy during this month the start treatment was postponed to May. There were two critical incidents between the months of March to May and on average the crisis occurred at a frequency of four/month. The patient then commenced the first cycle of IGF1 treatment on the 25th of May 2010. The epileptic treatment during this time was Valproate. From the time of the previous visit to the next visit on the 31st of August 2010, there were five critical episodes reported while on the Valproate therapy, on average the crisis during IGF1 administration have the same intensity and frequency than before the treatment. More frequent critical incidents occurred between the second and third visit, where the patient was on Valproate. Following this visit, there was the suspension of IGF1 on the 27th of November in 2010. On the fourth visit, 15th of February 2011, after the suspension of IGF1 administration, the crisis appeared in clusters on several occasions. At this point, the epileptic therapy was adjusted to Valproate plus Frisium (for when the patient has many crises). By the fifth visit on the 24th of May 2011, critical episodes became more frequents and occurred on almost a daily basis. The therapy was Valproate, while Frisium was suspended. Visit six, on the 21st of October 2011, indicated critical episodes occurring upon awakening. Still the patient remained on Valproate.

The second trial start date occurred on the 20th of November of 2012. Critical episodes are daily and the epileptic therapy was Valproate and Levatiracetam. The second visit showed similar critical episodes on a daily basis. Suspension of IGF1 occurred on the 28th of February 2013. The third visit took place on the 12th of March 2013, where the critical episodes remain daily. The crisis did not change with the administration of IGF1 treatment and the epileptic therapy remained on Valproate and Levatiracetam. On the 24th of September 2013, the critical episodes were reported the same on a daily basis. During this fourth visit, the patient was on the same epileptic treatment. On the 28th of October 2013, the epileptic therapy was changed: Levitiracetam was removed and Carbamazepine was added. The sixth visit, on the 3rd of November 2014, reported that Carbamazepine caused a thinning out of critical episodes, but they were clustered. The epileptic therapy was carbamazepine and valproic acid.

DISCUSSION

The use of IGF1 represents an appealing therapy for RTT. Due to IGF1 affecting multiple symptoms of RTT in animal models, its noticeable benefits from pediatric use, and its physical properties allowing it to cross the blood brain barrier; pilot trials (12, 13), indicated that IGF1 is safe and tolerable for treatment of RTT patients.

Considering the finding that any treatment for RTT should be administered for life (8), our research addresses the issue of multiple IGF1 treatments and tolerability in children with RTT syndrome. During our first study (12), we observed the major improvements during the first weeks of IGF1 treatment, and for this reason, we decided to reduce the time of the second treatment to 20 weeks. These findings are in line with the study by Khwaja et al. (13), where the amount of IGF1 in the serum decreases during the administration period, and the authors suggest that an adjustment of the dosage and administration time could be more beneficial to the Rett patients.

Although our study is mostly oriented in confirming the safety and tolerability of IGF1 through multiple cycles, we observed an overall improvement of autonomic function during IGF1 treatment, and then a lessening of improvement upon suspension of IGF1 (**Figure 3**; **Table 5**). We recorded no hypoglycemia, which indicates that IGF1 shows good toleration in patients. This is important because it is one of the main risks associated with IGF1 treatment (13).

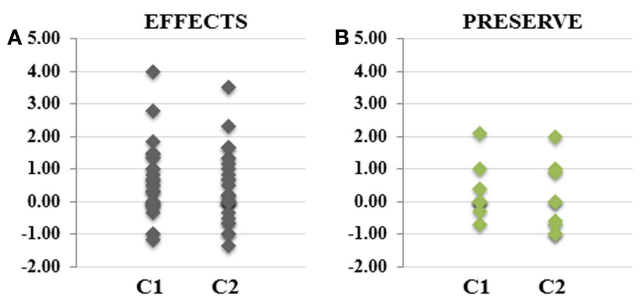

FIGURE 4 | The effects of IGF1 treatment for each cycle are comparable. The plots represent the differential scores of growth parameters, ISS scores and Social and cognitive scores, before and after IGF1 Administration [EFFECTS **(A)**], and in follow-up studies [PRESERVE **(B)**]. Although we observe a slightly major efficacy of IGF1 during the first cycle of treatment, the differences are limited and they are not significant.

Table 4 | Differential scores of growth parameters during cycles.

Growth parameters	Cycle 1		Cycle 2	
Differential score	**Effect**	**Preserve**	**Effect**	**Preserve**
Weight	2.8	−0.3	1	−0.6
Height	4	2.1	3.5	0.9
Head circumference	0	0.4	0.2	0
BMI	1.5	−0.7	0.2	−0.7

*This table summarizes the differential scores for each component of the growth (listed on the left), considering the difference at the end versus the beginning of the treatment (effect) and the difference after 6 months from the last administration versus the scores on the day of the last administration (preserve). The original scores are those reported in **Tables 1** and **2**. The improvements are highlighted in green, and the worsenings are highlighted in red.*

In this study, similarly to what reported in the past and by other researchers (12, 13), we did not observed any specific influence of IGF1 treatment on epileptic episodes: the appearance of the epileptic crisis preceded the first cycle of IGF1 administration, and the intensity and frequency of the episodes remained on average the same before and after the treatment. The intense episodes reported between the second and third week during the first cycle of treatment, can be due to an endogeneous evolution of the crisis, as suggested also by the increased frequency after the interruption of the treatment (which required an adjustment of the therapy). Before starting the second IGF1 administration, the crisis had a daily frequency and they remained unaltered during the whole cycle of treatment. These observations are in accordance to the literature in pediatric syndromes, where IGF1 is not associated to an increase in seizures, but rather epileptic phenotype is associated to decreased IGF1 levels in the serum (14, 15). On the contrary, it is possible that the seizure medication hindered the positive effects of IGF1. It would be interesting to compare the effects of IGF1 administration in patients treated with valproate versus patients treated with alternative AEDs.

Insulin-like growth factor 1 has also been considered to be more effective when given in acute intermittent pulses, versus chronic dosing. One noted side effect from IGF1, was that long term IGF1 administration has linkage with potential puberty effects; which is already accelerated in RTT (13). Being that IGF1 has a strong feedback system, patient tolerance over cycles is worth exploring into for future studies. This implies the evaluation of lower doses in life-long treatment. An assessment of total effects on single analyzed relative parameters (ISS scores, cognitive and social abilities, and growth parameters) showed that the effects observed with the first cycle of treatment were not significantly different from those in the second cycle of treatment (**Figure 4**, two tailed t-test p-value $= 0.28$).

While there were some phenotypic benefits shown by the assessment of social and cognitive abilities, nine of the improvements remained after the first cycle was over, and no phenotypic improvement remained in the second trial as different phenotypes improved aside from attention. However, we observed motor control as another form of improvement. However, our research insight into motor control improvement may be a result of subjectivity further research is suggested to elucidate the validity of this potential benefit. Our analysis confirms the results of Zoghbi's team, that whatever treatment is decided for RTT syndrome patients, it must be for life (8).

The two studies of IGF1 safety and tolerability in RTT patients (12, 13), showed similar results: IGF1 did not produce hypoglycemia and it is not associated to seizures, and overall is well tolerated by patients. In addition, we observed an improvement in autonomic function, and Khwaja and colleagues concluded that motor ability showed no measurable improvement, but also found

Table 6 | Differential scores of ISS scores during cycles.

ISS components	Cycle 1		Cycle 2	
Differential score	**Effect**	**Preserve**	**Effect**	**Preserve**
Growth and development	−1	1	−1	2
Locomotor apparatus	0	0	0	0
Movement	0	0	0	−1
Cortical function	1	0	−1	1
Autonomic function	−1	0	0	−1

This table summarizes the differential scores for each component of the ISS (listed on the left), considering the difference at the end versus the beginning of the treatment (effect) and the difference after 6 months from the last administration versus the scores on the day of the last administration (preserve). The improvements are highlighted in green, and the worsenings are highlighted in red. Note that the decrease in the scoring are improvements for the ISS; on the contrary, increases in the scoring represent worsening.

Table 5 | Differential scores of social and cognitive abilities during cycles.

(T1–T0) score	Negative features		(T1–T0) score	Positive features	
	Cycle 1	**Cycle 2**		**Cycle 1**	**Cycle 2**
Hand wringing	0.67	2.33	Pointing	0.00	0.00
Biting	1.33	1.67	Manipulating	−0.17	0.83
Rocking	0.50	−0.33	Reaching for something	0.50	0.50
Hitting	1.83	1.33	Ability to mimic/imitate	−0.33	0.67
Indiscriminate moaning	0.67	−0.67	Yes/No with head gesture	1.00	0.00
Tongue chewing	0.33	0.17	Reactivity to a call	−0.17	0.00
Vacant staring	−1.17	1.67	Reactivity to an object	0.00	1.33
Bruxism	1.50	−0.67	Smiling in response to a Stim	0.83	0.00
Breath-holding/apnea	1.50	−1.33	Deliberate vocalization	0.67	1.17
Valsalva maneuver	0.33	−0.50	Attention	0.33	0.67
Overall	7.83	3.67	Overall	3.17	5.17

This table summarizes the differential scores for negative and positive features of the social and cognitive abilities assessment. Each number represents the difference of the score for the specific ability at the end of the treatment (T1) versus the beginning of the treatment (T0). Improvements are therefore positive numbers and they are highlighted in green, while worsening are negative numbers and are highlighted in red.

that respiratory complications associated with RTT syndrome showed improvement (13).

One factor to consider in our findings is the environmental surrounding that the patient experienced during and after the IGF1 treatment. The patient lived with the mother during the time of treatment. The mother suffers from anxiety and seizures, which may have limited the benefits of IGF1 treatment due to high stress levels being perceived by the patient. The consideration is due to a follow-up communication, post IGF1 second cycle treatment, where the grandparents noted an immediate amelioration of the patient's phenotypic mannerisms. The stressful environment could have caused misperception of the severity level to phenotypic hindrances, which are RTT associated.

The personal environmental surrounding, together with the onset and progression of the epileptic crisis are two of the limits of this case study for assessing the benefits of IGF1 therapy. Appropriate double blind and placebo controlled studies are necessary to define the efficacy of IGF1 treatment. However, considering the rarity of RTT (1:10,000), and the recent discovery of IGF1 as possible Rett treatment, data coming from a single case study are nonetheless useful to further develop the treatment.

Our research show that repeated treatments with IGF1 is safe and tolerable for the patient. This finding along with other findings on IGF1 treatment with RTT, should encourage further research in IGF1 treatment for additional neurodevelopmental disorders.

CONCLUDING REMARKS

Our research shows that repeated treatments with IGF1 are safe and tolerable for the patient. We observed improved autonomic function and social and cognitive abilities in each cycle but the benefits were lost between cycles. The results in this clinical case study suggest that IGF1 is beneficial for our patient, but a larger cohort of patients and proper clinical trials are requested to establish the overall efficacy in RTT.

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New experimental treatments for core social domain in autism spectrum disorders

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Current therapeutics in autism spectrum disorders (ASD) only treat the associated symptoms, without addressing core social dysfunctions. A paradigm shift in research of the pathogenesis of ASD, its synaptic abnormalities and altered signaling in multiple dynamic systems, have led to new experimental treatments for treating the core social abnormalities of ASD. NMDA antagonists, especially memantine, have been introduced in clinical trials addressing glutamatergic transmission in children and adolescents with ASD. GABAergic signaling has been targeted in trials using the $GABA_B$ receptor agonist arbaclofen for ASD patients with promising results. Oxytocin has been recognized as implicated in social development and affiliative behaviors. Preliminary findings from clinical trials using oxytocin in children with ASD show encouraging improvements in social cognition, but larger studies are needed. In two of the single gene disorders associated with ASD, Insulin Growth Factor (IGF-1) is a new treatment that has been tested in Rett syndrome and Phelan-McDermid syndrome (Chromosome 22 deletion syndrome). IGF-1 has been demonstrated to reverse the reduction in the number of excitatory synapses and the density of neurons that characterize these conditions in animal studies and it is being introduced as an experimental treatment. As a novel approach to verify treatment efficacy, neural processing modifications were recently evaluated by fMRI after a pivotal response training intervention. Another study of neural changes in response to treatment examined variations in EEG signaling in patients after an Early Start Denver Model (ESDM) intervention.

Keywords: autism spectrum disorders, experimental treatments, preclinical models, clinical trials, childhood and adolescence

INTRODUCTION

Autism spectrum disorders (ASD) are early-onset neurodevelopmental disorders characterized by major difficulties in social interaction, communication, and repetitive or restricted interests and behaviors. Autism is defined as a spectrum disorder due to the heterogeneity of clinical presentation, the degree of social impairment, intellectual ability, associated symptoms, and possible etiology. ASD are included in the diagnostic category of neurodevelopmental disorders in the Diagnostic and Statistical Manual of Mental Disorders V (1). The diagnosis of ASD is based on two major symptoms: social-communication deficits, restricted and repetitive interests, and behaviors. By definition, these symptoms must occur during the early childhood of individuals with ASD.

The currently accepted prevalence of ASD, based on consistent reports from multiple sources in different populations, is about 1% worldwide. ASDs are therefore among the most common pervasive developmental disorders and there is great concern regarding its growing incidence (2–4).

To date, the only FDA-approved treatments for ASD are the atypical antipsychotics risperidone and aripiprazole, which are mainly directed at treating the associated symptoms and not the core social dysfunctions that characterize this heterogeneous group of disorders. Treatments with these two medications have been demonstrated to reduce and attenuate irritability (e.g., tantrums, aggression, hyperactivity, and self-injurious behaviors) in children and adolescents with ASD (5–8). Improvements in social interaction and reciprocity have been observed as well, but this is probably a secondary effect of an overall reduction in maladaptive behaviors and not a primary therapeutic effect of these medications.

Targeted treatments for ASD are developed through the understanding of molecular and cellular abnormalities that guide specific interventions, hypothesizing that the wide variety of genetic variants in ASD converge in a core set of molecular pathways that mediate phenotypic expression in some identifiable core symptoms (9). Most research for new therapeutics currently uses preclinical models, such as "knockout" mice displaying specific molecular abnormalities. Genetic studies of ASD and related neurodevelopmental disorders have provided classes of potentially useful compounds. Proof-of-principle assays with agents that reversed phenotypes in mouse models have paved the way for clinical trials (10).

EXCITATORY/INHIBITORY IMBALANCE IN ASD AND NEW TREATMENTS

An imbalance of excitatory (glutamate) to inhibitory (GABA) neurotransmission (E/I imbalance) is thought to be implicated in the pathogenesis of ASD (11–13). Excessive excitatory glutamatergic neurotransmission with a loss of inhibitory GABA transmission, as well as abnormalities in synaptic plasticity due to dysfunctions

in the NMDA, AMPA, and/or GABA receptor systems, have been detected in mouse models and support this conceptualization of ASD pathogenesis (10). Pharmacological evidence emerged from mouse models with deletions in synaptic genes Fmr1, Mecp2, and Shank2, and the BTBR inbred strain, which have demonstrated favorable outcomes from treatments with glutamatergic agents, including memantine (14). After these preclinical investigations, clinical trials were carried out to test the potential role of glutamatergic and GABAergic agents in reversing core social dysfunction in ASD.

GLUTAMATERGIC TARGETING IN ASD

Altered glutamatergic excitatory transmission involves different receptors, including down-regulation of AMPA receptors, abnormalities of NMDA receptor-mediated plasticity, and altered metabotropic glutamate receptor subtype 5(mGluR5) signal transduction. The mGluR5 antagonists have been tested in preclinical models of autism with variable effects on social and stereotypic behaviors (15). MPEP, an antagonist of the mGluR5 receptor, reduced repetitive self-grooming in BTBR mice. BTBR is an autism mouse model with behavioral abnormalities in social interaction and communication, as well as repetitive movements and behaviors. Risperidone also reduced repetitive self-grooming in BTBR, but only at doses that induced sedation (16). GRN-259, a selective negative allosteric modulator of the mGluR5 receptor, was tested in BTBR mice. GRN-529 reduced repetitive behaviors in three cohorts of BTBR mice and it attenuated social withdrawal with an associated increase in communication, providing overall improvement in behavioral conditions (17). Treatment with the two AMPAKINE compounds CX1837 and CX1739, agents active on AMPA receptors that enhance excitatory glutamatergic action, succeeded in recovering social impairment but it did not attenuate the high levels of repetitive self-grooming in BTBR (18). In 4-week-old Balb/c mice, the NMDA receptor agonist D-cycloserine was found to improve both sociability and repetitive behaviors, emphasizing the potential role of NMDA agents in modulating the core social dysfunctions of ASD (19). Studies of Shank3 heterozygous mice detected a reduction in basal neurotransmission, reflecting reduced AMPA receptor-mediated transmission, which was targeted with insulin growth factor (IGF-1) treatment (20, 21).

Clinical trials followed the preclinical phase of investigation with glutamatergic agents. Memantine, an NMDA receptor antagonist, was tested in children and adolescents with ASD. Overall, the memantine trials conducted so far have yielded inconsistent findings due to methodological flaws, thus they did not allow for firm conclusions. No recommendations on the use of memantine or of other glutamatergic agents in ASD treatment are warranted as yet (22).

GABAergic TARGETING IN ASD

GABAergic signaling has been demonstrated to be implicated in E/I imbalance in ASD (23). Engrailed2 (En2) knockout mice have been proposed as a model for ASD. In this mutant, the cerebellum and hippocampus have been shown to have abnormal synaptic transmission, altered developmental processes and defective GABA transmission convergent with ASD pathways. A link between altered function of En2, deficits of GABAergic forebrain neurons, and the pathogenesis of ASD has been postulated (24, 25). In addition, social and cognitive abnormalities have been detected in En2 knockout mice, further highlighting its role in ASD. Deficits in reciprocal social interactions growing over time were found in En2 null mutants (26). Interestingly, mice lacking Mecp2 from GABA-releasing neurons demonstrated Rett syndrome and autistic features, including repetitive behaviors. In these mice, MeCP2-deficient GABAergic neurons showed a presynaptic reduction in glutamic acid decarboxylase 1 (Gad1) and glutamic acid decarboxylase 2 (Gad2) levels, and GABA immunoreactivity (27).

In clinical trials, GABA signaling has been probed with specific compounds. Arbaclofen, a $GABA_B$ receptor agonist that acts upstream of mGluR5 receptor signaling and is thought to augment inhibitory neurotransmission, was investigated. Initially, a preclinical study (28) followed by a clinical trial with arbaclofen was carried out in Fragile X syndrome, the most common cause of intellectual disability and ASD (29), and favorable findings were reported regarding social domains (30). A recent open-label trial with STX209, a form of Arbaclofen, was conducted in ASD patients (31). Safety, tolerability, and efficacy were tested in ASD individuals in an 8-week open-label trial enrolling 32 children and adolescents with a score of $\geq$17 on the aberrant behavior checklist (ABC)-irritability subscale. STX209 was generally well tolerated. Improvements were reported on several outcome measures, including the ABC-Irritability (the primary endpoint) and the Lethargy/Social Withdrawal subscales, and the Social Responsiveness Scale. Randomized controlled trials are needed to confirm these preliminary positive findings.

mTOR TARGETING IN ASD

The mammalian target of rapamycin (mTOR) pathway is central to synaptic protein synthesis and it integrates inputs from different sources, including NMDA and metabotropic glutamate receptors. In addition, it is directly involved in the maintenance of the physiological synaptic E/I ratio. Abnormalities in mTOR signaling have been found in ASD (32). Activation of mTORC1 promotes the formation of the eIF4F initiation complex. Mutations in genes upstream of mTOR, as detected in tuberous sclerosis complex (TSC1/2), neurofibromatosis 1(NF1), phosphatase and tensin homolog (PTEN), or the Fragile X syndrome gene (FMR1) cause hyperactivity of the mTORC1–eIF4E pathway and lead to syndromic forms of ASDs (33, 34). Accordingly, mTOR inhibitors are first line candidates for the treatment of ASD in TSC (35). Everolimus, an mTOR inhibitor, is currently being studied in a double-blind controlled trial in children and adolescents (6–21 years old) with TSC, ASD and seizures (NCT01289912).[1] The primary aim of this trial is improvement in cognition, while ASD symptoms attenuation and seizure frequencies are secondary objectives. Rapamycin, an mTORC1 inhibitor and immune-suppressor, was investigated in a recent preclinical study as an agent to ameliorate social behaviors and attenuate stereotypies in BTBR mice. Rapamycin was found to improve many components of sociability in the BTBR mouse,

[1] http://clinicaltrials.gov

pointing to mTOR overactivation as a therapeutic target in ASD. However, no effects of rapamycin on stereotypic behaviors were detected (36).

An important regulatory action was recently described in the other component of this pathway, the downstream effectors of mTOR. Signaling molecules in the downstream of the mTOR pathway have been demonstrated to play crucial roles in ASD pathogenesis, further highlighting its role (37, 38). Furthermore, 4E-BP2 inhibits protein translation by competing with eIF4G for eIF4E binding. The deletion of the gene encoding 4E-BP2 (EIF4ebp2) leads to autistic-like behaviors in mice. It was demonstrated that the removal of 4E-BP2, or overexpression of eIF4E, enhances protein translation; increased translation of neuroligins (NLGNs) is the next step that in turn causes an increased synaptic E/I ratio, which may eventually lead to ASD phenotypes. Pharmacological inhibition of eIF4E reversed social behavior deficits in EIF4ebp2 knockout mice and restored the E/I balance (37). These findings thus established a strong link between eIF4E-dependent translational control of NLGNs, E/I balance, and the development of ASD-like behaviors. Pharmacological targeting of downstream effectors of mTOR may represent promising experimental therapeutics in ASDs.

SINGLE GENE DISORDERS WITH ASD AND EXPERIMENTAL TREATMENTS

Single gene disorders associated with high rates of comorbid ASD, such as Rett syndrome and Phelan-McDermid syndrome (PMS) have provided significant models of targeted interventions with novel experimental treatments.

IGF-1 IN PHELAN-McDERMID SYNDROME (CHROMOSOME 22q13 DELETION SYNDROME)

The loss of a functional copy of the SHANK3 gene leads to 22q13 deletion syndrome, a complex clinical condition known as PMS. Haploinsufficiency of SHANK3 accounts for about 0.5% of cases of ASD and/or developmental delay (39). There is mounting evidence for a wider role of SHANK3 and glutamate signaling abnormalities in ASD and related conditions. IGF-1 regulates synapse formation, neurotransmitter release, and neuronal excitability via posttranslational modification of NMDA and AMPA receptors (40).

Therapeutic approaches with IGF-1 aimed at reversing deficits in SHANK3-haploinsufficiency have been carried out in mice. IGF-1 has been demonstrated to reverse the reduction in excitatory synapse numbers and density of neurons. Administration of daily intraperitoneal injections of human IGF-1 over a 2-week period was found to reverse deficits in hippocampal AMPA signaling, long-term potentiation (LTP), and motor performance in Shank3-deficient mice (41). These findings led to a current trial of IGF-1 as a treatment in PMS, which is still underway and expected to provide important clues on its role in the treatment of this ASD subtype (see text footnote 1 NCT0152590).

In a recent study, induced pluripotent stem cells (iPSCs) from individuals with PMS and autism were used to produce functional neurons. It was demonstrated that PMS neurons have reduced SHANK3 expression and major defects in excitatory, but not inhibitory, synaptic transmission. IGF-1 treatment promoted the formation of mature excitatory synapses that lack SHANK3 but contain PSD95 and NMDA receptors with fast deactivation kinetics. These findings provided evidence for a disruption in the ratio of E/I in PMS neurons, and they point to a molecular pathway that can be recruited to restore it (42).

IGF-1 IN RETT SYNDROME

Studies in mouse and human neuronal models of Rett syndrome also demonstrated benefits with IGF-1 administration. In 90% of cases, Rett syndrome is caused by abnormalities on the MECP2 gene, by either deletions or mutations. IGF-1, highly expressed during brain development and very important for spine maturation, has been tested to overcome MECP2 deficit. Synaptic abnormalities have been observed in Rett syndrome related to genetic defects on the MECP2 gene (43). Treatment with IGF-1 in a mouse model of Rett syndrome has been shown to increase synaptic growth and to rescue phenotype defects (44). A phase II randomized placebo-controlled trial is underway to evaluate the safety and efficacy of IGF-1 in treating ASD symptoms and respiratory/autonomic dysfunction in patients with Rett syndrome (see text footnote 1 NCT01777542).

INTRANASAL OXYTOCIN IN ASD

There has been growing interest in oxytocin as it is recognized to be implicated in social development and affiliative behaviors. Intranasal oxytocin has been shown to increase social initiative and motivation (45), as well as social cognition (46), and to reduce repetitive and restricted behaviors in ASD. Previous studies with oxytocin in ASD were conducted by single-dose administration to adults, therefore, the long-term effect of nasal oxytocin and its effect on children must still be clarified (47).

There have been only a few trials with intranasal oxytocin in children with ASD, and most were pilot studies on small numbers of patients. In one of the first double-blind studies, oxytocin nasal spray was found to improve emotion recognition in 16 male youth aged 12–19 with ASD (48). In a recent pilot study, 15 children and adolescents with ASD with verbal IQs of $\geq$70 participated in a trial on the safety and tolerability of intranasal oxytocin. Repeated measures of regression analysis controlled for week, dose, age, and sex were employed. The highest dose evaluated, 0.4 IU/kg, was found to be well tolerated. Over 3 months of treatment, social cognition, repetitive behaviors, and anxiety were improved with the maintenance of effect for 3 months after discontinuation of treatment (49). In an open-label study, oxytocin was administered intranasaly over a long term of 7 months to eight male youths with ASD (10–14 years of age; IQ: 20–101). Six of the eight participants had improvement in the communication and social interaction domains at ADOS-G evaluation and at *T*-scores of the CBCL; however, no statistically significant improvement was found in the ABC (50). In a double-blind study with a controlled design, a group of 38 male youths (7–16 years old) with ASD were administered 24 or 12 IU, depending on weight, of intranasal placebo or oxytocin once daily over four consecutive days. The oxytocin or placebo was administered in a dynamic social condition, i.e., during parent–child interaction training sessions. In this study, intranasal oxytocin did not significantly improve social interaction skills, however it must be

mentioned that it was an untested behavioral intervention, limiting the interpretation of results (51). Collectively, these studies demonstrated mixed results as to the outcome of core social impairment, with a tendency toward an improvement in social domains. Large-scale, double-blind placebo-controlled studies are needed to confirm the role of intranasal oxytocin in core social ASD dysfunction.

A randomized, double-blind cross over fMRI trial with intranasal oxytocin was conducted in a group of 17 ASD children and adolescents (age 8–16.5 years) to evaluate neural circuit modifications hypothesized after oxytocin administration (52). The Reading the Mind in the Eyes Test (RMET), a social cognition test (53) was used to investigate the brain areas under examination, and a remarkable variation was observed 45 min after oxytocin administration. Structures and circuits within the so-called social brain, such as the dorsal and ventral striatum, premotor cortex, posterior cingulate, and posterior–superior temporal sulcus, demonstrated enhanced activity after oxytocin administration. Moreover, changes in salivary oxytocin concentrations from baseline to 30 min post administration were positively associated with increased activity in the right amygdala and orbito-frontal cortex during social vs. non-social judgments. Furthermore, it was highlighted that oxytocin enhanced the salience of social stimuli, thus positively interfering with social cognition. These changes in the relevant brain areas of interest were observed following a single oxytocin administration and further research to test the persistence of these changes over time in long-term treatments is needed.

NEW APPROACHES TO VERIFY TREATMENT EFFICACY

Neural circuit modification following treatment for ASD is a novel field of research that could shed light on the mechanisms involved in neural changes and in the search for the biomarkers of treatment response (54). Functional MRI is the main instrument for a dynamic investigation of the neural substrate. A case study in two children with ASD was carried out with functional MRI performed before and after an intensive intervention of 4 months of pivotal response training (PRT). PRT is an empirically validated behavioral treatment that has widespread positive effects on communication, behavior, and social skills in young children with ASD. Neural processing modifications during a biological motion task, a form of social information, were evaluated by fMRI. Outcome measures included the evaluation of dysfunctional areas identified in children with ASD, compared to typically developing children (right amygdala, bilateral fusiform gyri, left ventrolateral prefrontal, and right posterior superotemporal sulcus). After treatment, ASD children showed increased activation in the above listed areas, indicating that neural modifications had occurred and they overlapped with the brain area activations of their typically developing peers (55).

Another study, investigating neural changes in response to treatment, examined variations in EEG signaling after an intervention with the Early Start Denver Model (ESDM) in a group of 48 children with ASD, aged 18–30 months. Children with ASD were randomized into two groups; one received an ESDM intervention for 2 years and the other received only their previous treatment in the community. A typically developing group of children served as controls. Event related potentials (ERP) and EEG activity (spectral power) were detected during the presentation of faces versus objects. The ESDM group demonstrated a better outcome in ASD symptoms, IQ, language, and adaptive behavior than the others. Increased cortical activation, i.e., a reduction in alfa power and increased theta band, was detected in this group of children as well as in typically developing children when viewing faces, in contrast to the community intervention group that showed the opposite pattern with greater cortical activation when viewing objects. The limitation of this research was that it lacked a baseline evaluation (56).

CONCLUSION AND FUTURE DIRECTIONS

Currently available medications mostly act upon selective symptoms of ASD, possibly offering a chance to further explore the specific mechanisms and circuits involved in etiological factors. Synaptic or circuit mechanisms associated with circumscribed aspects of ASD, e.g., social and communication domains, need to be thoroughly evaluated at the molecular and synaptic levels and possibly separated from the other symptoms/domains. Core mechanisms that cover distinct fractions of ASD would parallel the concept of core symptoms in ASD, based on the hypothesis that a group of protein factors converges on common pathways to be targeted (13). The next generation of animal models carrying human mutations will be of the utmost importance in uncovering the neural and molecular bases of ASD and to pave the way for clinical trials of treatments for core social domains. A homogeneous group of ASD individuals with phenotype and synaptic defects should be enrolled in pharmacological trials to test the efficacy and the safety of specific compounds. These trials would measure outcomes in the core social domain of ASD and also identify specific biomarkers for a wider perspective of treatment outcomes. Investigations of dynamic brain changes in ASD patients, using fMRI after treatments, would be extremely valuable in order to shed light on the neural underpinnings of core social impairment in these individuals.

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Autism as a disorder of biological and behavioral rhythms: toward new therapeutic perspectives

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There is a growing interest in the role of biological and behavioral rhythms in typical and atypical development. Recent studies in cognitive and developmental psychology have highlighted the importance of rhythmicity and synchrony of motor, emotional, and interpersonal rhythms in early development of social communication. The synchronization of rhythms allows tuning and adaptation to the external environment. The role of melatonin in the ontogenetic establishment of circadian rhythms and the synchronization of the circadian clocks network suggests that this hormone might be also involved in the synchrony of motor, emotional, and interpersonal rhythms. Autism provides a challenging model of physiological and behavioral rhythm disturbances and their possible effects on the development of social communication impairments and repetitive behaviors and interests. This article situates autism as a disorder of biological and behavioral rhythms and reviews the recent literature on the role of rhythmicity and synchrony of rhythms in child development. Finally, the hypothesis is developed that an integrated approach focusing on biological, motor, emotional, and interpersonal rhythms may open interesting therapeutic perspectives for children with autism. More specifically, promising avenues are discussed for potential therapeutic benefits in autism spectrum disorder of melatonin combined with developmental behavioral interventions that emphasize synchrony, such as the Early Start Denver Model.

Keywords: autism spectrum disorder, biological rhythms, motor, emotional and relational rhythms, synchronization of rhythms, melatonin, Early Start Denver Model, therapeutics

INTRODUCTION

Endogenous physiological variations involved in biological rhythms reflect adaptation to the environment. Thus, the sleep–wake rhythm associated with biological circadian rhythms can be viewed as an adaptation to the day–night cycle. Circadian rhythms allow temporal organization of biological functions in relation to environmental changes (1). The periodicity of activities applies to all biological, physiological, and psychological functions; recently, the science of biological rhythms, chronobiology, has emerged with its own theory, science, and education (2).

Furthermore, recent studies in the field of cognitive and developmental psychology have highlighted the importance of rhythmicity and synchrony of motor, emotional, and relational rhythms in early development of social communication. Given the major role of the sleep hormone melatonin in the ontogenetic establishment of diurnal rhythms, the synchronization of peripheral oscillators (also termed clocks) and the regulation of human circadian rhythms (1), melatonin might be involved in the synchrony of motor, emotional, and relational rhythms.

Indeed, relationships might exist, based on the hypothesis of ergodicity (3), between cellular communication networks involving a cellular synchrony (synchronization of cellular oscillations by melatonin) and early social communication development involving a synchrony of motor, emotional, and interpersonal rhythms. Autism spectrum disorder (ASD) – a developmental disorder characterized by social communication impairments associated with repetitive interests and behaviors – provides an interesting and challenging model of abnormal melatonin production in early developmental disorders and its possible relationship with autistic behavioral impairments.

This article proposes a central role of rhythmicity and synchrony of rhythms in typical child development and offers a new integrative approach, which considers autism as a disorder of biological and behavioral rhythms. In this perspective, promising avenues will be discussed in this article for potential therapeutic benefits in ASD of melatonin and developmental behavioral interventions that emphasize rhythms and synchronization, such as the Early Start Denver Model (ESDM).

PHYSIOLOGICAL AND BEHAVIORAL RHYTHM DISTURBANCES IN AUTISM

AUTISM AS A DISORDER OF BIOLOGICAL RHYTHMS

Alterations in circadian sleep–wake rhythm have frequently been reported in autism (4, 5). More specifically, reduced total sleep and longer sleep latency as well as nocturnal and early morning awakenings are often observed in individuals with ASD (6–12). Furthermore, prior studies on melatonin in autism have all reported abnormalities in melatonin secretion (see **Table 1**). In addition, abnormalities in cortisol circadian rhythm have also been reported in autism [for a review, see Tordjman et al. (13)]. In particular, significantly higher frequency of absence of circadian variation in melatonin and cortisol levels was observed in individuals with autism compared to typically developing controls. Golombek et al. (14) described the effects of circadian desynchronization that can enhance susceptibility to certain disorders (metabolic, immune, cognitive, and somatic disorders including cancer). It is noteworthy that congenitally blind children with consequently abnormal melatonin secretion and synchronization [the production of pineal-derived melatonin depends on the light acting through the retinohypothalamic tract (15)] very frequently display autism [up to 42% (16)], whereas hearing impaired children, including hearing loss, show autism less frequently [up to 10% (17)]. More specifically, abnormally low daytime and nighttime melatonin secretion was associated with an absence of melatonin circadian variation in some individuals with autism (18, 19), which in turn, given the role of synchronizer of melatonin, also has consequences on the circadian rhythms network, including the cortisol circadian rhythm (13, 20).

This blunted circadian rhythmicity with no or little variability might be related to the difficulties in adapting to changes typically observed in individuals with autism. Thus, children with autism who are confronted with physiological continuity due to absent circadian rhythms may have difficulties adapting to changes in either their external or their internal environment (26). Indeed, as previously underlined, the circadian clocks network, synchronized by melatonin and involving an internal system of continuity/discontinuity, allows adaptation to environmental changes.

Similarly, blunted circadian rhythmicity may explain the difficulty observed in many children with autism in adapting to

Table 1 | Studies of melatonin levels in individuals with autism.

Study	Sample	Study group	Measured variable	Results
Ritvo et al. (21)	Urine	Young adults with autism ($N = 10$)	Melatonin concentration	Increased daytime values compared to typically developing controls Similar nighttime values compared to typically developing controls
Nir et al. (22)	Serum	Young men with autism ($N = 10$)	Melatonin concentration	Increased daytime values compared to typically developing controls Decreased nighttime values compared to typically developing controls
Kulman et al. (19)	Serum	Children with autism ($N = 14$)	Melatonin concentration (24-h circadian rhythm)	Decreased nighttime values compared to typically developing controls No circadian variation in 10/14 (71.4%) children with autism Inverted rhythm in 4/14 (28.6%) children with autism
Tordjman et al. (23)	Urine	Children and adolescents with autism ($N = 49$)	6-Sulphatoxymelatonin excretion rate (12-h collection)	Decreased nighttime values compared to typically developing controls
Melke et al. (24)	Plasma	Adolescents and young adults with autism ($N = 43$)	Melatonin concentration	Decreased daytime values compared to typically developing controls
Mulder et al. (25)	Urine	Children and adolescents with autism ($N = 20$)	6-Sulphatoxymelatonin excretion rate (24-h collection)	Trend to lower 24-h melatonin excretion rate in hyperserotonemic compared to normoserotonemic individuals with autism
Tordjman et al. (18)	Urine	Post-pubertal adolescents and young adults with autism ($N = 43$)	6-Sulphatoxymelatonin excretion rate (split 24-h collection)	Decreased daytime values compared to typically developing controls Decreased nighttime values compared to typically developing controls No circadian variation in 10/43 (23.2%) individuals with autism

changes in rhythms of their external and internal environment. Thus, rapid rhythms of sensory stimuli in their external environment (e.g., repeated visual stimuli provided by a stroboscopic light) can provoke epileptic seizure in some individuals with autism [approximately one-third of children with autism have epilepsy (27)]. Interestingly, EEG changes tended to be correlated with an abnormal rhythm of melatonin in young adults with autism (22). Furthermore, some parents have reported that their daughters with autism experience epileptic seizures toward the 14th day of their menstrual cycle, which is when luteinizing hormone (LH) levels peak (Tordjman, personal communication), suggesting quite tentatively that individuals with autism may have difficulties adapting to periodic hormonal changes in their internal environment. We can state the following hypothesis: a change in rhythm associated with excessive environmental stimuli might strongly increase arousal and lead to physiological stress which, for some individuals with autism, can disturb the rhythmic activity of a particular brain area, leading it to fall out of sync with the rest of the brain and causing its population of neurons to fire (depolarization and occurrence of an epileptic seizure). This underlines the importance of stable physiological rhythms.

Finally, significant relationships have been found between lower nocturnal melatonin excretion and increased severity of autistic social communication impairments, especially for verbal communication and social imitative play (18, 23). These findings are in agreement with studies suggesting an association between reduced melatonin production and language impairment (22, 28). Along the same line, the systemic administration in the animal model of Zebra Finch of a melatonin-1B receptor antagonist at the beginning of the night shortens the song and motif length and affects the song syllable lengths produced the next day (29). Reduced melatonin activity might create timing problems in biological clocks with physiological and psychological effects that might be, according to Boucher's model of autism (30) and Wimpory's theory (31), involved in autistic impairments, notably in autistic social communication impairments. It is noteworthy that deficiency in oxytocin [oxytocin is considered a bonding hormone (32, 33)] has also been reported in autism (34, 35) and bonding is involved in the development of very early social interaction in infants. Interestingly, the release of oxytocin by the posterior pituitary gland follows a robust circadian rhythm in mammals. Further studies are needed to better understand the underlying mechanisms of oxytocin anomalies in autism and to explore, in particular, possible oxytocin rhythm disturbances in ASD. The importance of the synchrony of rhythms in the development of social interaction and communication is detailed in the next section.

IMPORTANCE OF SYNCHRONY OF RHYTHMS FOR SOCIAL COMMUNICATION IN TYPICAL DEVELOPMENT AND AUTISM SPECTRUM DISORDER

Several studies, based on animal models and human perinatal development, suggest that stable patterns of repeated stimuli in the form of maternal physiological rhythms, involving cross-modal perception such as regular cardiac rhythm, which provides the fetus with auditory and vibratory stimuli, allow the fetus to integrate sensory information facilitating prenatal perceptual learning and develop a coherent representation of his or her internal and external environment (36–38). Fluctuations in the physiological rhythms (variants), such as variations in the maternal cardiac rhythm and also variations in hormone levels involved in the circadian rhythms that are already present during the fetus life (the fetus' circadian rhythms are the mother's ones), occurring in a background of regular repetition of identical sequences (invariants), may help the fetus to develop the ability to adapt to change in an environment characterized by high regularity. As previously emphasized (38), very early mother–infant relations provide a secure environment based on the repetition of invariants, while at the same time promoting adaptation to change through the presence of variants. It is through the regular repetition of identical sequences of discontinuity, such as circadian rhythms, that a continuum is constructed associated with the development of adaptation to changes.

The development of the very earliest form of communication relies on the sharing of emotions between mother and infant, when, for example, the infant is suckling in his or her mother's arms, through emotional synchrony that enhances the integration of sensory inputs (39, 40). Concerning memory processes, emotions enable to "fix" events, just as a photographic fixing agent sets images (38). Cortisol (a stress and arousal neurohormone) crosses the placental barrier. The cortisol circadian rhythm, as well as the melatonin circadian rhythm in the fetus and infant after birth, are those of maternal cortisol and melatonin. Indeed, the infant's circadian cortisol and melatonin rhythms are only established between 2 and 3 months of age in typical development, at the same time that infants begin to have more regular sleep–wake cycles associated with nighttime sleep lasting 6–8 h (41, 42). Interestingly, this period coincides in typical development with the emergence of social smiling by the second month of life (43), the advent of mirror self-recognition at around 3 months of age (44), and increased brain activation to speech occurring between 3 and 4 months of age (45).

At birth, the human immaturity of the cerebral cortex allows initial learning to influence the neural architecture through perceptual-action mapping (46, 47). The infant's social skills, especially including imitation, shared attention, and empathic understanding, also contribute to the development of learning (46, 48). Social synchrony can be defined as the dynamic and reciprocal adaptation of the temporal structure of behaviors between interactive partners (49). In typically developing children, the quality of social interaction depends on an active dialog between the parent and the infant (50, 51). Numerous studies have been emphasizing the importance of parent–infant synchrony and the construction of shared timing in social communication development (52).

Also, biological markers were associated with relational synchrony. First, oxytocin administration to parent enhances infant physiological and behavioral readiness for social engagement and parallels an oxytocin increase in infants (32). Second, neural correlates were found using hyper-scanning recordings of EEG brain activity and measures of neural synchronization between distant brain regions of interacting individuals through a free imitation task (53). Dumas and colleagues' study (53) was the first to record dual EEG activity in dyads of subjects during spontaneous non-verbal interaction. Eleven same-sex pairs were scanned. They found that interpersonal rhythmic oscillations were correlated

with the emergence of synchronization in the brain's alpha–mu band between the right centro-parietal regions (an area involved in social interaction) of both participants. Developmental psychologists now study interaction not only as the addition of two behaviors but also as a global phenomenon in which synchrony is considered as social *per se*. To describe the dialog between two partners engaged in behavioral and affective exchange, developmental psychologists more and more take into consideration rhythm and temporal course of both behavior and affect, regarded as key expressions of adaptation during interaction (49, 52).

Only a few studies have addressed the importance of infant–caregiver synchrony/reciprocity in the development of social communication involving infants who subsequently are diagnosed with autism. It appears appropriate in the field of autism to consider the combined domain of social communication, as the most recent version of the ADOS scale does, as well as the recently released DSM-5 American classification. Methods to investigate this issue include studies using early home videos (54), parental interviews focusing on early abnormalities (55), and prospective assessment of children at risk of ASD (e.g., siblings) (56, 57). Studies have revealed a pervasive developmental course in infants who were later diagnosed with ASD. Thus, the first signs were abnormalities in eye contact, imitation, disengagement, joint attention, orienting to name, and body language. These behaviors are parts of the affective tuning disturbances; the term affective tuning, defined by Stern (58) as the "execution of behaviors expressing the emotional ownership of a shared affective state," refers to infant–caregiver emotional communication based on rhythmic similarity from the second semester of life onward. Also, these behaviors are important precursors of later-developing symptoms. However, whether these first signs impair early infant–parent interactions and whether they reflect already autistic behavioral impairments in the infant remain to be explored. In two related studies based on home movies of children later diagnosed with autism, Saint-Georges et al. (54) and Cohen et al. (59) showed that motor and emotional asynchrony was present between infants and parents before 12 months of age, and parents perceived weaker initiatives from their children. In addition, parents experienced weaker interactive responsiveness from their children and increasingly tried to compensate this perceived deficit by soliciting behaviors through touching the infant. This was particularly observed after 12 months of age for the fathers of infants who were later diagnosed with autism.

Many authors have studied imitation in children with autism (imitation of other people's faces, gestures, or vocal signals) in order to better understand the development of autistic social communication impairments. This specific type of imitation is referred to as "spatial" imitation to highlight the capacity to produce an instantaneous copy of the form of the signal. However, another way to communicate with others is to perform a "temporal" imitation of their behavior (60). This is what humans do through rhythmic finger or foot taping, dancing, singing, and drumming in synchrony with others (61). Xavier et al. (62) highlighted the importance of rhythmicity and synchrony in the development of children's imitative exchanges with peers. From birth, a child has a predisposition to engage, intersubjectively, with the rhytmic actions and awareness of other persons, and to move in synchrony with them (44, 63). Synchronic imitation is an important preverbal way to communicate among peers (64, 65). This reciprocal experience concerns two children able and motivated to coordinate their behavior with the non-ritualized behavior of the other, in both form and timing and to alternate turns between model and imitator (66). The impression of fluidity in the coordination of movements between partners is underlined by mutual attention, engagement, continuous adaptation, and turn taking (67). This rhythmic process made of ludic spontaneous imitation reveals moments of discontinuity occurring in a background of continuity. Neural bases of this coupling activity are constituted by the neuron mirrors system (68), with the same evidence showing that the neuronal structures involved when a mental state is experienced, are also recruited during the observation of others. Guionnet et al. (69) designed a free imitation paradigm in an fMRI study to examine some neural correlates of social interaction. Their results agree with those previously evidenced (70, 71) concerning the core circuit of imitation, but they found different activations between the situation of imitating and that of being imitated.

A special quality of temporal imitation is the ability to use different motor movements in order to communicate. Thus, simple finger tapping can be synchronized with another's head nodding or trunk movements, whether entrained by one of the movements or in response to external synchronizing stimuli such as music. Although animals and humans can perceive rhythms and produce rhythmic motor patterns, only humans can adapt their rhythmic movements to external rhythms (72) [with the exception of the cockatoo (73)]. The ability to be rhythmically synchronized with the environment appears important for infant development in the emotional, cognitive, social, and sensorimotor realms (44, 74). It has been demonstrated that the human fetus and newborn already have the capacity to perceive and produce rhythms (75). The ability to produce temporally adapted motor patterns comes later and depends on the specific motor system involved and the relationship between the beat presented and the spontaneously occurring motor tempo of the infant (76, 77). It should be fruitful to longitudinally examine children with autism in terms of ability to adapt their own rhythm to external rhythms. Interestingly, clinical observations suggest that some children with autism are able to respond to an external rhythmic vocalization by a similar rhythmic motor pattern such as hand flapping (Tordjman, personal communication). However, previous studies reported disorganized rhythms, stereotypies, and poor synchrony in most of these children (78), which might be related to the low melatonin levels reported to be associated in autism with the severity of verbal communication and social imitative play impairments (18, 23). Melatonin, as a regulator of physiological rhythms and oscillations, might enhance the capacity of children with ASD to synchronize their movements with movements of others (this synchronization of movements is needed for imitative play) and with external rhythmic auditory stimuli (such as music and/or human voice enhancing their verbal skills). Interestingly, in a study of social smiling in infants (79), there was no difference in frequency of smiling between 2- and 5-month-old infants with and without ASD during infant–caregiver face-to-face interactions. However, whereas typically developing infants showed a significant increase

in smiling rate when caregivers were smiling, smiling in infants later diagnosed with ASD was not synchronized with smiling in caregivers and was not contingent upon caregiver behavior (caregiver facial expressions and vocalizations). Furthermore, it is noteworthy that, as previously indicated in this section, the infant's melatonin circadian rhythm is established between 2 and 3 months of age, and a study (80) reported that eye contact was normal in 2-month-old infants later diagnosed with autism but declined between 2 and 6 months of age, suggesting an additional argument in favor of a possible relationship between the well-replicated nocturnal melatonin deficit in autism (see **Table 1**) and the development of autistic social communication impairments.

REPETITIVE BEHAVIORS AND INTERESTS

Repetitive behaviors and interests are defined, according to DSM-5 criteria (81), as a repetition of identical sequences of behaviors for motor stereotypies (motor stereotypies involve repetitive maladaptive movements) or thoughts for restrictive patterns of interests (restrictive and repetitive interests involve fixated interests, adherence to routines, or ritualized and rigid thinking patterns). Thus, repetitive behaviors and interests can be viewed as behavioral responses to the need to create discontinuity that is repeated at regular intervals, which could have been fundamentally lacking in the physiological development of children with autism due to the melatonin deficit reported in autism. Our finding (18) observed in a sample of 43 adolescents and young adults with autism (nocturnal excretion of 6-SM was significantly negatively correlated with repetitive use of objects), taken together with improvement of stereotyped behaviors following administration of melatonin in 24 children and adolescents with ASD (82), supports this hypothesis.

The autistic deficit in melatonin secretion might lead to physiological rhythm disturbances in autism impairing biological circadian rhythms and even, in certain cases, to an "endless" physiological continuity provoked by the absence of variation in melatonin levels. From this perspective, stereotyped behaviors and interests can be seen as offering to children with autism rhythmic forms providing rhythmicity and discontinuity through the creation of repeated identical patterns. Albert Goldbeter (83), director of the Chronobiology Unit at the Brussels Sciences University, underlines that life is rhythm. It is noteworthy that the word pineal comes from pine cone that is a Mesopotamian symbol representing the source of life and the power of regeneration. In Asia, the pineal gland is considered as the 7th chakra and represents the location of the soul and the highest level of consciousness.

Donald Winnicott (84) emphasized that "the main problem for a typically developing child is to be able to create a continuum out of discontinuity." According to him, the first optimal container ("holding environment") for the newborn is the progressive internalization of the rhythmic structures of feeding (rhythmic ebb and flow corresponding to the kinesthetic experience of suckling), providing a sense of continuing existence. We can nevertheless hypothesize, as we did in the previous section and in a prior article (26), that this internalization process starts far earlier, in the womb, through maternal physiological rhythms; conversely, based on clinical observations in autism, children with autism create discontinuity out of continuity. We can also state the hypothesis that children with autism, confronted with an "endless" physiological continuity (or at least with an absence of physiological discontinuities repeated at regular intervals), would develop anxiety and stress due to the lack of secure stable rhythms, and would therefore try to create by themselves rhythmicity in order to control them through stereotyped behaviors. However, the relationships between stereotyped behaviors and stress responses or anxiety need to be clarified given discrepant results reported in some studies (85, 86).

Finally, it should be noted that several studies have also recently opened new behavioral, neurobiological, and pharmacological perspectives on autistic repetitive behaviors, especially on self-injurious behaviors [for a review, see Adler et al. (87), Minshawi et al. (88), and Stigler (89)]. Thus, research on potential therapeutic effects for repetitive behaviors and interests should investigate benefits of melatonin in ASD (see next section) but should not be limited to the administration of melatonin.

THERAPEUTIC BENEFITS OF MELATONIN IN AUTISM SPECTRUM DISORDER

Published melatonin treatment studies in autism are presented in **Table 2**. Many have been most concerned with effects of melatonin on sleep. Reviews, commentaries, and meta analyses of melatonin treatment studies are presented in **Table 3**. These two tables come from a prior article of our team (26).

As previously discussed (26), the treatment studies have a number of limitations. In some studies individuals covering a wide age range were included with pre-pubertal, pubertal, and post-pubertal individuals being studied (102, 103, 105). Reported differences in pineal melatonin secretion according to age and pubertal stage, coupled with the clearly developmental nature of autism, suggests that therapeutic effects of melatonin might vary considerably with age and pubertal status (23). Studies have also been limited by small sample sizes (90–93, 95, 96, 98, 102, 104). In larger studies, heterogeneous groups are often examined and have included blind individuals and individuals with various neurological disabilities with concomitant intellectual disability. In such studies, often results seen for the autism subgroup are not separately presented (97, 100, 103–107). The specificity and interpretation of the results with respect to autism are often unclear. Additional research is needed to determine whether and which findings might be specific to autism or whether melatonin's effect might be similar across groups. It can be hypothesized that melatonin's effects are due to actions on certain behavioral dimensions and that they can be observed across disorders. However, it should be pointed out that in our studies (18, 23), lower melatonin excretion was significantly associated with social communication impairments rather than with sleep problems. Future studies of melatonin in ASD should simultaneously examine melatonin levels, sleep problems, autistic behavioral impairments, and level of functioning so that a more complete picture can emerge.

It is worth noting that just a few of the therapeutic trials of melatonin have assessed effects on autistic behavioral impairments. These include reports of improved communication (105), reduced social withdrawal (82, 99), decreased stereotyped behaviors and rigidity (82, 102), and reduced anxiety (99, 103). Furthermore, at time the improvements noted were not sufficiently detailed. For example, Wright et al. (105) reported significant

Table 2 | Studies on potential therapeutic benefits of melatonin in autism.

Study	Journal	Population	Design	Duration of treatment	Melatonin (formulation, dose)	Time of intake	Main outcome measures	Effects on sleep	Other outcomes	Comments
SINGLE CASE REPORTS										
Horrigan and Barnhill (90)	*J Am Acad Child Adolesc Psychiatry*	17-year-old boy with Asperger's Syndrome (AS)	–	Not given	3 mg	20–30 min before bedtime (BB)	Sleep	Sleep improvement. No side effects	Daytime behavior improvement	–
Hayashi (91)	*Psychiatry Clin Neurosci*	14-year-old boy with autistic disorder, severe intellectual disability and phase delay with polyphasic sleep	–	4 months	Immediate release (IR) 6 mg	11:00 p.m.	Sleep	Melatonin increased sleep duration. No side effects	None	–
Jan et al. (92)	*Dev Med Child Neurol*	12-year-old boy with AS and complex sleep disturbance (phase delay and parasomnias)	–	6 months	Controlled release (CR) 5 mg	30 min BB	Sleep	Normalization of the sleep–wake rhythm and disappearance of parasomnias. No side effects	None	–
RETROSPECTIVE STUDIES										
Gupta and Hutchins (93)	*Arch Dis Child*	9 cases of children with autistic disorder (AD) aged from 2 to 11 years. Chronic sleep problems	Not given	1 week to 1 year	IR 2.5–5 mg	45 min BB	Parental evaluation of sleep	56% showed improvement in total sleep duration	None	No standardized collection of sleep variables
Andersen et al. (94)	*J Child Neurol*	107 children and adolescents aged from 2 to 18 years with ASD (DSM-IV): 71% AD, 5% AS, 19% PDDNOS (pervasive developmental disorder not otherwise specified)	Not given	Mean duration: 1.8 years	IR in 91% of the cases. Dose escalation protocol from 1 to 6 mg based upon age	30–60 min BB	Parental evaluation of sleep	Parents reported full (25%) or partial (60%) improvement. Beneficial effects of melatonin seem to stop after 3–12 months despite the use of higher doses. Side effects observed in 3 children: sleepiness, fogginess, increased enuresis	None	No standardized collection of sleep variables. The loss of response to melatonin treatment is discussed in the text
Galli-Carminatti et al. (95)	*Swiss Med Wkly*	6 adult patients with AD (CIM-10) and intellectual disability, aged from 19 to 52 years	Not given	6 months	IR. Dose escalation protocol from 3 to 9 mg if clinically required	45 min BB	Sleep (CGI-S and CGI-I)	Improvement in sleep onset latency, night and early morning awakenings. No side effects	None	No standardized collection of sleep variables. Two to four associated psychotropic drugs per patient
OPEN-LABEL TRIALS										
Jan et al. (96)	*Dev Med Child Neurol*	15 children with multiple neurological disabilities and severe sleep disorders	Not given	Not given	2–10 mg	Bedtime	Not given	Partial improvement in sleep disorders. No side effects	Behavior and social improvement	Heterogeneous sleep disorders and neurological disabilities
Ishizaki et al. (97)	*No To Hattatsu*	50 children and young adults with autism ($n = 27$) or mental retardation ($n = 20$) or severe motor/intellectual disability ($n = 3$) aged from 3 to 28 years with sleep disorders	Not given	Not given	Not given	Not given	Sleep disorders and emotional/behavior disturbances	34 patients experienced improvement in response to melatonin. Side effects reported in 17 patients	Improvements in excitability when sleep also improved. No change in contrariness, stereotyped behavior and in school/workshop refusal	Various types of insomnia and diagnoses

(Continued)

Table 2 | Continued

Study	Journal	Population	Design	Duration of treatment	Melatonin (formulation, dose)	Time of intake	Main outcome measures	Effects on sleep	Other outcomes	Comments
Paavonen et al. (98)	*J Child Adolesc Psychopharmacol*	15 children with AS (DSM-IV) aged from 6 to 17 years with severe sleep problems for at least 3 months	Not given	14 days	IR 3 mg	30 min BB	Sleep (72 h-period actigraphy, sleep diaries), daytime behavior (Karolina Sleepiness Scale; KSS), Child Behavior Checklist (CBCL)	Melatonin treatment was associated with significant decrease in sleep onset latency and nocturnal activity. Discontinuation of melatonin led to a significant decrease in sleep duration and more nocturnal activity. Side effects in 20% of the cases: tiredness, headaches, severe sleepiness, dizziness, diarrhea	Significant improvement of daytime behavior (CBCL)	No principal outcome specified. KSS is not validated in children nor in ASD
Giannotti et al. (99)	*J Autism Dev Disord*	29 children with AD (DSM-IV) aged from 2 to 9 years with current sleep problems	Controlled-release melatonin	6 months	Dose escalation protocol from 3 mg (1 mg of IR + 2 mg of CR) to 6 mg when clinically required, based upon age (max 4 mg under 4 years old and max 6 mg over 6 years old)	08:00 p.m.	Sleep (diaries and Children's Sleep Habits Questionnaire CSHQ), daytime behavior, Childhood Autism Rating Scale (CARS)	Melatonin treatment was associated with improvement in sleep onset latency, night awakenings, and sleep duration, which vanished after melatonin discontinuation. No side effects	Parents reported less irritability, less anxiety, and better mood. Significant improvement of depression, anxiety, and withdrawal symptoms during melatonin treatment in children with AS. No effect was reported on the CARS	No principal outcome specified. Missing data: analyses on 25 patients
De Leersnyder et al. (100)	*Pediatr Neurol*	88 children with heterogeneous neurodevelopmental disorders (Smith-Magenis syndrome, mental retardation, encephalopathy, Angelman syndrome, Rett syndrome, Bourneville syndrome, blindness, and autism) aged from 5 to 20 years. Seven patients with autism, mean age 12 years old	6 years of open-label follow up	3 months	CR 2–4 mg (<40 kg) or 6 mg (>40 kg) based upon weight	60 min BB	Parental evaluation of sleep and mood (self-constructed questionnaire)	According to parental reports, both sleep latency and sleep duration improved within 3 months such as night awakenings, sleep quality, and daytime napping. Eleven children experienced adverse events (daytime nap, difficulties in swallowing tablets) that the parents attributed to melatonin treatment	12% of the parents reported improvements of mood in their children	Heterogeneous neurodevelopmental disorders. Results cannot apply to a population with autism spectrum disorders. No standardized collection of sleep and mood parameters. Mean dose for patients with autism: 5.7 mg
Malow et al. (82)	*J Autism Dev Disord*	24 children with ASD (DSM-IV, ADOS): AD, AS, and PDDNOS aged from 3 to 9 years. Sleep onset delay of 30 min or longer confirmed on actigraphy. Exclusion of neurodevelopmental disabilities such as fragile X, Down, and Rett syndromes	Before treatment families received structured sleep education and children underwent a treatment acclimatation phase in order to be sure the melatonin will be taken	14 weeks	CR. Dose escalation protocol from 2 to 9 mg when clinically required	30 min BB	Sleep (actigraphy, Children's Sleep Habits Questionnaire; CSHQ, diaries), daytime behavior (Child Behavior Checklist; CBCL, Repetitive Behavior Scale-Revised), parental stress (Parenting Stress Index Short Form), side effects (Hague Side Effects Scale)	Significant improvement in sleep latency within the first week of treatment but not for other sleep parameters such as night awakenings and sleep quality	Significant improvement in children's behavior (withdrawal, affective problems, attention-deficit hyperactivity, stereotyped, and compulsive behaviors). Significant improvement in parental stress	No placebo

(Continued)

Table 2 | Continued

Study	Journal	Population	Design	Duration of treatment	Melatonin (formulation, dose)	Time of intake	Main outcome measures	Effects on sleep	Other outcomes	Comments
PLACEBO-CONTROLLED TRIAL										
McArthur and Budden (101)	*Dev Med Child Neurol*	9 children and adolescents with Rett syndrome aged from 4 to 17 years. Mean age:10 years old	Randomized double-blind crossover trial	2 periods of 4 weeks with a wash out period of 1 week	2.5–7.5 mg based on weight	60 min BB	Sleep (actigraphy, diaries)	Significant improvement in total sleep time. No side effects	None	–
Garstang and Wallis (102)	*Child Care Health Dev*	11 children and adolescents with ASD aged from 5 to 15 years with chronic sleep disorders resistant to behavioral treatment	Randomized double-blind crossover trial	2 periods of 4 weeks with a wash out period of 1 week	IR 5 mg	60 min BB	Sleep (diary)	Melatonin and placebo were associated with significantly decreased sleep latency and nocturnal awakenings, increased total sleep time. No side effects	Several parents and class teachers commented that their children were easier to manage and less rigid in their behavior while taking melatonin	ASD criteria were not consensual. Only 7 children completed the trial. Investigators found that some of the placebo capsules were empty. Missing data
Wasdell et al. (103)	*J Pineal Res*	51 children and adolescents with neurodevelopmental disabilities (16 patients with ASD) aged from 2 to 18 years. Sleep delay phase syndrome and impaired sleep maintenance with resistant to sleep hygiene intervention	Randomized double-blind crossover trial. Three weeks trial followed by a 3-month open-label study. Behavioral sleep treatment before inclusion	2 periods of 10 days with a wash out period of 3–5 days	Dose escalation protocol based on unspecified conditions: from 5 mg (1 mg FR + 4 mg CR) to 15 mg	20–30 min BB	Sleep (actigraphy, diaries, CGI-S, CGI-I), familial stress (family stress scale)	Significant improvement in total sleep duration and sleep latency as well as reduced stress levels in parents in the melatonin arm	Half of the patients with ASD had their dose increased during the open-label phase with no additional improvement in sleep latency or sleep duration, but caregivers reported less anxiety	Unspecified ASD criteria. Fifty patients completed the trial and 47 completed the open-label phase. Selection bias due to previous melatonin treatment (25% of the cases). At the end of the trial, 29 patients received a dose of 10 or 15 mg. Higher doses were necessary in patients with bilateral cerebral lesions
Wirojanan et al. (104)	*J Clin Sleep Med*	12 children and adolescents with unspecified sleep problems, aged from 2 to 15 years: 5 patients with AD (ADOS and ADI-R), 3 patients with fragile X syndrome with AD, 3 patients with AD and fragile X syndrome, and 1 patient with fragile X premutation	Randomized double-blind crossover trial	2 periods of 2 weeks. No wash out period	IR 3 mg	30 min BB	Sleep (actigraphy, diary)	Significant, but mild improvement in total sleep time (+21 min) and decrease in sleep latency (−28 min)	None	Missing data: only 12 patients completed the trial (order bias). No subgroup analysis in AD patients. No side effects
Wright et al. (105)	*J Autism Dev Disord*	22 children and adolescents aged from 3 to 16 years with ASD (ICD-10, ADOS, ADI-R): AD (70%), AS (10%), and AA (20%). No fragile X or Rett syndrome. Current sleeplessness (confirmed on a 1-month-diary) and resistant to behavioral treatment	Randomized double-blind crossover trial	2 periods of 3 months separated by 1 month of washout	IR. Dose escalation protocol from 2 to 10 mg when clinically required	30–40 min BB	Sleep (sleep difficulties questionnaire, diary), daytime behavior (Developmental Behavior Checklist), side effect questionnaire	Significant improvement in sleep latency (−47 min) and total sleep duration (+52 min) in the melatonin arm. No improvement in night awakenings. The side effect profile was not significantly different between the 2 groups	Improvement in children's behavior in the melatonin arm that was significant for communication ($p = 0.045$)	Missing data. Analysis on 16 patients. No actigraphy. Mean melatonin dose: 7 mg

(Continued)

Table 2 | Continued

Study	Journal	Population	Design	Duration of treatment	Melatonin (formulation, dose)	Time of intake	Main outcome measures	Effects on sleep	Other outcomes	Comments
Cortesi et al. (106)	*J Sleep Res*	160 children with ASD (DSM-IV, ADI-R, ADOS) aged from 4 to 10 years with sleep onset insomnia and impaired sleep maintenance	Randomized placebo-controlled. Randomization in 4 groups: (1) melatonin alone (2) melatonin + cognitive behavioral therapy (CBT) (3) CBT alone (4) placebo	12 weeks	CR 3 mg	09:00 p.m.	Sleep (actigraphy, Children's Sleep Habits Questionnaire, diaries)	144 patients completed the trial and 134 were analyzed. Combination group showed greater significant improvements on sleep followed by the melatonin alone and the CBT alone compared to placebo group. No side effects	None	–
Gringas et al. (107)	*BMJ*	146 children aged from 3 to 15 years with neurodevelopmental disorders (60 patients with ASD) and severe sleep disorders that did not respond to standardized sleep advice	Double-blind randomized multicentre placebo-controlled phase III trial	12 weeks	Immediate release melatonin (dose escalation protocol from 0.5 to 12 mg) or matching placebo	45 min before bedtime	Total sleep time after 12 weeks (sleep diaries and actigraphy); sleep onset latency; child behavior (Aberrant Behavior Checklist); family functioning; adverse events	Melatonin increased total sleep time by 22.4 min (diaries) and 13.3 (actigraphy); reduced sleep onset latency by 37.5 min (diaries) and 45.3 (actigraphy). Children in the melatonin group woke up earlier than the children in the placebo group. Melatonin was most effective in children with longest sleep latency. Adverse events were similar between the 2 groups	Child behavior and family functioning outcomes showed some (but not significant) improvement and favored use of melatonin	The results are not specified by category of developmental disorder

communication improvement without reporting separate verbal or non-verbal scores. In other instances, investigators did not use validated instruments; thus, Garstang and Wallis (102) reported improvements in rigidity based solely on parents' and teachers' comments, while Wasdell et al. (103) reported reductions in anxiety based on caregivers' comments. All of the published clinical trials of melatonin in autism/ASD have used sleep measures as their main outcome variable and none have studied the dose–response relationship for melatonin. To reiterate, there is a real need for further studies on the therapeutic effects of melatonin that are conducted on large, relatively homogeneous samples and that employ validated behavioral assessments.

SYNCHRONIZATION OF MOTOR, EMOTIONAL, AND RELATIONAL RHYTHMS: THERAPEUTIC BENEFITS OF THE EARLY START DENVER MODEL IN AUTISM SPECTRUM DISORDER

Although there is a growing interest in the role of motor, emotional, and interpersonal rhythms in autism, there have been only a few attempts to focus on behavioral synchronization of social communication during therapeutic and educational intervention in autism. The ESDM, developed by Sally Rogers and Geraldine Dawson (119, 120), is a comprehensive behavioral and developmental early intervention approach designed for delivery to 12- to 60-month-old children. The model uses the knowledge of how typical infants, toddlers, and pre-schoolers develop, in addition to knowledge of the ways in which autism affects early development, in order to facilitate an appropriate developmental trajectory in young children with autism beginning at the earliest ages. The goals of the ESDM are to reduce the severity of disabling autism symptoms in very young children, and to accelerate children's developmental rates in a multitude of domains, including cognitive, social–emotional, and language domains. In order to do so, there is a strong focus on interpersonal engagement marked by synchrony and reciprocity during teaching, as research demonstrates that these are closely tied to successful developmental outcomes (121–124). Within the ESDM, the therapist and child work together to develop and sustain coordinated, synchronous activity routines into which teaching opportunities are embedded. The ESDM reflects an understanding of the importance of synchrony of motor, emotional, and relational rhythms for the early development of young children with autism.

Thus, the ESDM allows one to initiate interaction with the child by synchronizing the therapist's motor, emotional, and relational rhythms with the child's rhythms (providing invariants/continuity). For example, if the child uses an object with a rhythmic motor pattern, the therapist will reproduce this behavior with exactly the same rhythmic pattern. The evolution of the child is then facilitated by introducing new and different rhythms (providing variants/discontinuity). In addition, the adjustment of the therapist's behaviors, movements, level of arousal, and posture (for example, standing up, lying down, or sitting) to those of the child contributes to foundational invariants necessary during the initial phase. Furthermore, the ESDM requires the program to be implemented in different environments (such as home, school, and caregiver settings), which also contributes to the introduction of variants.

Table 3 | Review, meta-analysis, and discussion of therapeutic uses of melatonin in autism.

REVIEW/META-ANALYSIS		
Jan and O'Donnel (108)	*J Pineal Res*	Review based on100 individuals with chronic sleep disorders, aged from 3 months to 21 years. Half of these 100 patients presented visual impairment or blindness. Melatonin dose ranged from 2.5 to 10 mg. Higher doses were needed in patients with impaired sleep maintenance. Partial or total improvement in sleep parameters was found in 82% of the cases. No side effects
Jan et al. (109)	*Dev Med Child Neurol*	Systematic review of studies on melatonin in children. Twenty-four studies found, most of them were case reports or uncontrolled studies with small samples. Mean age: 10 years old. Associated diagnosis: blindness and neurodevelopmental disabilities, 1 single case of an adolescent with AS (76). Doses ranged from 0.5 to 20 mg. Improvement in sleep in all the studies
Phillips and Appleton (110)	*Dev Med Child Neurol*	Only three studies, reporting a total of 35 children, fulfilled the criteria for inclusion (randomized controlled clinical trials). Two of them reported a significant decrease in time to sleep onset
Braam et al. (111)	*Dev Med Child Neurol*	Meta-analysis of placebo-controlled randomized trials of melatonin in individuals with intellectual disabilities and sleep problems. Nine studies were included. Various doses and formulations of melatonin were given. Melatonin decreased sleep latency by a mean of 34 min ($p < 0.001$), significantly decreased mean number of wakes per night ($p = 0.024$), and increased total sleep time by 50 min ($p < 0.001$). Specified reports on adverse effects were given in four studies. Adverse effects were minor and their incidence in both melatonin and placebo phases were the same. Patient groups in studies included in this meta-analysis were very heterogeneous
Guénolé et al. (112)	*Sleep Med Rev*	Systematic review of efficacy and safety of exogenous melatonin for treating disordered sleep in individuals with autism spectrum disorders: 4 case reports, 3 retrospective studies, 2 open-label clinical trials, 3 placebo-controlled trials. All studies supported the existence of a beneficial effect of melatonin on sleep in individuals with ASD with minor side effects. Limitations are: small sample, clinical heterogeneity of ASD and sleep disorders, varying methods used to measure sleep, confounding factors such as behavioral interventions and cross over design (no analysis of intention to treat). Melatonin doses ranged from 0.75 to 10 mg/day. The authors propose that future research on the efficacy of melatonin in children with ASD should include daytime functioning as a principal outcome measure. Only 6 patients of 205 presented side effects: daytime sleepiness, fogginess, dizziness, nocturnal enuresis, tiredness, headache, and diarrhea
Doyen et al. (113)	*Eur Child Adolesc Psychiatry*	Systematic review on pharmacokinetics data on melatonin and its role in sleep disorders and autism spectrum disorders. Authors reviewed 17 studies on effectiveness and side effects of melatonin in patients with AD, AS, PDDNOS, and Rett syndrome. Effectiveness on sleep disorders was found in all the studies, side effects were reported in 5 studies. Melatonin doses ranged from 0.5 to 10 mg. Melatonin seems to have anxiolytic properties. Most frequent reported side effects: infections, flu, epilepsy, intestinal disorders, and agitation
Rossignol and Frye (114)	*Dev Med Child Neurol*	Aim of the study: investigate melatonin-related findings in ASD including AD, AS, Rett syndrome, and PDDNOS. Eighteen studies on melatonin treatment on ASD patients were identified (5 RCT), 12 of them reported improvement in sleep with melatonin in 67% to 100% of the patients. Six studies reported improvement in daytime behavior (less behavioral rigidity, ease of management for parents and teachers, better social interaction, fewer temper tantrums, less irritability, more playfulness, better academic performance, and increased alertness). Melatonin doses ranged from 0.75 to 15 mg, age of patients ranged from 2 to 18 years, treatment duration ranged from 2 weeks to 4 years. Twelve studies explored side effects (headache, tiredness, dizziness, and diarrhea) in which 7 studies reported no side effects. Nine studies found low levels or abnormal circadian rhythm of melatonin in ASD. A correlation between these abnormal levels and autistic behaviors was found in 4 studies. Night time urinary excretion of melatonin metabolite (6-SM) was reported to be inversely correlated with the severity of impairments in verbal communication, play, and daytime sleepiness in patients with ASD. Five studies found genetic abnormalities of melatonin receptor and enzymes involved in melatonin synthesis
Reading (115)	*Child Care Health Dev*	Correlation between plasmatic levels of melatonin and autistic behaviors was found. Melatonin groups showed improvements in total sleep duration and sleep onset latency versus placebo groups but not on night awakenings

(Continued)

Table 3 | Continued

LETTER TO THE EDITOR		
Guénolé and Baleyte (116)	*Dev Med Child Neurol*	Response to the Rossignol and Frye review (73); Authors proposed that studies should separately explore sleep disorders in patients with ASD and sleep disorders in patients with Rett syndrome
Guénolé and Baleyte (117)	*Pediatr Neurol*	Response to the De Leersnyder et al. study (86) of open-label trial. The definition of «chronic sleep disorder»did not refer to international classifications. Half of the children manifested Smith-Magenis syndrome that involves specific abnormalities of melatonin secretion. Thus, results cannot apply to a population with ASD. The effects of melatonin should be studied separately in each neurodevelopmental disorder and with specific sleep diagnoses
DISCUSSION/COMMENTARY		
Jan and Freeman (92)	*Dev Med Child Neurol*	Discussion on melatonin use in children with ADHD, ASD, neurodevelopmental disabilities, epilepsy, and blindness. Exogenous melatonin seems to regulate endogenous melatonin secretion. It seems to be more effective in sleep–wake cycle disorders with sleep onset delay disorders. Night and morning awakenings seem to be more difficult to treat, such as sleep problems associated with cerebral lesions. The more the child shows mental or motor comorbidities, the more the melatonin dose is high
Lord (118)	*J Autism Dev Disord*	General brief discussion of melatonin and its potential for treating sleep problems in autism

The ESDM is both a curriculum and a set of teaching practices. A specific developmental curriculum, administered every 3 months in typical practice, defines the skills to be taught at any given time. In addition, a manual of teaching practices outlines the ways in which these skills are to be taught. Embedded within both the curriculum and the set of teaching practices is a focus on rhythms and synchrony. In other words, when therapists are working within the model, a focus on rhythms permeates both what they teach and how they teach it.

The ESDM curriculum outlines skills to be taught in multiple areas, including cognitive, social–emotional, and language domains. The curriculum focuses heavily upon the teaching of skills that promote engagement with other people in a synchronous, rhythmic way. Many young children with autism enter an ESDM program with weaknesses in these skills, such as imitation, joint attention, orienting to name, and eye contact, and these have broad influences upon the manner in which these children can engage with others. It is well recognized that learning occurs within a social context and that social skills, such as imitation and shared attention, provide a foundation for many aspects of learning, including language, cognitive, and social–emotional abilities. Thus, the ESDM focuses heavily on the development of social engagement and interactional synchrony early on in order to further support learning of a wide range of skills.

One area of focus within the ESDM, which is closely tied to the concept of rhythmicity, is imitation. Young children with autism often exhibit deficits in their imitation skills, including those of imitating others' facial expressions and movements, gestures, body actions, and actions on objects. From the beginning of a young child's ESDM program, teaching of imitation is stressed, from basic imitation of actions on objects to more nuanced imitation of sound effects produced in play. Spontaneous and appropriate imitation of others is typically rhythmic and marked by mutual attention, continuous adaptation, and turn taking. The beginning stages of facilitating imitation and social engagement often start with imitating the child, thereby entering into a rhythmic interaction with the child. By imitating the child's movements and establishing a synchronous interaction, eye contact and mutual engagement in the interaction are promoted (125).

Rhythmicity is also involved in the way teaching occurs within the ESDM upon multiple levels, from the basic structure of each interactive routine to the ways in which adults engage with children during interaction. Social communication is about sharing moments of synchrony with others, and the ESDM supports the emergence of interactional synchrony. The therapist facilitates the occurrence of synchronous moments and strings them together into routines into which the therapist can naturally embed teaching opportunities. The primary vehicle through which all teaching is accomplished in this model is the joint activity routine, which is permeated by moments of rhythmic, synchronous interaction, and enriched with positive affect. Adult and child are attuned to one another, both taking the lead, both following the other's lead, taking turns, and creating a positive and motivating activity in conjunction with one another.

The joint activity routine, although flexible and naturalistic, adheres to a four-part structure: (1) opening, (2) theme, (3) elaboration, and (4) closing. This structure provides a set of invariants, against which multiple variants can occur at differing levels. In order to begin engagement within a joint activity routine, the adult works hard to *find the child's smile*. This can often be accomplished by getting into the child's own rhythm (imitating a child's actions is a very powerful tool to facilitate motivated response and interaction) or by finding a rhythm that the child likes. For example, perhaps a child finds a toy drum and begins banging on it with his hand. In the ESDM, the therapist would join the child and would likely take her/his own drum and imitate the child's actions, joining his rhythm. This would be considered the opening phase of the joint activity routine.

Next, the child and adult would develop a theme. This can be considered a continuation of the rhythm introduced during the

opening phase – in our example, banging a drum slowly with hands. The adult and child would take turns doing so, each leading and each following. The skillful adult would embed teaching opportunities into these turns – perhaps a focus on eye contact during dyadic engagement, or on imitation of actions on objects, or on giving and taking objects with eye contact. Both partners then play within this rhythm for a while, until one introduces a variant, or elaboration. In the ESDM, elaboration occurs by introducing a change into the joint activity routine, a change in the rhythm which often allows different teaching targets to be practiced. In this example, the child and adult might start to play peek-a-boo behind the drums. A new rhythm would need to be established, and both partners would work together in order to do so. An elaboration of this sort is a rhythmic fluctuation occurring against a background of invariants (e.g., the same interactive partner, the same material, the same setting), which allows the child to learn to adapt to change and teaches the child how to engage with people and materials differently. Finally, this rhythm would likely begin to fade, the teaching value of the activity would start to diminish, and/or the child would begin to lose motivation. The fourth stage of the joint activity routine – the closing – is the last part of the joint activity routine. Adult and child might help one another clean up the materials, and then both partners would begin an entirely new joint activity routine. This could often involve a change in location, activity level, or teaching domain, and an entirely new set of coordinated interactions would be developed.

Within the joint activity routine, synchrony pervades within several different levels and in several different areas. Adult and child are attuned to one another and responsive to one another in terms of sensory input and output, motor actions, and emotions. Coordinated dyadic engagement, well-balanced in terms of leader and follower, pervades a high-quality joint activity routine. It is quite natural to view this ever-present synchrony in terms of a focus on shared nuanced rhythms.

There is also a focus on rhythmicity within some of the broader teaching practices of the ESDM; for example, in the way in which a teaching session is constructed. As mentioned above, teaching is delivered within joint activity routines. For a therapist-delivered session, these routines are strung together within a session to occupy a full 1- or 2-h time period. Within that time period, therapist and child move around to occupy several spaces. In a well-coordinated way, they engage together in a joint activity routine at the table, and then may move onto the floor for the next. They may sit together, then they may stand or walk or run. The child's level of arousal is carefully monitored, and the session is marked by alternations of quiet thoughtfulness and active play, all coordinated artfully by a skilled adult. All of these changes occur against a stable background, as the session is marked by stability and continuity, with the introduction of variants when appropriate. The ESDM, a comprehensive, behavioral, and developmental early intervention designed for infants and toddlers with autism, is rooted within a sense of and appreciation for rhythmicity and synchrony at multiple levels, ranging from the specific skills that are taught to how those skills are taught, woven together and supported by a broader structure focused on maintaining a well-coordinated, synchronous set of dyadic interactions.

CONCLUSION: TOWARD AN INTEGRATIVE APPROACH COMBINING THE USE OF MELATONIN WITH THE ESDM

Taken all together, ASD could be seen as a disorder of rhythmicity with, more specifically, impairments in the synchrony of rhythms. Alternatively, such asynchrony might play an important role in a possibly large subgroup of individuals that forms part of the heterogeneous ASD category. In this article, we proposed an integrative approach to study desynchronization in biological and psychological rhythms in ASD and develop an etiopathogenic hypothesis as well as therapeutic perspectives for ASD based on this integrative approach. Indeed, this integrated physiological and psychological approach opens important therapeutic perspectives for ASD based on regulation of physiological rhythms (in particular, through the use of chronobiotics such as melatonin, and also through light exposure, use of regularly scheduled bedtime, wake up, meals, or activities) combined with synchronization of motor, emotional, and relational rhythms through developmental behavioral intervention such as the ESDM. Further studies are required to better ascertain the underlying mechanisms of physiological alterations induced by temporal desynchronization and to better understand the role of biological rhythms and rhythmicity in the development of social communication, repetitive behaviors, and interests or adaptation to changes, and therefore in the development of autism involving impairments in these domains.

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Reducing maladaptive behaviors in preschool-aged children with autism spectrum disorder using the Early Start Denver Model

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The presence of maladaptive behaviors in young people with autism spectrum disorder (ASD) can significantly limit engagement in treatment programs, as well as compromise future educational and vocational opportunities. This study aimed to explore whether the Early Start Denver Model (ESDM) treatment approach reduced maladaptive behaviors in preschool-aged children with ASD in a community-based long day care setting. The level of maladaptive behavior of 38 children with ASD was rated using an observation-based measure on three occasions during the intervention: on entry, 12 weeks post-entry, and on exit (post-intervention) over an average treatment duration of 11.8 months. Significant reductions were found in children's maladaptive behaviors over the course of the intervention, with 68% of children showing a treatment response by 12 weeks and 79% on exit. This change was accompanied by improvement in children's overall developmental level as assessed by the Mullen scales of early learning, but not by significant changes on the Vineland Adaptive Behavior Scales-II or Social Communication Questionnaire. Replication with a larger sample, control conditions, and additional measures of maladaptive behavior is necessary in order to determine the specific factors underlying these improvements; however, the findings of the present study suggest that the ESDM program may be effective in improving not only core developmental domains, but also decreasing maladaptive behaviors in preschool-aged children with ASD.

Keywords: autism spectrum disorder, Early Start Denver Model, maladaptive behavior, early intervention, efficacy

INTRODUCTION

Autism spectrum disorder (ASD) is a life-long neurodevelopmental disorder characterized by impairments in social interaction and communication, and restricted, repetitive patterns of behavior, activities, or interests (1). The prevalence of ASD appears to be rising worldwide (2), with ASD estimated to affect around 1 in every 88 persons (3).

Autism spectrum disorder is recognized as a major public health concern because of its early onset, life-long persistence, and high levels of associated impairment (4). This impairment is attributable not only to the core symptoms of ASD, but also to the range of co-existing conditions that individuals with ASD often experience, including emotional and behavioral problems, sleep, feeding and eating problems, sensory sensitivities, learning and intellectual disabilities, as well as co-morbid health and mental health diagnoses (5). These co-existing conditions can be of equal or greater concern for parents and teachers of children with ASD than the core features of ASD, and have a significant impact on behavior management, learning acquisition, and the development of social relationships (6).

Problem behaviors (or maladaptive behaviors as they are referred to in this paper), characterized by disruptive, destructive, aggressive, or significantly repetitive behaviors, are prevalent in young children with ASD (7). For example, Dominick et al. (7) found that 32.7% of children with ASD displayed aggressive behaviors including hitting, kicking, biting, and pinching others. More than three-quarters of these children showed aggressive behaviors both at home and outside the home, and aggression was directed toward more than one person in 92% of cases. Self-injurious behavior, including head banging, hitting oneself, and biting oneself, was present in almost one-third of children with ASD (7). Furthermore, 70.9% of children with ASD had experienced a period of severe temper tantrums and, for 60% of these children, tantrums occurred on a daily basis and were a constant (rather than episodic) problem during the period in which they were present (7).

Several authors have noted a strong negative relationship between the ability to communicate and the prevalence of maladaptive behavior in young children with ASD (8). Self-injurious behaviors among children with ASD have also been linked to their receptive and/or expressive communication deficits (9). It follows that when treatment programs focus on developing the young child's communication skills to the extent that they can serve as effective replacement behaviors, a reduction in the maladaptive behavior may result (10).

Maladaptive behaviors are particularly problematic in group settings, such as early intervention services, childcare services, and preschools, as they can be disruptive to the learning program and pose significant challenges to the children with ASD themselves, their peers, and staff. For these reasons, maladaptive behaviors are amongst the most commonly identified barriers to the inclusion of children with ASD in group settings (11).

Further, once maladaptive behaviors become an established part of a child's behavioral repertoire, they are unlikely to decrease and, according to Berg et al. (12), will typically remain or worsen without intervention. If left untreated, these behaviors can significantly reduce a child's social and educational opportunities by limiting their access to available treatments, learning activities, interactions with others, community experiences and, in particular, their ability to transition to, and participate in, school programs (13). In addition to having a negative impact on children with ASD themselves, a number of studies have shown that parents' level of psychological distress is associated with the severity of their child's maladaptive behaviors as well as their ASD symptoms (14–20).

Therefore, early interventions for young children with ASD should incorporate the management of maladaptive behaviors (21). Given the relationship between maladaptive behaviors and deficits in communication and social skills, it is important that intervention approaches target these core deficits. Myers and Johnson (22) argue that the primary goals of intervention for children with ASD should be to maximize the child's functional independence and quality of life by reducing the core symptoms of ASD; facilitate development and learning; promote socialization; reduce maladaptive behaviors; educate and support families. They suggest that, in addition to targeting communication and social skills, contemporary comprehensive intervention approaches for ASD should target a reduction in disruptive or maladaptive behavior by using empirically supported strategies, including functional behavior assessment (FBA). FBA is "the process of determining the intent an inappropriate behavior serves for obtaining a desired outcome and replacing that behavior with a more appropriate one that accomplishes the same goal" [Ref. (23), 136 p.].

The general importance of early intervention for ASD is widely recognized, and is supported by studies showing better outcomes with earlier treatment (24, 25). Early intervention for ASD, especially that commencing before the age of 3 years, results in significantly improved outcomes relative to intervention commencing later in life (26–28). Early intervention in the first years of life offers the best potential for children as brain plasticity is greatest during this period, enabling the establishment and reorganization of neuronal networks in response to environmental stimulation (26).

A review of research conducted by Prior et al. (29) to identify the most effective models of early intervention for children with ASD classifies approaches into three main categories.

Each target maladaptive behaviors differently:

- Biologically based interventions, including medication, have been used to treat the co-morbid symptoms of ASD such as anxiety and hyperactive behavior with varying degrees of effectiveness.
- Psychodynamic interventions target the emotional component of behavior only. However, because ASD is considered a neurodevelopmental, rather than emotional, disorder, there is little empirical evidence demonstrating their effectiveness.
- Educational interventions including behavioral interventions such as applied behavior analysis (ABA); the Lovaas program; Pivotal Response Training; developmental and relationship-based interventions including ESDM; communication-focused interventions and sensory–motor interventions tend to have a positive treatment response. Each of these approaches uses different mechanisms to target maladaptive behaviors, and some advocate for the use of FBA as part of this process. These interventions usually approach behavior modification directly, focusing on the behavior itself.

Programs such as the Early Start Denver Model (ESDM) focus on building communication skills, especially by following the child's lead and increasing the reinforcement value of social interaction, thereby teaching children adaptive ways of getting their needs met (30). Given that the ESDM is designed to enhance the social attention and communicative abilities of young children with ASD, with particular focus on the critical skills of social attention, affect sharing, imitation, and joint attention, it is conceivable that a significant reduction in maladaptive behavior may result.

Several meta-analyses conducted in recent years have tended to conclude that early intensive behavioral intervention (EIBI), generally defined as intervention that is delivered at an intensity of 15–20 h/week, incorporating the principles of ABA, is the treatment of choice for young children with ASD [cf. (8, 31)]. The literature indicates that superior outcomes are associated with entry into EIBI at the earliest possible age (32, 33).

The only comprehensive EIBI program available for children aged <30 months that has been empirically evaluated is the ESDM (27). The ESDM is specifically designed for children aged 12–60 months and is a manualized, comprehensive intervention that integrates ABA into a developmental and relationship-based approach (30). The ESDM is an intensive and comprehensive early intervention model that aims to reduce the severity of ASD symptoms and accelerate children's development in all areas, with particular emphasis in the cognitive, social–emotional, and language domains.

The ESDM draws from teaching practices developed in the original Denver Model such as relationship-based aspects of the therapist's work with the child, using play as a foundation for learning, and using communication intervention principles from the field of communication science (30). Positive behavior approaches focus on replacement of unwanted behaviors with more conventional behaviors and FBA is used when behaviors are more challenging.

The first and only randomized controlled trial of the ESDM demonstrated that, compared with children receiving community intervention, children receiving the ESDM showed significant gains in visual processing and improvements in language abilities, with subsequent gains in IQ and adaptive behaviors (27). In that study, children received 20 h/week of one-to-one ESDM intervention in a University clinic setting. There was also a separate parent training module. Two further studies (34, 35) have investigated the efficacy of delivery of the ESDM in group settings. Both studies reported significant developmental gains following

the intervention and Eapen et al. (34) also found a significant decrease in autism-specific symptoms.

While Dawson et al. (27) and Eapen et al. (34) investigated the impact of the ESDM on children's adaptive behavior, no studies of the ESDM to date have focused on the effect of the ESDM on children's maladaptive behaviors. Given the adverse effect that maladaptive behaviors have on children with ASD, as well as their parents, it is important to investigate the impact of interventions on these behaviors. This was the primary aim of the present study.

MATERIALS AND METHODS

The study was approved by the local institutional and University ethics committees and all families recruited to the study provided informed consent to participate.

STUDY DESIGN AND PARTICIPANTS

A pre–post study of children treated with ESDM was conducted. Note that clinical outcomes, but not ESDM clinician behavioral ratings data, for a portion of the cohort have been described previously in Eapen et al. (34). Participants were 38 children attending an Autism Specific Early Learning and Care Centre (ASELCC) in metropolitan Sydney, Australia. The center is one of the six ASELCCs established by the Australian Government within the setting of a long day child care center for children aged 2–6 years. All children had a DSM-IV-TR diagnosis of Autistic Disorder, made by a community-based physician, with the exception of one child who had a diagnosis of pervasive developmental disorder not otherwise specified. These children would all have met criteria for a DSM-5 diagnosis of ASD. Exclusion criteria included neurological (e.g., uncontrolled epilepsy) disorders, and significant vision, hearing, motor, or physical impairment.

The average age of children at the time of study commencement was 52.2 months (SD 5.4, range: 38.8–63.7 months), and 35 (92%) were male. English was the primary language spoken at home in 82% of families, although 60% of families reported a cultural background other than Australian.

None of the participants were receiving an EIBI outside of the ESDM intervention offered as part of this program. No families withdrew from the study during the course of the intervention; however, there were instances of missing data due to families not completing measures within the necessary timeframes.

INTERVENTION

The study employed the ESDM curriculum and teaching principles within a group setting. Other than accommodations to allow translation to the group context, which are described in Eapen et al. (34), no modifications were made to the ESDM curriculum.

Rogers and Dawson (30) outline a specific teaching approach in the ESDM that was followed in this study. ESDM teaching principles are embedded in play and in natural daily routines within elaborated joint activity routines that address multiple objectives across multiple developmental domains. The main focus is on teaching imitation; developing awareness of social interactions and reciprocity; teaching the power of communication; teaching more flexible, conventional, and creative play skills; making the social world as understandable as the world of objects. Rogers and Dawson (30) contend that just as typically developing children spend their waking hours engaged in the social milieu and learning from it, children with ASD need to be drawn into a carefully prepared and planned social milieu that they can understand, predict, and participate in.

Whilst a primary focus on maladaptive behaviors is not central to the ESDM curriculum, the general approach in this model for children whose level of maladaptive behavior has not improved after 3 weeks of intervention follows the principles of positive behavior supports (36, 37). This is a method of applying the principles of ABA that focus on the use of reinforcement strategies to teach children adaptive and conventional behaviors for meeting their needs and expressing their feelings, as well as promoting independent functioning (30). There were only two children in the current sample whose behavior had not improved after 3 weeks of intervention. For these children, FBA was conducted by the Behavior Analyst on the Intervention team. This process determined the functions of the child's behavior and the consequence that was reinforcing the behavior. This is based on the premise that the behavior is in the child's current repertoire because it leads to a rewarding consequence; therefore the effects of a range of consequences must be tested to determine how the behavior is being reinforced or maintained. The FBA also enabled the Behavior Analyst to identify replacement behaviors that would serve a similar function for the child, but were more conventional behaviors for the child to learn. The ESDM then identifies the skills that can be converted into objectives and targeted in the child's individualized program, so that he/she can quickly learn, master, and generalize the new adaptive behaviors to become part of their behavioral repertoire.

During their attendance at the center, participants received an hour of intensive individualized ESDM therapy each week, in addition to an hour of intensive small group ESDM therapy daily, and ESDM-driven learning experiences throughout the day. Each child also received between 15 and 20 h/week of group ESDM intervention. The one-to-one sessions were conducted by the child's key worker, who carried a caseload of five-to-six children across the period of the intervention. Each child had an individualized treatment plan that incorporated a range of objectives dependent on the child's level of functioning. These objectives were developed from the child's initial assessment using the ESDM curriculum checklist, which includes a list of skills spanning receptive communication; expressive communication; social skills; joint attention behaviors; fine motor; gross motor; imitation; cognition; play skills; behavior; and personal independence (eating, dressing, grooming, and chores).

All interventions were delivered by therapists with tertiary-level qualifications who were trained to certification in the ESDM by an accredited trainer. In order to be certified in direct delivery of this model, therapists were required to achieve: (1) a fidelity rate of 80% or more with the ESDM trainer on each of the 13 ESDM teaching principles across multiple children and sessions, and (2) to achieve the same level of concordance on the individualized written treatment plans they had developed and data they collated on each child. That is, 80% or more concordance was required in both the clinical delivery and data recording aspects of the ESDM, including on the ESDM behavior checklist, which formed a key measure in this study. There were six key workers, each trained in

this way, involved in the study. Therapists also continued to receive clinical supervision in their delivery of the ESDM by an Accredited Trainer.

MEASURES

Pre- and post-measures included the (1) ESDM behavior rating as well as the (2) Vineland Adaptive Behavior Scales, second edition (Parent Form) [VABS-II; (38)]; (3) Social Communication Questionnaire [SCQ; (39)]; and (4) Mullen Scales of Early Learning [MSEL; (40)]. The ESDM behavior rating was also completed 12 weeks after entry to the program.

The rating of children's maladaptive behavior was completed during the child's individual 1 h ESDM session using the ESDM Behavior Coding system. The coding system allows therapists to quantify the child's behavior for each 15-min period, as well as for the hourly session as a whole. The rating for the session as a whole was used in this study. This rating measured the level of maladaptive behavior that was typically present over the hour rather than the best or worst behavior observed during the session.

The Behavior Coding system designed by Rogers and Dawson (30) for measurement of maladaptive behaviors is described below:

1. *Severe problem behaviors* including aggression, self-injurious behavior, frequent and intense tantrums;
2. *Mild problem behaviors* including non-compliance, some tantrums, but able to participate to some extent;
3. *Some problem behaviors* including fussy, whining, some non-compliance, but able to participate in most of the activity;
4. *No problem behavior* but difficulty staying on task;
5. *Compliant* on task, working at ability level;
6. *Above average* performance for that child; pleasant, excited about the activity.

Rating of behavior codes was completed by each child's key worker who conducted their individual ESDM therapy and was responsible for collecting their data within the group program also. These data were then discussed and peer reviewed in daily Key Worker meetings. Discrepancies were discussed with the ESDM trainer. Senior ESDM trainers working in the UC Davis MIND Institute were available to discuss significant discrepancies; however this was not required for any behavioral ratings. All child data, including the behavior codes, were reviewed on a quarterly basis by the ESDM trainer, including through the use of videos of therapy sessions or live viewing (from the observation room) of therapy sessions to ensure ongoing fidelity.

A pre-intervention behavior score was coded on entry to the program (in the therapy session following the initial assessment), a second behavior score was coded after the first 12 weeks of intervention, and a post-intervention behavior score was coded before the child exited the program.

Parents of participating children completed two measures. The VABS-II (38) assesses parents' perceptions of their child's everyday adaptive functioning in the domains of Communication (including expressive and receptive language), Daily Living Skills, Socialization, and Motor Skills. For each domain, including an overall Adaptive Behavior Composite, a norm-referenced standardized score with a mean of 100 and SD of 15 is calculated. *V*-scale scores with a mean of 15 and a SD of 3 and age-equivalent scores are calculated for each sub-domain, including Internalizing Behavior, Externalizing Behavior, and the Maladaptive Behavior Index. The VABS-II has well-established strong psychometric properties (38). The SCQ (39) is a 40-item measure of autism-specific symptoms where scores of 15 or more indicate probable ASD. The SCQ has robust psychometric properties (41–43). These measures were administered at two time points (on entry to and exit from the program). Parents also completed a demographic questionnaire at the start of the study.

In addition, children were assessed at entry to and exit from the program using the MSEL (40), a widely used, standardized measure of early development for children aged from birth to 68 months, yielding standardized *T* Scores and age-equivalent scores on the following subscales: Visual Reception, Fine Motor, Receptive Language, Expressive Language, and Gross Motor. The Gross Motor subscale was not administered in this study. Given the majority of children in the current sample did not receive MSEL subscale raw scores that were high enough for calculation of a meaningful *T* score (i.e., they were performing at a level <0.1 percentile), standardized developmental quotients (DQs) were calculated for each subscale of the MSEL by dividing each child's age-equivalent score by their chronological age at the time of testing and multiplying by 100, as is common practice. In this regard, a child who was aged 48 months, but who had an age-equivalent score of 24 months, would receive a DQ of $(24/48) \times 100 = 50$. An overall DQ was also calculated for each child by taking the average of the child's DQs for the four completed subscales in order to provide an estimate of overall intellectual ability. Note that the sum of the *T* scores for these four subscales (i.e., Visual Reception, Fine Motor, Receptive Language, and Expressive Language) is used to calculate the Early Learning Composite Score of the MSEL. It should also be noted that the DQs calculated in this study are not equivalent to *T* scores or the Early Learning Composite Score of the MSEL, but represent an attempt by the study team to standardize scores for the purpose of making comparisons over time.

STATISTICAL ANALYSIS

Paired samples *t*-tests were conducted to compare children's scores pre- and post-intervention on the aggregate measures of clinician ESDM child behavior ratings; Vineland Adaptive Behavior Composite score; Vineland Maladaptive Behavior Index Score; SCQ total score; and overall MSEL DQ. Cohen's *d* effect sizes were also reported. It is widely accepted that Cohen's *d* values of 0.2–0.49 denote small-sized effects; 0.5–0.79 denote medium-sized effects; and >0.8 denote large effect sizes. To explore change, pre- and post-intervention in the subscales of measures used, a series of repeated measures MANOVA analyses were conducted using the Pillai's Trace criterion. The aggregate scores noted above were not included in these MANOVA analyses as these scores were not independent of the subscale scores. Partial eta values were reported as a measure of effect size for MANOVA analyses. Correlations were also computed to investigate relationships between children's behavior and baseline demographic and clinical variables. Analyses were conducted using SPSS statistical software. Alpha was set at 0.05 for the majority comparisons, following recommendations by

Saville (44) who argues for this per-comparison level rather than a family wise approach when conducting research in novel areas. An exception to this was in the instance where multivariate effects detected in the MANOVA analyses were further explored using paired samples t-tests. In those cases, a Bonferroni correction was applied.

RESULTS

The average time between pre- and post-intervention assessment was 11.8 months (SD 5.8). As shown in **Table 1**, a significant reduction in clinician-rated ESDM behavior rating was found, $t(37) = -16.6$, $p < 0.001$. The size of this effect was Cohen's $d = -3.7$, which is large. There was also a significant increase to children's overall MSEL DQ, $t(17) = -5.0$, $p < 0.001$, $d = -0.41$, which approaches a medium-sized effect. There was, however, no significant change in children's VABS-II Adaptive Behavior Composite, VABS-II Maladaptive Behavior Index, or SCQ total scores.

To explore changes in core subscales of the VABS-II, a repeated measures MANOVA was performed with VABS-II standard domain scores as the dependent variables (Communication; Socialization; Daily Living Skills; and Motor Skills). The within-subjects independent variable was time, with two levels (pre-intervention and post-intervention). There was no significant multivariate effect of time $F(1, 11) = 0.18$, $p < 0.05$ or VABS-II subscale scores $F(3, 9) = 2.8$ $p > 0.05$, nor a domain scores by time interaction. With respect to the VABS-II Maladaptive Behavior subscales, a repeated measures MANOVA was performed with Internalizing Behavior and Externalizing Behavior as the dependent variables and time as the within-subjects independent variable. The multivariate effect of time $F(1, 13) = 0.67$, $p < 0.001$ and the time by VABS-II Maladaptive Behavior subscale score interaction $F(1, 13) = 0.18$, $p > 0.05$ were not statistically significant. However, the multivariate effect for subscale scores was significant $F(1, 13) = 23.1$, $p < 0.001$, $\eta_p{}^2 = 0.64$. When explored further using paired sample t-tests with an adjusted alpha rate of

Table 1 | Pre- to post-intervention scores in a cohort of preschoolers treated with group ESDM.

	Pre-intervention		Post-intervention		t[a]	df	p	Cohen's d[h]
	Mean	SD	Mean	SD				
ESDM behavior rating	1.8	1.0	5.1	0.8	−16.6	37	<0.001**	−3.67
VINELAND ADAPTIVE BEHAVIOR SCALES-II STANDARD DOMAIN SCORES								
Communication[b]	62.4	15.2	64.8	19.7				−0.14
Socialization[b]	66.8	14.2	63.7	13.6				0.22
Daily Living Skills[b]	62.1	14.7	62.2	16.6				−0.01
Motor Skills[b]	69.4	20.7	65.3	23.2				0.19
Adaptive Behavior Composite[b]	62.2	14.8	62.5	14.7	−0.2	12	0.84	−0.02
VINELAND ADAPTIVE BEHAVIOR SCALES-II MALADAPTIVE BEHAVIOR								
Internalizing Behavior[c]	19.4	1.8	18.9	4.0	0.5	13	0.60	0.17
Externalizing Behavior[c]	16.0	2.2	15.1	3.0	1.0	13	0.34	0.35
Maladaptive Behavior Index[c]	18.8	1.4	18.8	1.8	0.0	13	1.0	0.00
SCQ total score[d]	18.3	6.3	17.0	7.3	1.0	13	0.34	0.19
MULLEN SCALES OF EARLY LEARNING								
Visual Reception DQ[e]	37.2	19.9	48.3	27.3	−2.7	19	0.013[g,*]	−0.47
Fine Motor DQ[e]	46.3	24.3	50.6	21.2	−1.4	21	0.17[g]	−0.19
Receptive Language DQ[e]	30.4	22.3	39.7	24.4	−3.5	17	0.003[g,**]	−0.40
Expressive Language DQ[e]	33.4	18.4	40.7	20.0	−4.5	20	<0.001[g,**]	−0.38
Overall MSEL DQ[f]	37.9	19.8	46.5	22.2	−5.0	17	<0.001[g,**]	−0.41

*$p < 0.05$, **$p < 0.01$, SCQ, Social Communication Questionnaire.

For the SCQ total score, lower scores are indicative of fewer ASD symptoms. Similarly, for VABS-II Maladaptive Behavior subscales (Internalizing Behavior, Externalizing Behavior, and Maladaptive Behavior Index), lower scores denote fewer symptoms. For all other measures, higher scores are indicative of better functioning.

[a] *Paired samples t-tests were conducted a priori for aggregate scores of ESDM behavior rating, VABS-II Adaptive Behavior Composite, VABS-II Maladaptive Behavior Index, SCQ total and overall MSEL DQ. In other instances, paired samples t-tests were conducted only following significant results in multivariate repeated measures MANOVA analyses.*

[b] *Standard score (mean: 100, SD: 15).*

[c] *V-scale score (mean: 15, SD: 3).*

[d] *Range = 0–40. Scores of 15 or more denote probable ASD.*

[e] *DQ (developmental quotient) = (age-equivalent score/chronological age) × 100.*

[f] *Overall MSEL DQ = (Visual Reception DQ + Fine Motor DQ + Receptive Language DQ + Expressive Language DQ)/4.*

[g] *Bonferroni adjusted $\alpha = 0.013$.*

[h] *Following the recommendations of Dunlap et al. (45), Cohen's d scores were calculated using the pooled standard deviation score uncorrected for the correlation between pre-post scores.*

0.05/2 = 0.025 neither of the Internalizing or Externalizing scores changed significantly over time, however effect sizes were non-trivial (see **Table 1**). In the case of the MSEL, a repeated measures MANOVA was performed with Visual Reception DQ, Fine Motor DQ, Receptive Language DQ, and Expressive Language DQ as the dependent variables and time as the within-subjects independent variable. The multivariate effects of MSEL subscale scores $F(3, 15) = 6.5$, $p = 0.005$, $\eta_p^2 = 0.57$ and time $F(1, 17) = 24.69$, $p < 0.001$, $\eta_p^2 = 0.59$ were significant; however, the subscale scores by time interaction were not. When explored further using paired sample t-tests with an adjusted alpha rate of 0.05/4 = 0.013, the Visual Reception DQ, Receptive Language DQ, and Expressive Language DQ all showed significant improvement from pre- to post-intervention with effect sizes approaching medium size (see **Table 1**).

To further explore the speed with which improvement in the level of maladaptive behaviors occurred, *post hoc* analyses were conducted using ESDM clinician-rated behavior checklist data obtained at entry, 12 weeks post-entry, and exit from the ESDM program. It emerged that, at entry to the program, only one of the 38 children had a behavior score of 5 or 6 (indicating compliant or above average behavior). This number increased to 26 of 38 children (68%) after 12 weeks of intervention, and to 30 of 38 children (79%) by the end of the intervention.

A related analysis involved examining the number of participants whose scores improved by three points or more on the six point scale (taken to denote a conservative estimate of meaningful change) at the different time points. One participant had an entry score that would preclude improvement by three points; hence subsequent analyses were conducted on the remaining 37 children. After 12 weeks of intervention, 25/37 children (68%) had improved by three or more points (rapid responder sub-group), whereas 32% of children had not responded in this way (non-responder sub-group). By program exit, the non-responder group had dropped to 24% of the sample.

A series of independent samples t-tests were conducted to examine whether the rapid responder sub-group differed from the 12-week non-responder sub-group according to baseline variables. Given the relatively small sample size in these analyses, MANOVAs were not performed and Cohen's d effect sizes were also inspected for cases where the effect size was of medium size or larger (Cohen's $d > 0.50$). Analysis revealed that the SCQ score for the rapid responder sub-group (mean = 16.1) was lower than that of the non-responder sub-group (mean = 21.5) at a level that approached significance $t(27) = 1.86$, $p = 0.07$, Cohen's $d = 0.77$, that is, the rapid responder group tended to have lower baseline ASD symptoms than the non-responder group. Other areas where the difference between rapid responder and non-responder groups was above Cohen's $d = 0.5$ at baseline were VABS-II Communication, Daily Living Skills, and Motor Skills Standard Scores. In all instances, the rapid responder group performed better than the non-responder group at baseline.

Correlations between baseline clinical variables and pre- and post-intervention behavior ratings as well as change in behavior ratings are presented in **Table 2**. As shown in **Table 2**, clinician-rated behavior at entry was not significantly related to any baseline clinical variables, including DQs, autism severity, or adaptive behavior (all ps > 0.05).

Table 2 | Correlations between clinician-rated behavior scores and baseline clinical variables.

	Entry behavior	Exit behavior	Change in behavior
Entry behavior rating	–	–	–
Exit behavior rating	0.14	–	–
Change in behavior rating	−0.72**	0.59**	–
VABS-II Communication	0.22	0.26	−0.04
VABS-II Socialization	0.22	0.14	−0.12
VABS-II Daily Living Skills	0.25	0.42*	−0.02
VABS-II Motor Skills	0.12	0.36	0.10
VABS-II ABC	−0.23	0.31	−0.02
VABS-II Internalizing	−0.17	−0.09	0.08
VABS-II Externalizing	−0.05	0.37*	0.28
VABS-II Maladaptive	−0.13	0.02	0.10
SCQ total	0.08	−0.20	−0.20
SCQ Communication	0.16	−0.18	−0.26
SCQ Restricted Social Interaction	−0.13	−0.24	−0.06
SCQ Repetitive Behavior	0.13	−0.09	−0.17
VRDQ	0.07	0.37	0.18
FMDQ	0.17	0.55**	0.21
RLDQ	0.26	0.48*	0.10
ELDQ	0.32	0.46*	0.02
Overall DQ	0.23	0.53**	0.16

*$p < 0.05$, **$p < 0.01$, *VABS-II, Vineland Adaptive Behavior Scales-II; ABC, Adaptive Behavior Composite; SCQ, Social Communication Questionnaire; VRDQ, Visual Reception DQ Score; FMDQ, Fine Motor DQ Score; RLDQ, Receptive Language DQ Score; ELDQ, Expressive Language DQ Score.*

Clinician-rated behavior at exit was shown to be significantly and positively correlated with Fine Motor, Receptive Language, Expressive Language, and overall DQs at entry ($r = 0.46$–0.55, ps < 0.05). Clinician-rated behavior at exit was also positively correlated with baseline daily living skills ($r = 0.42$, $p < 0.05$), that is, the better a child's daily living skills at entry, the better their clinician-rated behavior at exit. Finally, clinician-rated behavior at exit was found to be positively correlated with baseline externalizing behavior, as measured by standardized VABS-II scores ($r = 0.37$, $p < 0.05$), suggesting that the more problematic a child's externalizing behavior at entry, the better their clinician-rated behavior at exit.

Change in clinician-rated behavior was not found to be significantly associated with any baseline variables (all ps > 0.05).

DISCUSSION

Children with ASD frequently engage in maladaptive behaviors such as aggression, self-injurious behavior, and stereotyped behaviors (7). These behaviors are problematic in group settings, as they disrupt the learning program and place children at increased risk for social exclusion, making it very difficult for them to transition to and access mainstream education settings (13). These behaviors also correlate positively with levels of stress in caregivers (17).

While the genesis of these maladaptive behaviors is thought principally to reside in communication and social skills difficulties, there is some uncertainty in the literature as to whether maladaptive behaviors are best managed via direct behavioral intervention; via treatments targeted primarily at improving pro-social and communicative skills; or via a combination approach. This study sought to examine the behavioral benefits to maladaptive behaviors of the ESDM, an early intervention focused predominantly on improving communication and pro-social skills, within natural daily play and care routines.

Several key findings were obtained. Principally, the level of maladaptive behaviors in the cohort of children studied, as assessed by clinician rating, reduced substantially following the 11-month ESDM intervention period. Moreover, for 68% of the children studied, substantial positive change was observed within the first 12 weeks of intervention. This group, who we have described as "rapid responders" tended to have less severe ASD symptoms at baseline and had a higher level of communication, daily living, and motor skills at baseline compared with children whose level of maladaptive behavior did not respond quickly to the ESDM. The behavior rating obtained at entry was not associated with any of the other baseline variables, which together with the finding that only 1 out of the 38 participants had ratings of good behavior at baseline, suggests that maladaptive behaviors occurred relatively uniformly within the sample. Across the whole sample, the degree of change in behavior rating from pre- to post-intervention was not associated with any baseline variables. However, clinician-rated behavior at exit was shown to be significantly and positively correlated with Fine Motor, Receptive Language, Expressive Language, and overall DQs; daily living skills; higher level of externalizing behavior at entry. In general terms, we would contend therefore that while maladaptive behaviors appear to have been ubiquitous in our cohort, children with relatively better adaptive functioning and fewer ASD symptoms at baseline seemed more likely to show rapid and subsequent improvement in their level of maladaptive behaviors. Overall, however, more than three-quarters of participants showed improvements (of three points on the six point scale) in maladaptive behaviors by the end of the intervention. Given the negative consequences of maladaptive behavior on children's learning (6), the ESDM's ability to bring about reductions in maladaptive behaviors – early in the intervention for around 3/4 of participants – may have allowed children to access and gain from the intervention program more effectively.

Significant improvements were also found following ESDM intervention in MSEL Visual Reception, Receptive Language, Expressive Language, and overall DQs. This is consistent with previous research (27, 34, 35). It is possible that, by promoting child development across domains, particularly receptive and expressive communication, and by using appropriate behavior management strategies, the ESDM resulted in an increase in conventional behaviors and a reduction of maladaptive behaviors. This is consistent with research showing a strong relationship between communication skills and the presence of maladaptive behavior in young children with ASD (8), and provides support for the suggestion by Myers and Johnson (22) that contemporary comprehensive intervention approaches for ASD should target communication and social skills in addition to disruptive or maladaptive behavior.

Furthermore, a child who is highly motivated is also more likely to learn at a faster rate (8). The ESDM works to increase child motivation by incorporating components such as child choice, turn taking, reinforcing attempts, and interspersing maintenance with acquisition tasks (30). The ESDM therapist is also highly trained in managing child attention; delivering clear antecedent, behavior, consequence sequences; modulating child arousal; creating interesting routines; building dyadic engagement though joint activity routines; responding with sensitivity to all child communicative attempts. The teaching principle that targets modulation of child arousal equips ESDM therapists to recognize and respond immediately to changes in child arousal levels and modulate these in the moment, potentially preventing maladaptive behaviors from developing in the first place.

Despite improvements in clinician-rated behavior and developmental skills, maladaptive behavior ratings on the VABS-II did not show improvement from pre- to post-intervention. It is interesting to observe that the externalizing behavior score on the VABS-II did show the largest Cohen's d effect size change of any VABS-II score ($d = 0.35$), but this was not statistically significant. One possible explanation for this finding is offered by Weiss et al. (46), who question the validity of the Maladaptive Behavior domain of the VABS-II in assessing levels of maladaptive behavior among children with ASD. It is also important to note that normative data on the VABS are only available for much older children than those in the current sample, and is not available for those with ASD. It is also possible that, while children's behavior during ESDM therapy sessions improved, this improvement did not generalize to the home environment and therefore no changes were found in parent-reported maladaptive behavior. Mastering the teaching principles of the ESDM equips adults to engage, modulate, and motivate the child into an optimal state for learning, hence promoting pro-social behavior. It is possible that the optimal behavior elicited during ESDM sessions was not replicated in other settings as parents or other caregivers were not similarly equipped with the skill set to elicit these pro-social behaviors. This suggestion highlights the potential importance of training parents and other professionals, such as those in school settings, in the ESDM model in order to provide the child adequate opportunities to generalize their newly acquired skills, and ideally of future research to explore the relative outcome for children in groups where parents had, or had not received intervention. We note that there was no specific parent training component to the ESDM intervention applied in this study; however, optional parent education evenings were offered at the center. Similarly, no significant improvements were found in the VABS-II standard domain scores or on the SCQ. This could again be attributable to these measures being parent reports, and skills not generalizing to the home setting; however, the lack of change observed in the current study on the SCQ is inconsistent with findings of significant improvement on this measure by Eapen et al. (34).

Findings of reduced clinician-rated maladaptive behavior and accelerated developmental rates in the present study are promising; however, due to the design of the current study, it is not possible to make conclusions about the mechanisms behind these improvements. That is, it is not possible to determine whether the reduction in maladaptive behavior observed in the present

study was a consequence of the ESDM's focus on social attention, affect sharing, imitation, and joint attention, or whether it was due to the use of behavioral techniques that are not specific to the ESDM, such as FBA and positive behavior supports, which have previously been shown to be effective in managing behavior within the framework of multiple treatment approaches. While ABA principles, FBA, and positive behavior supports are integral components of the ESDM, it is important to note that their specific implementation was only required for 2 of the 38 children in the present study whose behavior had not significantly improved after 3 weeks of intervention. It is therefore unlikely that the improvements in maladaptive behavior observed in the present study were directly and solely attributable to these behavioral strategies. Nonetheless, it is necessary to replicate the present study using a larger cohort and control conditions (both a different treatment condition and a non-treatment condition) in order to establish whether the reductions in maladaptive behavior occurring during ESDM intervention are significantly different to reductions that may occur in the context of a different treatment program or by maturation alone.

Regardless of the exact mechanisms behind the improvements in maladaptive behavior in the present study, our findings suggest that the ESDM program may be an effective tool in improving not only core developmental domains, but also decreasing maladaptive behaviors in preschool-aged children. This finding is important, given previous research demonstrating the negative impact of maladaptive behaviors and developmental delays on the child's learning acquisition and the development of social relationships (6). The relatively quick reduction in maladaptive behaviors observed in the present study (68% of children showed a significant decrease in maladaptive behavior by 12 weeks) may allow children to more effectively participate in and benefit from learning opportunities, including the intervention itself, and may be a key factor in the developmental gains observed in the present study and previous research (27, 34, 35). It is hypothesized that these developmental gains, particularly in the areas of receptive and expressive communication, may then provide children with adaptive means of getting their needs met, thereby further reducing maladaptive behaviors.

LIMITATIONS

This pilot study was limited by the use of a clinician-rated behavior score as the main dependent variable, particularly given that there were no blind raters on any measures. The fact that significant improvements were found in this rating over the course of the intervention, despite no change in VABS-II Internalizing, Externalizing, or Overall Maladaptive Behavior, raises questions over the reliability and validity of the ESDM clinician-rated behavior score. However, as noted previously, the validity of the VABS-II in assessing levels of maladaptive behavior among children with ASD has been questioned (46). Furthermore, the achievement of inter-rater reliability is fundamental to becoming certified as an ESDM therapist, with a requirement of initial and ongoing consistency of ratings with peers and the ESDM trainer. The fact that 32% of children did not show a change of three or more points in clinician-rated behavior over the first 12 weeks of the intervention is also an argument against rater bias.

A further limitation of the present study was the lack of a control group, which makes it difficult to determine whether the observed behavioral and developmental improvements were the effect of maturation or the intervention. Literature suggests, however, that maladaptive behaviors, once they become part of a child's behavioral repertoire, will typically remain or worsen without intervention (12). Moreover, the size of the improvement in maladaptive behaviors observed was large $d = 3.67$, which suggests that maturation alone is unlikely to be the causative factor. Similarly, the common course among children with severe ASD presentations without intervention is for IQ to remain the same or regress (47). The children in the current study had relatively severe presentations, including MSEL DQs <47 and VABS-II adaptive behavior scores within the range of 62–70 at baseline. Therefore, it appears that the behavioral and developmental improvements observed from pre- to post-intervention in this study are unlikely to arise as a result of maturation. The uncontrolled design of the present study also means that it is not possible to determine whether the observed reductions in maladaptive behavior were the result of ESDM-specific principles or to behavioral techniques that are not specific to the ESDM. Therefore, replication of the present study using a larger cohort and control conditions is necessary. Follow-up studies are also required to determine whether the behavioral and developmental improvements observed in the present study are maintained, which has the potential to foster ongoing educational opportunities and improve quality of life for children with ASD and their families.

Since maladaptive and challenging behaviors often pose a barrier to inclusion and community participation with significant consequences on social and educational opportunities, harm or injury to self or others, and family distress, it is critical to address these behaviors in the comprehensive management of children with ASD. The findings of the present study are promising, suggesting that the ESDM delivered in a community setting with relatively minimal one-to-one intensive therapy has the potential to reduce children's maladaptive behaviors which, in turn, may increase their capacity to participate in intervention and educational programs and make gains in other developmental domains.

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Predictors of outcomes in autism early intervention: why don't we know more?

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Response to early intervention programs in autism is variable. However, the factors associated with positive versus poor treatment outcomes remain unknown. Hence the issue of which intervention/s should be chosen for an individual child remains a common dilemma. We argue that lack of knowledge on "what works for whom and why" in autism reflects a number of issues in current approaches to outcomes research, and we provide recommendations to address these limitations. These include: a theory-driven selection of putative predictors; the inclusion of proximal measures that are directly relevant to the learning mechanisms demanded by the specific educational strategies; the consideration of family characteristics. Moreover, all data on associations between predictor and outcome variables should be reported in treatment studies.

Keywords: autism, early intervention, outcomes, predictors, individual differences

With increasing advances in autism research over the past decades, it has become clear that clinical heterogeneity is one of the most significant features of autism spectrum disorder (ASD) as diagnosed today. Genetic research indicates that hundreds of genes are implicated in ASD (1); neuropsychological research suggests that multiple neurocognitive mechanisms, rather than a single impairment, might underlie ASD symptoms (2); clinical research points to remarkable heterogeneity at the behavioral/phenotypic level (3, 4). Concomitantly, substantial individual differences are apparent with regard to treatment outcomes in this population (5).

On the basis of group-level data, research suggests that behavioral programs that are implemented as early as possible and in an intensive manner [often referred as early intensive behavioral interventions (EIBI)] can be efficacious in improving cognitive, adaptive, and social–communicative outcomes in young children with ASD (6, 7). However, analysis of treatment response at the individual level indicates that whilst some children show dramatic improvements, some show moderate gains, and others only show minimal or no treatment gains (8). Moreover, there is marked variation in the type and magnitude of positive outcomes seen between studies assessing effectiveness of the same intervention (5, 9–13).

Current knowledge of the factors associated with such individual differences in response to early intervention is limited. Therefore, the positive impact of these programs is hampered by lack of knowledge on the critical issue of "which children benefit from which program" (14). In the absence of this crucial information, the issue of which interventions should be chosen for a particular child is a common dilemma facing both families and clinicians. This question needs to be answered so the most effective early interventions can be provided for every child and to allow service and policy level decision making that will make best use of resources.

Recent literature indicates that choice of early intervention and education programs by families and clinicians is currently linked to factors such as regional proximity to services, anecdotal reports on effectiveness, or persuasive sales pitches/marketing, rather than based on the knowledge of which specific service is likely to result in best outcomes given the individual characteristics of the child (15, 16). This gap in knowledge creates the risk of enrolling children in programs from which they will not gain benefit, leading to a profound emotional and economic burden for affected children, their families, and the community. Given the tremendous implications of this issue at the personal, family, and societal level, in our opinion the identification of predictors of treatment outcomes should be at the top of the autism research agenda. However, advances in this area require a different lens for assessing individual differences as well as the adoption of more fine-grained research methodology, including new theoretical frameworks to analyze profiles of "responders" and "non-responders" under different types of early intervention.

In this paper, we shall argue that information about predictors of treatment response is limited because the current theoretical and methodological approaches are not adequate to address this issue. Furthermore, we will offer some directions for research, and discuss the implications for clinical practice.

CURRENT KNOWLEDGE ON PREDICTORS OF EARLY INTERVENTION OUTCOMES

While there are a number of treatments in the field of ASD, only a small proportion of these have scientific evidence for their efficacy. EIBI programs are currently recommended as the "treatment of choice" for children diagnosed with an ASD (17, 18), with most early intervention research focused on this type of approach. EIBI is characterized by the active engagement of the child for many hours per week (usually 20+) in a planned educational intervention delivered primarily in direct 1:1 child–adult instruction, with specific goals derived from assessment results, manualized/operationalized instructional procedures, and a data collection system to facilitate progress and outcome measurement (11). Within this framework there are different programs, which vary according to the specific curriculum and teaching procedures (19–21).

A number of reviews and meta-analyses have examined group-level outcomes in response to early intervention programs (6, 10, 22), indicating that EIBI programs appear to be effective for increasing adaptive behavior and IQ in young children with ASD. Nevertheless, there are limitations to available research, including lack of randomization in most published studies, small sample sizes, and possible biases due to awareness of treatment status in parents and providers. Moreover, the definitions of EIBI vary across studies, ranging from very specific definitions (e.g., including only programs that strictly adhere to the behavioral procedures originally described by Lovaas) [e.g., Ref. (23)] to very broad ones (including programs that are implemented early and intensively but use teaching procedures that significantly depart from the work of Lovaas, such as Pivotal Response Training) (22).

Importantly, available evidence suggests that EIBI programs (and early educational programs more in general) are not equally beneficial for all treated children (8). In the following, we focus on the factors associated with individual-level variability in response to current early educational intervention approaches, including strictly defined EIBI programs based on the work of Lovaas, as well as programs that are based on behavioral techniques but are not necessarily implemented at a high level of intensity [e.g., Reciprocal Imitation Training (24)], and interventions that meet the intensity requirement but do not have an explicit behavioral orientation [e.g., the PACT program (25)].

Analysis of available evidence indicates that pre-treatment cognitive abilities (IQ) and language abilities are the most often reported correlates of gains in early intervention studies (26–33), although not all studies concur in their conclusions (34–36). Several studies also indicate pre-treatment level of adaptive behaviors as a relevant correlate of treatment gains (6, 37, 38).

Whilst there is some evidence that children who are younger and less severely affected might be more responsive to early intervention, other studies report mixed or negative findings [(31, 39–43); see also Ref. (10, 44)].

A number of studies have identified more specific abilities associated with positive treatment outcomes, including play skills (24, 45–47), interest in objects (48–50), joint attention (46, 47, 50), imitation (38), low social avoidance (51), and response to social versus non-social reinforcement (52). In addition, there have been mixed reports on family factors such as the level of maternal education (53, 54) and family stress (55, 56).

LIMITATIONS WITH CURRENT APPROACHES TO RESEARCH ON PREDICTORS OF OUTCOME

Despite such findings on predictors, knowledge of the factors underlying positive treatment outcomes is limited and inconclusive. The major limitations to current research approaches are outlined below.

1. Variables associated with change in treated groups do not necessarily reflect actual predictors of outcomes (i.e., moderators or mediators of treatment response), as not all the observed change can be attributed to treatment (57, 58). If factors other than treatment that might contribute to change are not considered and controlled, correlates of changes within a treated group might or might not tell the full story about prediction of treatment response.
2. The selection of predictor variables is often not theory-driven (59, 60). In most papers reporting on predictors of outcomes, no rationale is provided on why specific variables are selected for analyses. It is apparent that, in most cases, the predictor variables are the measures used to characterize samples at trial commencement, with the purpose of identifying any important differences between randomized groups. Equally, the types of measures used often indicate broad constructs and thus lack the specificity required for a predictor variable. Therefore, the current findings of IQ/language variables as the most consistent predictors of response to early intervention programs tell us more about how commonly these measures are used in baseline assessments in intervention research than about their strength as predictors.
3. Analyses of specific behavioral predictors that could account for individual or subgroup differences are rarely included in research on intervention and prognosis (61). Most intervention studies focus on overall group-level outcome data, and analyses on those factors associated with identifiable subgroups of children or individual differences are either not conducted or not reported, despite the fact that treatment response is so variable in ASD. In medical research, the evaluation of intervention effectiveness often follows a progression from an initial focus on overall group-level outcomes ("does the treatment work for this condition?") to a subsequent focus on predictors of outcomes for identifiable subgroups or at an individual-level ("what are the factors associated with outcomes?"), particularly when treatment response is variable, of insufficient benefit compared to risk to warrant treatment for some, or when advances are still needed to improve outcomes. In research on autism intervention, most trials are designed to only address the question of group-level efficacy/effectiveness. However, as current research emphasizes the heterogeneity of ASD at every level of analysis, and the results of trials and meta-analyses show variable effect sizes, there is a clear need to move to the next stage of intervention research, with a focus on the specific individual predictors of treatment outcomes, and a focus on moderators and mediators of treatment response.

4. Few studies compare predictors of outcomes across different intervention programs (58). The impact of child characteristics is likely to vary according to the intervention program implemented, so that a child who is not responsive to program X (e.g., discrete trial training) might be more responsive to program Y [e.g., pivotal response training (14, 49)]. Since different programs utilize different instructional techniques (for example, different emphasis on external versus social reinforcements, different emphasis on verbal versus non-verbal instructions), it is likely that children with varying intrinsic cognitive and learning profiles will respond preferentially to different teaching approaches, just as do children who do not have ASD (60, 62, 63). However, the majority of studies on predictors of outcomes include data on response to one program only. Moreover, the inclusion of only one treatment leaves open the possibility that the change predicted by the pre-treatment factors is due to factors other than treatment (59).
5. Intervention studies recruit different samples from those that present at clinical services. Exclusion criteria in many intervention studies result in children with associated medical conditions, seizures, or a low IQs [e.g., IQ < 35 in Ref. (64)], or developmental age [e.g., <12 months in Ref. (25)] being excluded. While these exclusion criteria are set to create homogeneous samples for research purposes, they reduce generalizability to those who are seen in clinical and community-based settings, providing little information about which programs should be recommended based on different pre-treatment characteristics in the wider ASD population. Children with medical conditions, seizures, and/or severe intellectual disability make up a significant proportion of the ASD population (65, 66), and they are more likely to be referred to treatment programs compared to children with no identifiable comorbid features (67, 68). Therefore, research on their responses to different programs is crucial to inform clinical practice, so that enrollment to an intervention service is most likely to offer benefits and cause no harm.

   Current guidelines in clinical trials suggest that excluding patients with associated disorders from research should not occur when these comorbidities are common or when they affect treatment response and prognosis (69). As current research reports an increasing number of comorbid conditions associated with ASDs (66), including dysfunctions that are not diagnosable as specific disorders, the attempt to isolate "pure autism" in the ASD population by excluding "autism complicated by comorbid features" appears to be increasingly impracticable and unrealistic [see Ref. (35)].
6. Standard predictor measures currently used in research are very broad. Omnibus factors such as tested IQ, speech and language assessments, and adaptive behavior have predominated as both predictors and outcome measures in intervention research (53, 60). The use of such broad measures in intervention studies as predictors is problematic for a number of reasons. Low scores in IQ, language, and adaptive behaviors reflect a variety of distinct underlying processes, making it difficult to understand the specific mechanisms underlying the intervention response. Given that performance on IQ tests is, in itself, a measure of learning abilities (e.g., inefficient information processing), it is not surprising that children with lower IQ have more difficulties in learning from educational treatments. To avoid circular reasoning (children who have more difficulties in learning, as measured through pre-treatment IQ testing, are the ones who will have more difficulties in learning from educational treatments), research needs to focus on more proximal predictor variables. These should reflect specific and clearly defined processes that might explain difficulties in responding to educational strategies [e.g., response to social reinforcements in (52); lack of spontaneous imitation in (70)]. Moreover, broad variables such as IQ and communication scores are not robust measures in children younger than 3 years (71), and different tools used to measure IQ might provide different results depending on the instruction formats (e.g., verbal instructions versus demonstration) in the ASD population.
7. Family factors are seldom considered as predictors in outcomes studies. There is very little systematic data on how the characteristics and behaviors of parents influence children's responses to intervention (72). The motivation of parents to pursue and persist with intervention programs, which involve considerably more effort than administering medications or using complementary and alternative therapies, may be an important factor in treatment outcomes. Moreover, the parents' personal strengths (e.g., communication style, flexibility) may contribute to the developmental gains of their child. Lack of research in this area is surprising, given that (1) parents are frequently expected to engage as the main therapists for their children in many interventions, even those that are not called parent-mediated interventions, and (2) family factors have been found to impact on treatment response across a number of intervention programs for children with other conditions [see Ref. (73)]. Moreover, family factors such as higher parenting stress, negativity and depression, and low SES are ubiquitous factors in poorer outcomes across a range of child mental health interventions (74–76). The few studies that have investigated family factors in ASD indicate the relevance of parent's education, responsivity, and beliefs about the importance of child independence (77, 78). Another study (79) reported that higher distress in mothers pre-treatment was associated with lower adaptive behavior outcomes post-treatment, although the effect was not statistically significant [see also Ref. (30)]. Similarly, Osborne et al. (55) reported that high levels of parenting stress reduced the intervention gains in their children, particularly for high intensity interventions. Despite the relevance of this literature, the available evidence on family characteristics moderating treatment outcomes is limited and inconclusive.

RECOMMENDATIONS

On the basis of the limitations in the intervention outcome literature to date, a number of recommendations are made here.

1. Selection of putative predictors should be theory-driven, and predictors should be proximal and specific rather than broad. In order to match specific learning profiles to specific teaching programs, it is important to conduct a fine-grained analysis of child characteristics. Doing so will enable us to determine not

only what the child needs to learn (which will inform treatment objectives) but also how the child learns (which will inform treatment strategies). In addition, we also need to develop a fine-grained understanding of how each treatment works; that is, what are the processes (or the active ingredients) of the intervention that interacts with the child characteristics to promote learning in that child? With time it is likely that an understanding will emerge about the role of different factors on the pathway to effective intervention, such that some will be seen as "first order" or "primary" and required for certain educational or behavioral approaches to be effective, while others will be "second or later order" indicating modification that need to be made to optimize effectiveness of intervention techniques.

The analysis of the active ingredients of treatment involves a conceptual distinction between moderators of treatment effects, and mediators through which the intervention supposedly works (80). Moderators of treatment outcomes are the pre-treatment characteristics that might determine the degree of effectiveness of treatment versus control, but do not change as a consequence of the intervention, such as chronological age, gender, or maternal education. Conversely, mediators of treatment outcomes are the factors through which a treatment exerts its effects: they are subject to change as a consequence of the treatment, and these changes, in turn, affect treatment outcomes. For example, based on the theoretical tenet and the educational strategies of the Early Start Denver Model (a program in which the therapists are instructed to follow the child's lead), it is plausible that changes in the spontaneous propensity to initiate social interactions and engage in joint activities mediate outcomes in this program (81). Similarly, it is plausible that changes in the propensity to imitate others mediate outcomes in Reciprocal Imitation Training (24), and that changes in the ability to understand and follow visually mediated task instructions would be relevant in response to the TEACCH program (82).

The study of mediators of treatment outcomes requires the knowledge of the learning processes upon which the instructional techniques of the teaching program are based, or, in other words, understanding of the active ingredients underlying treatment-related changes. Without such knowledge, selecting among the many variables that are potentially associated with response to treatment in ASD is a difficult task. Importantly, intervention programs should have manualized guidelines and fidelity procedures to ensure that the active ingredients supposedly involved in the therapy are not "diluted" when the programs are translated into community practice.

A clear definition of the processes through which the child is able to learn in response to the particular instructional techniques is therefore one starting point for defining a specific set of putative moderators and mediators. Furthermore, it is crucial to focus on proximal factors that are known to support learning, with different predictor variables reflecting distinct and defined processes, so that the specific weight of these putative predictors mediating treatment response can be measured. Processes that are known to be foundational for social learning, and might be relevant predictors of outcomes for early educational programs include: social attention [paying attention to people and their actions (83)]; social motivation [which can be operationalized in terms of social approach versus social avoidance behavior, or response to social versus non-social rewards (52, 84)]; intentional communication [using language or non-verbal communication to communicate (85)]; receptive language/communication [understanding others' communication (86)]; joint attention [both initiation and response to joint attention (87, 88)]; goal understanding (89, 90); imitation (91, 92); functional play (35, 93). All of these processes reflect different facets of social cognition that are known to support social learning, and which are associated with developmental outcomes in ASD as well as in typical development [e.g., Ref. (94, 95)]. Preliminary evidence provides encouragement on the value of these factors in predicting response to intervention [e.g., Ref. (24, 35, 38, 45, 46)]. Another factor that might be associated with outcomes is the extent of restricted/repetitive behaviors (RRBs), since engagement in inflexible routines and insistence on sameness are likely to hinder acquisition of new skills and social learning. For example, Watt et al. (96) reported that prolonged engagement with RBBs was negatively related to social competence across the crucial developmental period from 2 to 3 years. Data on the relevance of RRBs in response to treatment are scant and equivocal (52, 97, 98), so more research is needed to investigate how individual differences in the extent of RBRs affect response to intervention.

Other factors that are not specific to ASD might also be associated with treatment outcomes across intervention programs. These include attention (in particular sustained attention, e.g., the ability to be focused on a task from the beginning to the end); memory; responsivity to instrumental learning/conditioning (the ability to associate contingent rewards to own behaviors); processing speed and efficiency; generalization (the ability to use what has been learned in non-training situations). Rate of learning (e.g., number of treatment goals achieved in the first 6 months of treatment) might also be a relevant predictor of outcomes (99). Research on the predictive value of these factors in response to treatment is scant.

As standardized tests are not available for many of the processes listed above, it is necessary to develop and utilize novel, fine-grained experimental measures and observational protocols that are suitable for young children with ASD across the spectrum of severity [e.g., Ref. (35, 52)]. **Table 1** summarizes a list of the factors that have been theoretically and/or empirically linked to positive treatment outcomes (including both child and family factors, discussed in the next section), and for which psychometric development is needed to advance a methodology for identifying predictors of outcomes.

2. Family factors should be investigated in treatment studies. As family involvement is a recommended component of early intervention (100), future research should systematically investigate the family characteristics associated with responses to treatment for children with ASD. Importantly, different early intervention programs involve instructional techniques (e.g., play-based versus highly structured strategies) that may or may not fit with a family's educational practice and cultural values. Different families might also respond differently to the parent training formats used by the different programs, which

Table 1 | Putative predictors of treatment outcomes that require standardization/psychometric development.

Child factors	Family factors
Functional communication (requesting, protesting)	Sociodemographic background (resource-poor versus resource-rich)
Social communication (sharing, commenting)	Family expectations about treatment
Social approach (versus avoidance)	Family sense of competence/self-efficacy
Joint attention	Parent/therapist alliance
Social understanding	Family stress and discord
Imitation	Father positive involvement
Level of RRBs	Social support
Functional play with objects	
Responsivity to reward learning	
Generalization	
Core neuropsychological functions (processing speed/efficiency, sustained attention, cognitive control, memory)	

vary from information sessions in small groups to 1:1 sessions in which the parent is asked to implement educational strategies under a therapist's supervision. Other potential family factors mediating response to treatment are the level of parent/therapist collaboration, maintaining positive expectations for child outcomes, lack of family conflict, positive father involvement, level of social support, and level of stress in the family (14).

High levels of child behavior problems are prominent in families with children with developmental disabilities and contribute to greater stress in families than cognitive delay (101, 102). Generally, mothers of children with ASD report higher levels of distress than those of non-disabled children or those with children with other disabilities, although there is considerable variability across families (103, 104). In a recent paper, Benson (105) has shown that social network attributes, including the range and function of emotional support, are related to perceived social support, which in turn can bring about a decrease in depressed mood in mothers of children with ASD. Formal and informal social support networks can have the effect of enhancing quality of life, confidence in parenting and optimism (106) for families with children with disabilities, and might be crucial factors predicting treatment outcomes.

Accurate measures that appropriately capture individual differences between families in terms of specific values, attitudes, and resources may need to be developed. Future research should investigate the role of family factors in a systematic way, selecting specific, theory-driven variables, developing novel fine-grained measures and comparing variables associated to outcomes across different programs. When examining a broad array of putative factors (e.g., family characteristics and attitudes, sociodemographic background) that may be associated with outcomes, it is important to consider that while a single factor might not be predictive of outcomes, the accumulation of multiple risks often is. Studies looking at longitudinal outcomes in at-risk populations often use cumulative risk approaches (107) in which a discreet number of risk indexes are created to capture the level of risk within a number of predefined theoretically driven domains. To illustrate, a number of studies [e.g., Ref. (108, 109)] have examined the predictive value of risk factors within the child (various child attributes), sociocultural (demographic characteristics), and parenting domains (parent beliefs/attitudes), showing that cumulative risk within each domain predicted outcomes in typically developing children. This approach can be helpful in examining whether risk factors in family related and sociodemographic domains play a role in response to treatment in the ASD population.

3. Studies on treatment efficacy/effectiveness should always report on individual differences in response to treatment as well as those factors associated with positive treatment outcomes. The analysis of individual differences (i.e., characteristics of responders and non-responders) should become a standard procedure in early intervention research [similarly to current procedures in pharmacological intervention research (110)]. Moreover, when predictor variables are measured, their association with the outcome measures should always be reported. The tendency to report on predictors of outcomes only when results indicate significant associations makes it hard to draw clear conclusions on the robustness and consistency of factors associated with treatment response. Thus, it should be mandated that all data on predictor and outcome variables are reported, as in the case of outcome measures in clinical trials (111). Reporting all available information on the association between predictors and outcomes can also be useful in studies with small sample sizes, which comprise the majority in the field of ASD, so that data from multiple small trials can be pooled for meta-analyses. While data on correlates of treatment gains *per se* do not allow for definite conclusions on predictors of treatment outcomes, this information can be critical to inform subsequent research designed to control for the predictive value of factors that appear to be relevant in relation to treatment changes.
4. Research on predictors of treatment outcomes should compare responses across different programs. As mentioned above, the analysis of correlates of gains in a treated group might not be informative on predictors of treatment outcomes, as not all observed change in a treatment study can be attributed to the treatment. Research on predictors of treatment outcomes should use designs that compare different programs, thus allowing for the analysis of the interaction between selected participant characteristics X treatment group. This approach is critical not only from a methodological point of view (distinguishing moderated treatment effects from factors that are associated with outcomes/changes in ASD more generally), but also from a clinical perspective. Different early intervention programs, while sharing many similarities, involve distinct instructional techniques that are based on different theories and which tap into different learning processes. For example, some programs involve an emphasis on teaching words/sentences that have the instrumental function of obtaining desired items, whilst in other programs language is targeted with an emphasis on its social versus instrumental function. Similarly, programs

vary across a number of teaching processes (e.g., verbal versus visually conveyed instructions, following the child's lead versus adult-directed models), reflecting different underlying theoretical frameworks. Since these different teaching practices require different learning processes on the part of the learner, it is possible that different types of learners will respond to different types of teaching procedures. Given that remarkable differences are present both in the teaching procedures of the different programs and in the social, cognitive, and learning profiles of children with ASD, it is imperative that future research compares the profiles of response to different early learning programs that use different instructional techniques. This approach would allow identification of the pre-treatment child characteristics that support learning when a specific set of teaching procedures is used. Only a few studies to date have provided information on the specificity of predictors of outcomes to one versus another treatment program [see Ref. (49, 50)].

5. Research on predictors of outcomes should involve large heterogeneous samples. It is important for studies on the predictors of response to treatment to include the full range/spectrum of children with ASD. Many children with comorbid conditions are typically excluded from research studies, such as those with severe intellectual disability and associated medical conditions or seizures. However, as discussed above, these children represent a substantial proportion of the ASD population referred to community early intervention centers. It is particularly important that research focuses on children with very low IQ to determine whether the currently available early intervention programs are appropriate for this population, and which specific factors are predictive of positive outcomes (e.g., short attention span, passivity, poor play skills). If research shows that there are child characteristics that indicate a lack of responsivity to any of the currently available early intervention programs, we need to develop novel approaches targeting the specific impairments of the "non-responders," rather than referring them routinely to programs from which they do not benefit.

CONCLUSION

Little is known about predictors, moderators, and mediators of treatment response in children with neurodevelopmental and neuropsychiatric conditions (112, 113), and the field of ASD is no exception. With recent advances in research documenting the positive impact of early intensive behavioral programs for children with ASD, the critical issue facing researchers, clinicians, and practitioners in the field is not as much a lack of evidence-based treatments, but rather an inability to predict which treatment will work best for each child. The variability of response to EIBI and other early intervention programs is a phenomenon that very likely reflects the heterogeneity of the ASD population. While some crude prognostic variables, such as IQ level, hold some value in predicting which children will respond best to early intervention, to date, the available information on moderators and mediators of treatment response is inconclusive. In this paper, we argue that lack of knowledge on "what works for whom and why" in ASD reflects a number of methodological and theoretical issues in ASD treatment research.

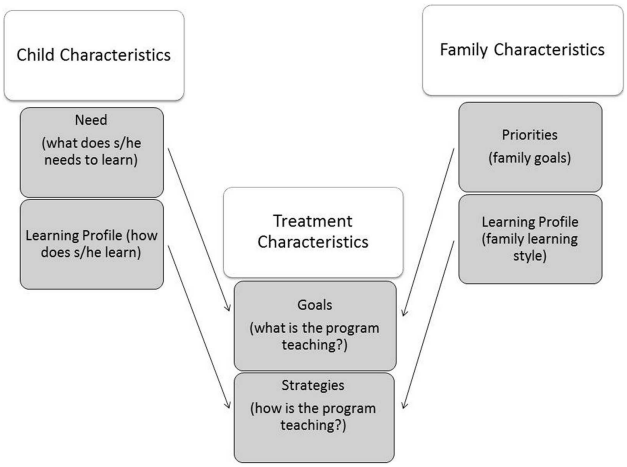

FIGURE 1 | Treatment goals should be selected on the basis of the child's needs and family priorities. Treatment strategies should be selected on the basis of the child learning profile and the family's learning style.

We argue that the selection of treatment objectives and treatment strategies should be informed by knowledge of: (1) child characteristics (how does the child learn best, and what does s/he needs to learn); (2) family characteristics (what are the family priorities/expectations, and what is the family learning style); (3) treatment characteristics (what are the treatment aims, and how does the treatment work). This model is illustrated in **Figure 1**. Current understanding on the interplay of these factors in determining treatment outcomes is limited, and we have offered here a number of recommendations to advance knowledge in the field. Given the complexity of the biological underpinnings of ASD, behavioral predictors might not be sufficient to predict treatment outcomes to a high degree of accuracy. Nevertheless, knowledge arising from this line of research can critically contribute to the development of decision-making guidelines in the field of ASD early intervention.

A number of questions remain, which need further critical evaluation. First, it is unclear whether predictors of intervention outcomes are specific to ASD. As reported above, a number of specific factors in the two areas of impairments that characterize ASD (social communication and repetitive behaviors domains) are likely to hinder positive responses to intervention; however, other factors that are not specific to ASD (e.g., attention span, memory) might also play an important role in predicting outcomes. For example, IQ and language abilities are associated with outcomes across a number of conditions [e.g., schizophrenia (114), depression (115), conduct disorder (116)] that are not related to ASD. Indeed, these factors might predict outcomes above and beyond receiving early intervention *per se* [see Ref. (117–119)].

It is possible that factors associated with positive responses to intervention are the same that support positive learning in children without an ASD. More research is needed, therefore, to achieve a more fine-grained understanding of mechanisms supporting early learning and cognitive development in typical development as well

as atypical development (e.g., neuropsychological processes such as attention and processing speed and social processes such as social engagement, sharing of affect, and joint attention). Sophisticated research designs are also needed to identify distinctions and overlaps and the interplay between variables moderating treatment changes, those mediating outcomes, and those affecting both processes.

Given the scarcity of standardized tools to assess social and non-social factors that might be relevant for response to intervention, measurement of putative predictors of outcomes poses relevant challenges. More efforts are currently needed to develop systematic observational and experimental tasks that provide reliable measurements of such predictors.

Another critical aspect concerns the definition of positive versus nil or minimal outcomes in ASD research. Outcome measures used in treatment research might not be sufficiently sensitive to capture gains in children making very slow or very small gains. However, these small progressions might have a relevant impact on the family and child's quality of life and may be the necessary "first order" changes that are needed to allow further positive response. Thus, the operational definition of "responders" versus "non-responders" should take into account different outcome criteria besides the standard/conventional measures used in ASD research.

Moreover intervention in ASD, while promoting gains in certain areas, can also be instrumental in preventing declines or worsening in other domains. For example, there is evidence that some repetitive behaviors might increase over time in ASD (120), and it is conceivable that a treatment program could aim to prevent such increase. In this case, the absence of change in repetitive behaviors in children undergoing treatment cannot be seen as evidence of a lack of treatment response. Therefore, definitions of responders and non-responders must be conceptualized and framed on knowledge of developmental patterns in the different treatment target domains.

A related issue is the question of when do we begin to classify children as "non-responders"? If children are not showing measurable gains after 1 year, is it possible that they will start responding in the second year? Currently, our knowledge on the timing of treatment response is limited. Different studies provide information on predictors of outcomes in relation to programs that vary in duration, making it difficult to compare results and to calibrate expectations about the timing of treatment response. In order for research to inform clinical decision-making, some consensus about the timing of expected response to treatment is therefore necessary, and approaches that allow time-limited intervention with ongoing follow-up to allow future intervention planning should be developed.

A further challenge is the clinical management of "non-responders" to early intervention programs. In the best-case scenario, future research will indicate that those children who do not respond to some early intervention programs will nonetheless respond to other available programs, so that each child can be matched to the most appropriate program. However, it is possible that specific child or family factors are associated with minimal or no responses across all available treatment options. If this is the case, it will be necessary to target the specific factors that are known to limit the efficacy of the program ["treating the constraints" approach (59)], and to conduct specific research focused on these "non-responders." Until research indicates successful strategies to address the factors limiting response to treatment in this subgroup, the question is: should children who present with a "non-responder" profile be referred to programs from which they will not benefit? This raises a number of ethical issues as well as concerns with regard to cost–benefits considerations, and individual rights to access services.

Finally, the research recommendations that we have outlined, which involve a focus on individual differences in large heterogeneous samples, the measurement of a variety of theory-driven predictor variables at baseline, and the comparison of prognostic indicators across different programs, are expensive ones. To have a sufficient number of participants to identify robust profiles of responders and non-responders to available early intervention programs and to replicate findings across sites requires large-scale collaborative multisite research. Nonetheless, the practical implications of such a research program surely justify the necessary investment, especially as ASD is currently diagnosed in more than 1 in every 100 children and the problems associated with ASD have a high impact for the individual, their family, and the community.

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Reducing auditory hypersensitivities in autistic spectrum disorder: preliminary findings evaluating the listening project protocol

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Auditory hypersensitivities are a common feature of autism spectrum disorder (ASD). In the present study, the effectiveness of a novel intervention, the listening project protocol (LPP), was evaluated in two trials conducted with children diagnosed with ASD. LPP was developed to reduce auditory hypersensitivities. LPP is based on a theoretical "neural exercise" model that uses computer altered acoustic stimulation to recruit the neural regulation of middle ear muscles. Features of the intervention stimuli were informed by basic research in speech and hearing sciences that has identified the specific acoustic frequencies necessary to understand speech, which must pass through middle ear structures before being processed by other components of the auditory system. LPP was hypothesized to reduce auditory hypersensitivities by increasing the neural tone to the middle ear muscles to functionally dampen competing sounds in frequencies lower than human speech. The trials demonstrated that LPP, when contrasted to control conditions, selectively reduced auditory hypersensitivities. These findings are consistent with the polyvagal theory, which emphasizes the role of the middle ear muscles in social communication.

Keywords: autism, auditory hypersensitivities, social engagement behaviors, listening, polyvagal theory

INTRODUCTION

Frequently accompanying a diagnosis of autism spectrum disorder (ASD) are speech and language delays, difficulties in extracting human voice from background sounds, auditory hypersensitivities, and a general compromise in social communication skills (1–8). In contrast to the prevalent reports of auditory processing deficits, most individuals with ASD, even those with noticeable auditory perceptual disorders, have normal hearing when tested on a standard audiogram (9).

Several mechanisms have been proposed as contributing to frequently reported deficits in auditory processing including damage or dysfunction to peripheral structures (i.e., middle ear and inner ear), neural pathways (e.g., auditory nerve), and central structures (e.g., brainstem nuclei and cortical areas) (e.g., Ref. (10–17)). A review (18) suggests that although atypical auditory processing and both hypo- and hyper-reactivity to auditory signals are frequently observed in autism, these atypical reactions cannot reliably be attributed to specific neural pathways. Thus, subjective methods remain the sole indicators of auditory hypersensitivities (19).

PHYSIOLOGY OF THE MIDDLE EAR

Borg and Counter (20) described a role of middle ear muscles in facilitating the extraction of human speech by dampening the transmission of low frequency noise from the external environment to the inner ear. The Borg and Counter model suggests that atypical neural regulation of middle ear muscles may contribute to the frequently observed auditory hypersensitivities and auditory processing deficits in ASD. Deconstructing the path through which sound is processed illustrates the role middle ear structures have in auditory processing and how atypical neural regulation of the middle ear muscles may contribute to auditory hypersensitivities and atypical auditory processing.

Sound enters the outer ear and travels through the external auditory canal to the eardrum where it is transduced by the structures of the middle ear (i.e., small bones comprising the ossicular chain), which connects the eardrum with the cochlea. The rigidity of the ossicular chain determines the stiffness of the eardrum. The middle ear muscles, via cranial nerves, regulate the position of the ossicles and stiffen or loosen the eardrum. When the eardrum is "tightened" higher frequencies are absorbed and transmitted to the inner ear and the energy of lower frequencies is attenuated (i.e., reflected) before being encoded by the inner ear (cochlea) and transmitted via the auditory nerve (cranial nerve VIII) to the cortex. Complementing the ascending pathways are descending pathways that regulate the middle ear muscles, which functionally determine the energy (i.e., attenuate, pass, or amplify) of specific frequencies that reach the inner ear. The features describing the transformation of sound intensity from outer to inner ear defines the middle ear transfer function. If the acoustic information in the frequency band associated with speech is distorted by an atypical middle ear transfer function, the information being coded by the inner ear and subsequently being transmitted to the cortex will

not contain sufficient information to enable accurate detection of speech sounds. In addition, there are descending pathways that regulate the hair cells in the cochlea to "fine tune" auditory perception, which is especially important in the development of language skills. If the acoustic information related to human speech that reaches the cortex via ascending pathways is distorted, then the descending pathways to the cochlea may also be atypical and will further distort the individual's ability to process speech and to produce language.

As proposed by Borg and Counter (20), atypical central regulation of peripheral middle ear structures may pass low frequency sounds that dominate the acoustic spectrum in our mechanized society (e.g., ventilation systems, traffic, airplanes, vacuum cleaners, and other appliances) resulting in both a hypersensitivity to sounds and distorting or "masking" the frequency components associated with human speech reaching the brain. This emphasis on the functional role of the middle ear muscles in the dampening of background noise and the extraction of voice is based on a literature documenting two points: (1) the neural regulation of the middle ear muscles modulates the transfer function of the middle ear (21, 22) and (2) the transfer function of the middle ear determines the acoustic energy from low frequencies that reach the inner ear (23). Thus, an atypical middle ear transfer function would be a potentially parsimonious explanation of both the auditory hypersensitivities and the difficulties in auditory processing frequently associated with autism.

DESIGNING THE LISTENING PROJECT PROTOCOL

The listening project protocol (LPP) is a theoretical departure from the disciplines frequently involved in the treatment of auditory processing disorders, which emphasize the role of central structures in the processing of speech (see Ref. (18) for a review). LPP was theoretically designed to reduce auditory hypersensitivities by recruiting the anti-masking functions of the middle ear muscles to optimize the transfer function of the middle ear for the processing of human speech. LPP is based on an "exercise" model that uses computer altered acoustic stimulation to modulate the frequency band passed to the participant. The frequency characteristics of the acoustic stimulation were theoretically selected based on the documented frequency band and weights associated with the index of articulation (24) and speech intelligibility index (25). These indices emphasize the relative importance of specific frequencies in conveying the information embedded in human speech. During normal listening to human speech, via descending central mechanisms, the middle ear muscles contract and stiffen the ossicular chain. This process functionally removes most of the "masking" low frequency background sounds from the acoustic environment and allows human voice to be more effectively processed by higher brain structures. Modulation of the acoustic energy within the frequencies of human voice, similar to exaggerated vocal prosody, is hypothesized to recruit and modulate the neural regulation of the middle ear muscles and to functionally reduce auditory hypersensitivities (see Ref. (23)).

The features of the intervention including the context, the duration of stimulation, and frequency band selected were theoretically determined and based on the following neurophysiological principles: (a) the transfer function of the middle ear serves as an anti-masking mechanism to dampen low frequency sounds and to facilitate extraction of human voice from background sounds (20), (b) acoustic energy is readily transmitted across middle ear structures, regardless of the neural tone to the middle ear muscles, at a resonance frequency in children between 800 and 1200 Hz (26), (c) middle ear muscles are primarily composed of fast-twitch muscles and are vulnerable to rapid fatigue (27), and (d) the phylogenetic convergence in mammals of a brainstem area involved in the neural regulation of striated muscles of the face and head including the middle ear muscles (see (23, 28, 29)). Principles (a) and (b) were used to design the acoustic stimuli, principle (c) informed decisions related to the duration of each session, and principle (d) provided the basis for the social support provided during the intervention (i.e., the neural regulation of the middle ear muscles is optimized in a "safe" context).

LPP applies computer altered vocal music (i.e., filtered music) designed to exaggerate the features of human prosody and hypothetically to exercise the neural regulation of the middle ear muscles. By modulating the frequency band associated with human vocalizations, it was hypothesized that the ascending pathways would be providing dynamically changing information that would feedback on the descending pathways regulating the middle ear muscles. Metaphorically, the procedure could be conceptualized as a "treadmill" exercise for the middle ear muscles during which the demands to "listen" and process the acoustic features of the intervention stimuli were dynamically changing. To test the primary hypothesis that the filtered music condition would reduce hearing sensitivities in children with ASD, two trials were conducted. Trial I contrasted a filtered music group to a headphones only group and Trial II contrasted a filtered music group to an unfiltered music group.

The intervention consisted of five daily sessions of approximately 45 min during which the participant passively listened to the acoustic stimulation through headphones in a quiet room, while researchers provided social support to insure that the participants remained calm. The frequency bands were temporally modulated within each session and, independent of amplitude, the band of frequencies that were modulated progressively increased across the five sessions. Theoretically, the changing frequency bands were presented to increase the neural regulation of middle ear structures to dampen the perception of background low frequency sounds and to potentiate the extraction of human voice. Although middle ear muscle regulation could not be assessed, the Borg and Counter (20) model provided the scientific basis to hypothesize that the exercises embedded in LPP would reduce auditory hypersensitivities.

METHODS: TRIAL I AND TRIAL II

PARTICIPANTS

Potential participants contacted the laboratory for initial inclusion screening. Participants were informed about the research project by clinicians, parents who previously participated in our research program, and via professional presentations and/or newsletters. Individuals with a suspected diagnosis of ASD, who did not have a history of seizures, were scheduled for a diagnostic assessment that consisted of the autism diagnostic interview-revised (ADI-R) (30). The ADI-R provides a diagnostic algorithm consistent with

the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (31) and International Classification of Diseases, tenth edition (ICD-10) (32). Informed consent was obtained from parents. The Institutional Review Boards at the University of Maryland, the University of Illinois at Chicago, and the University of North Carolina approved the project. The protocols are excluded from the requirement to be registered (e.g., ClinicalTrials.gov), since enrollment was initiated before January 1, 2001 and data collection was completed before December 26, 2007.

Parents of 178 children contacted the laboratory to participate in the research. Based on the ADI-R criteria, 146 children met the full criteria of autism. Of the children, who did not meet full criteria, 29 exceeded the ADI-R cut off on at least the qualitative impairments in reciprocal social interaction and/or communication scales. Three children, who did not meet the cut off on either the qualitative impairments in reciprocal social interaction and/or communication scales, were excluded from participating in the research.

Based on presentation at the laboratory the first 73 children were assigned to Trial I. In Trial I, data from nine children (two in the filtered music and seven in the headphone only groups) were lost due to technical problems. In Trial I, questionnaire data were scored for 36 children in the filtered music group and 28 children in the headphones only group. Following the completion of Trial I, 102 children, who had not participated in Trial I, were enrolled in Trial II. In Trial II, due to scheduling difficulties, families of six children withdrew before participating in the trial and one family withdrew after the second day of the intervention. In Trial II, data from one child who was diagnosed with Fragile X were excluded from the data analyses. In addition, data from 12 children in the filtered music group were lost due to parents not returning the questionnaires, or returning the questionnaires late, or health issues. Data are not available for documenting the specific causes for lack of compliance. Questionnaire data in Trial II were available from 50 participants in the filtered music group and 32 participants in the unfiltered music group. Descriptive statistics of demographic features of the subjects from Trial I and Trial II with questionnaire data are reported in **Table 1**.

Trial I and Trial II included 86 participants in the filtered music condition, 32 participants in the unfiltered music condition, and 28 participants in the headphones only condition (see **Table 1**). Although mental age of the participants was not formally assessed, all participants had either speech (at least five words apart from "mama" and "dada," used spontaneously and meaningfully) or followed verbal instructions. Approximately 80% of the participants were Caucasian and the remaining 20% included children from African–American, Latino, and Asian parents.

EXPERIMENTAL DESIGN

The intervention research was conducted as two sequential randomized controlled trials with parallel control groups. All participants were randomly assigned sequentially by presentation at the laboratory to either the filtered music group or a control condition group. No clinical or behavioral feature was used to determine group assignment. Trial I participants were randomly assigned to either a filtered music or a headphones only group, which consisted of children wearing headphones without music.

Trial I was initiated to evaluate whether the intervention had an effect beyond the contextual variables of supportive play and low intensity social interactions that characterized the experimental environment for both groups. Since data analyses of parent questionnaires indicated a treatment effect on auditory hypersensitivities, Trial II was conducted to evaluate whether the filtering of the music uniquely determined intervention effects. Trial II participants were randomly assigned to either a filtered music group or an unfiltered music group. To insure a sample size sufficient to test hypotheses related to auditory hypersensitivities, twice as many participants were assigned to the filtered music group.

Parents were not informed about their child's group assignment until the follow-up sessions were completed. Nor were parents informed about the features of the intervention (i.e., filtered music) or the control condition within each trial (i.e., headphones only in Trial I and unfiltered music in Trial II). Circumaural headphones were used, since they provide excellent sound quality, are comfortable to wear, and have excellent external noise rejection. The features of the headphone in combination with low intensity auditory stimuli precluded the parents from detecting whether their child was receiving the filtered music condition or a control condition. Based on our interactions with parents, it appeared that parents were not informed about the group assignment of their children. After the completion of the follow-up assessment sessions, the children in the unfiltered and the headphones only

Table 1 | Demographic information for subjects with complete data by group assignment and sex.

	Trial I		Trial II	
	Filtered music Mean age (SD)[b]	**Headphones only condition Mean age (SD)[b]**	**Filtered music Mean age (SD)[b]**	**Unfiltered music Mean age (SD)[b]**
Met at least partial criteria on ADI-R[a]				
Male	58.24 (10.14), $n=25$	49.46 (10.96), $n=23$	54.89 (14.83), $n=44$	56.20 (9.36), $n=27$
Female[c]	48.67 (11.99), $n=11$	61.00 (7.91), $n=5$	44 (20.66), $n=6$	60.33 (9.29), $n=5$
Total	55.37 (11.42), $n=36$	52.67 (11.30), $n=28$	53.33 (15.95), $n=50$	56.74 (9.25), $n=32$

[a] *Exceeded the ADI-R cut off on at least the qualitative impairments in reciprocal social interaction and/or communication scales.*

[b] *Mean age and standard deviation in months.*

[c] *Females in Trial I were significantly older in the headphone only group.*

conditions were given the opportunity to receive the filtered music. Since knowing group assignment might bias parental perceptions of the child's behavior, data from the children, who received the filtered music after participating in either the headphones only or unfiltered music conditions, were not included in the data analyses.

One week following the intervention, parent reports were obtained for all participants in both trials. None of the children who participated in Trial I participated in Trial II. In addition to the parent questionnaire, semi-structured play-based behavioral assessment sessions were conducted with the children and videotaped before and after the intervention.

CONDITIONS AND PROCEDURE

Each condition (i.e., the filtered music, unfiltered music, and headphones only conditions) consisted of approximately 45 min sessions conducted during five-consecutive days. During the intervention, regardless of group assignment, each child wore headphones in the same laboratory environment. The same vocal music selections were used for both the filtered music and the unfiltered music conditions. In the filtered music condition, the vocal music was computer processed based on a proprietary algorithm developed to remove low and high frequencies and to modulate the width of the frequency band associated with human voice. The intervention stimuli were stored on compact discs and played via high quality compact disc player (Marantz CC-4000) to high quality over the ear headphones (Beyerdynamic DT831). Maximum loudness was calibrated at a peak of 75 dBC before the intervention started. During the headphones only condition, no auditory stimulation was provided through the headphones, although the context was identical to the filtered music and unfiltered music conditions. The low volume of the intervention stimuli and the use of over the ear headphones insured that the intervention stimuli could not be distinguished from the ambient background sounds in the test room by the parents.

The sessions were conducted in a research room with toys (e.g., books, doll house and accessories, parking garage and cars, pretend kitchen and accessories, stuffed animals, coloring books, and crayons). During the intervention, the children were able to freely play with the toys. One experimenter stayed in the room during the intervention to assist the child with the headphones when needed. Parents were also allowed to be in the room with their child. The experimenter and the parents were instructed to be quiet and to interact with the child only to maintain and to support a calm behavioral state. Due to the nature of the study (e.g., checking the integrity of the headphones), the experimenter who conducted the intervention session was not always blind to the child's group assignment. In Trial I, since the headphones only group received headphones without sound, the experimenter was frequently aware of the child's group assignment. However, since only the experimenter adjusted the headphones, the parents remained blind. In Trial II, since acoustic stimulation was being presented to both groups, the experimenter and the parent were unaware of the child's group assignment. Accordingly, to avoid the possibility of rating bias, the experimenter who conducted the intervention sessions did not participate in the play-based assessments during which sharing behaviors were coded.

BEHAVIORAL ASSESSMENT

Parent questionnaire

Following the intervention and the play-based assessments, parents were given a structured questionnaire developed in our laboratory, targeting specific categories of their child's developmental and behavioral problems including auditory hypersensitivities. The parents of children in all groups were instructed to complete and to return the questionnaire to the laboratory in a week. The questionnaire focused on whether the child had difficulties in a specific behavioral area and whether there were any changes in this area following participation in the research. For each behavioral category, parents were required to document changes, if any, following the intervention by providing specific examples of observed new behaviors. The structured questionnaire focused on the behavioral domains listed in **Table 2**.

Table 2 | Behavioral domains and explanations for the structured parent questionnaire.

	Definitions
Hearing sensitivity	Exaggerated negative responses (e.g., crying or placing hands over the ears) to common noises (e.g., vacuum cleaner, garbage disposal, baby crying, and air conditioning)
Spontaneous speech	Non-prompted use of words and sentences to communicate thoughts and ideas
Receptive speech	Ability to understand instructions and phrases
Spontaneity	Non-prompted behaviors initiated by the child
Behavioral organization	Ability to occupy oneself (when left alone) in a productive and non-stereotypical way
Emotional control	Ability to calm quickly when upset, to respond to unexpected changes without getting upset, and to tolerate objections and contradictions of other people
Affection	Behaviors reflective of warm emotional state expressed by the child toward familiar people (e.g., hugging, kissing, and saying "I love you" to the parent)
Listening	Ability to focus on human speech without visual or contextual cues, to understand spoken words, and to follow verbal requests
Eye contact	Making and maintaining eye contact during social interactions
Relatedness	Non-prompted social behaviors that reflect understanding of a joint partnership in interactions and sharing the same goals during social interactions (e.g., looking at a partner, showing toys, sharing an idea or a thought, and directing emotions to the partner)

Questionnaire scoring

Each of the 10 items representing the behavioral domains described in **Table 2** was scored as a 1, 0, or -1. A score of 1 was assigned if the parents indicated that their child had a problem in the area of interest before the participation in the project and provided an example of a new behavior that could be considered as an improvement in this area. An item received a score of 0 if the parents indicated that their child had a problem in the area of interest, but provided no example of a change. Non-specific parental responses (e.g., "somewhat better" and "a lot better") that were not supported by concrete examples of the new behaviors also were conservatively scored as 0. An item received a score of -1 if the parent indicated that the behavior became worse after participating in the research and provided an example of the new worsened behavior. If the parent did not indicate a problem in the area of interest, the item did not receive a score. Each questionnaire was scored by two researchers, at least one of whom was blind to the child's group assignment. Only when both scorers agreed that the example provided by the parent constituted a new and relevant behavior, a score of 1 was given. Scores of -1 were rare and did not occur on any of the behaviors coded in Trial I and only three times in Trial II. Thus, separate analyses for scores of -1s were not conducted.

Social interaction coding scale

Prior to and following their participation in the intervention project, all children participated in a 10-min semi-structured play-based observational assessment of social engagement skills with the social interaction coding scale (SICS) (33). The SICS provides information regarding the child's social engagement activity. Similar to the autism diagnostic observational scale (ADOS) (34) and early social communication coding scales (ESCS) (35), the SICS requires a semi-structured presentation of standard tasks. Each task provides an opportunity for social engagement by requiring the child to engage in a joint activity. In the current study, the number of spontaneous sharing behaviors was quantified.

Coding social interaction coding scale

The frequency of sharing behaviors was coded from videotapes by trained coders. Coders obtained reliability with each other on training tapes before using the scale for research (i.e., 80% agreement on individual items, mean kappa > 0.60 for three consecutive joint scoring). Each tape was coded by two trained coders independently and compared for agreement. At least one of the coders was not aware of the participant's group assignment when coding. Consensus was used to establish the final code. If raters disagreed on the same item, the code of the unbiased coder was recorded. If coders were uncertain about the final code, the opinion of the third trained coder was requested and the code that received the consensus of at least two coders was recorded. If all three coders disagreed on the final code, the behavior was not coded.

DATA ANALYSES

Analyses of variance and non-parametric χ^2 analyses were used to evaluate group differences within each trial on each of the behavioral domains. Since both analysis strategies identified the same group differences within each trial, only the analyses of variance are presented. A Bonferroni correction adjusted significance levels for multiple comparisons.

RESULTS

QUESTIONNAIRE DATA

Global evaluation of problems

Confirming the effectiveness of the randomization procedures, there were no group differences in the representation of the behavioral problems reported via the parental questionnaire within each trial or across trials (see **Table 3**). For example, the representation of hearing hypersensitivities across the four groups across both trials ranged from 43 to 50%. When the number of problem dimensions was summed for each participant, more than 95% of the parents reported that their child had at least one behavioral problem. The percentage of parents reporting multiple behavioral problems decreased as the number of domains increased, with approximately 80% of the parents reporting problems in at least five behavioral domains.

TRIAL I: GLOBAL AND SPECIFIC EVALUATION OF IMPROVEMENT

To evaluate the effectiveness of the filtered music treatment, group differences were evaluated with analyses of variance for each of the 10 behavioral dimensions included in the questionnaire. As illustrated in **Figure 1**, significant improvements, relative to the headphones only group, were noted in the filtered music group in hearing sensitivity, $F(1,29) = 6.46$, $p = 0.017$; spontaneous speech, $F(1, 49) = 5.61$, $p = 0.022$; listening, $F(1, 52) = 8.25$, $p = 0.006$;

Table 3 | Distribution of initial behavioral problems (%) within each trial.[a]

	Trial I		Trial II	
	Filtered music (%)	Headphones only group (%)	Filtered music (%)	Unfiltered music (%)
Hearing sensitivity	50	43	46	50
Affect	44	61	64	59
Eye contact	75	61	60	63
Behavioral organization	53	57	56	53
Emotional control	50	43	66	59
Spontaneous speech	75	82	82	78
Receptive speech	72	82	90	81
Listening	81	86	74	66
Spontaneity	69	71	44	44
Relatedness	83	82	64	66
At least 1 problem	92	96	98	97
At least 2 problems	92	93	98	94
At least 3 problems	89	89	96	91
At least 4 problems	83	79	94	88
At least 5 problems	81	75	92	78

[a] *No significant differences were found among the groups on any behavioral dimension.*

and behavioral organization, $F(1, 34) = 5.39$, $p = 0.027$. The percent of the participants improving, who had a problem within each domain, is presented in **Table 4**. At 1-week post-intervention, analysis of variance confirmed that the filtered music group exhibited significantly more improvements summed across domains than the headphones only group (i.e., 2.36 versus 0.81), $F(1, 62) = 7.76$, $p = 0.007$.

TRIAL II: GLOBAL AND SPECIFIC EVALUATION OF IMPROVEMENT

Since the relative benefits observed during Trial I could be attributed to listening to music, independent of the computer modulation of the acoustic features, Trial II was conducted contrasting the filtered music condition to the same music in an unfiltered form. The unfiltered music condition was similar to the "structured listening" condition described by Bettison (36). As illustrated in **Figure 2**, significant improvements in the filtered music condition relative to the unfiltered music condition were observed in both hearing sensitivity, $F(1, 28) = 4.53$, $p = 0.040$, and emotional control, $F(1, 49) = 5.84$, $p = 0.019$. The percent of the participants improving, who had a problem within each domain, is presented in **Table 4**. Note that when unfiltered music is used as the control, several of the benefits of filtered music condition observed in Trial I (i.e., spontaneous speech, listening, and behavioral organization) appear to be due to listening to music (i.e., unfiltered music) and not to the algorithm used to filter the music. Consistent with this interpretation, there was no significant difference in the sum of improvements for the filtered music group (1.98) when contrasted with the unfiltered music group (1.53). These data suggest that the unique benefit of the filtered music is a significant reduction in hearing sensitivity.

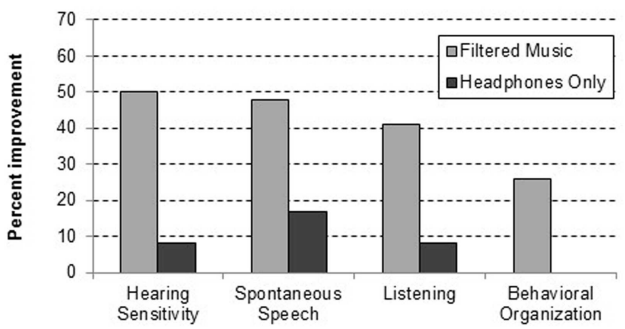

FIGURE 1 | Behavioral improvements at the 1-week post treatment assessment in Trial I. The data are reported as the precent of participants with a specific behavioral problem who improved.

CONTRASTS BETWEEN TRIAL I AND TRIAL II

Analyses of variance confirmed the similarity between the filtered music condition in Trial I and Trial II. The percent of participants improving on each domain was similar for the filtered music groups within Trial I and Trial II (see **Table 4**). Similarly, the number of problem domains was similar for all groups on entry into the protocol (see **Table 3**).

SHARING BEHAVIORS

Video data from a random subsample of children in the filtered music condition ($n = 61$) were coded. The subsample was partitioned into three groups: children who had no hearing sensitivity at the start of the study ($n = 34$), children who showed improvements on hearing sensitivity following the intervention ($n = 14$), and children who had no improvements on hearing sensitivity following the intervention ($n = 13$). A repeated measures analysis of variance identified a significant group × condition interaction, $F\ (2, 58) = 4.88$, $p < 0.011$. Consistent with the parental reports, only the subgroup of children with improvement on hearing sensitivity increased the amount of sharing behavior during the 10-min semi-structured play-based protocol. Descriptive statistics are reported in **Table 5**. *Post hoc* Bonferroni adjustment confirmed that only children who were reported to improve on hearing sensitivity increased the amount of sharing behavior during the 10-min

Table 4 | Percent[a] improving who had a problem within each behavioral domain at the 1-week follow-up.

	Trial I		Trial II	
	Filtered music	**Headphones only**	**Filtered music**	**Unfiltered music**
Hearing sensitivity	**50[b]**, $n = 18$	8, $n = 12$	**43[c]**, $n = 23$	13, $n = 16$
Affect	19, $n = 16$	18, $n = 17$	25, $n = 32$	21, $n = 19$
Eye contact	41, $n = 27$	24, $n = 17$	33, $n = 30$	40, $n = 20$
Behavioral organization	**26[b]**, $n = 19$	0, $n = 16$	29, $n = 28$	18, $n = 17$
Emotional control	17, $n = 18$	0, $n = 12$	**24[c]**, $n = 33$	0, $n = 19$
Spontaneous speech	**48[b]**, $n = 27$	17, $n = 23$	51, $n = 41$	44, $n = 25$
Receptive speech	31, $n = 26$	9, $n = 23$	9, $n = 45$	15, $n = 26$
Listening	**41[b]**, $n = 29$	8, $n = 24$	30, $n = 37$	29, $n = 21$
Spontaneity	48, $n = 25$	20, $n = 20$	36, $n = 22$	36, $n = 14$
Relatedness	30, $n = 30$	13, $n = 23$	34, $n = 32$	29, $n = 21$

[a] *Defined by the number of individuals who improved divided by the number of individuals with problems (n) within the behavioral domain.*

[b] *Significant improvement relative to headphones only in Trial I.*

[c] *Significant improvement relative to unfiltered music in Trial II.*

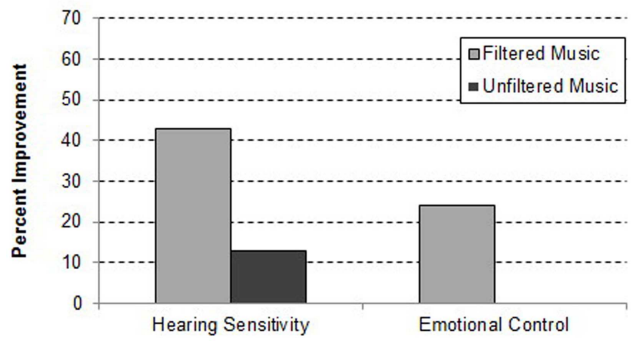

FIGURE 2 | Behavioral improvements at the 1-week post treatment assessment in Trial II. The data are reported as the precent of participants with a specific behavioral problem who improved.

Table 5 | Hearing sensitivity (HS) and total number of shares (*N*, mean, and SD).

	N	Pre-intervention		Post-intervention	
		Mean	SD	Mean	SD
Children who improved on HS	14	5.71	7.31	9.86	10.53
Children who did not improve on HS	13	7.46	7.33	7.62	6.74
Children who had no HS	34	5.82	8.50	6.32	7.97

semi-structured play-based protocol following the 5-day program relative to the initial assessment ($p < 0.005$).

TREATMENT EFFECTS ON PARTICIPANTS WITHOUT HEARING SENSITIVITIES

To investigate the effects of filtered music on the participants without hearing sensitivities, analyses of variance were calculated on each behavioral domain to identify possible behavioral domains that would improve in children without auditory hypersensitivities as a function of the filtered music. These analyses did not identify any specific behavioral domain that would reliably improve in the children without auditory hypersensitivities.

DISCUSSION

SUMMARY OF FINDINGS AND OTHER RESEARCH EVALUATING LPP

Two randomized controlled trials were conducted to evaluate the efficacy of LPP on auditory hypersensitivities and social behavior in children with ASD. Data from both trials confirmed that LPP (i.e., filtered music) selectively reduced auditory hypersensitivities. Trial I contrasted filtered music with a headphones only condition. The results of Trial I led to a more stringent Trial II in which filtered music was contrasted with an unfiltered music condition. In both trials, the LPP selectively reduced auditory hypersensitivities. In addition, within the filtered music groups the children with auditory hypersensitivities who improved following LPP significantly increased their spontaneous sharing behaviors. These findings, consistent with the polyvagal theory, support the hypothetical basis for designing the LPP as a neural exercise of pathways involved in regulating behavioral state, listening, looking, and other social engagement behaviors such as spontaneous sharing.

The current findings are consistent with a previous study (37), evaluating LPP with a more diverse sample of ASD children. In the previous study, the effectiveness of LPP was objectively assessed by evaluating auditory processing (assumed to be a function of the transfer function of the middle ear structures) and autonomic state (assumed to mediate behavioral state regulation). The study demonstrated that LPP significantly increased vagal regulation of the heart (i.e., increased amplitude of respiratory sinus arrhythmia) and normalized auditory processing on the filtered words and competing words subtests from the SCAN test for auditory processing disorder (38, 39). Collectively, the data from the current trials and Porges et al. (37) provide convergent preliminary support that LPP enhances function of the polyvagal "social engagement system" manifested in improved auditory processing, reduced auditory hypersensitivities, increased vagal regulation of the heart, and increased spontaneous social behaviors (e.g., sharing).

CONTRASTS WITH TRADITIONAL AUDITORY INTERVENTION THERAPIES

Since LPP delivers computer altered acoustic stimuli through headphones, it shares some of the features of auditory intervention therapies (i.e., AIT). However, although LPP is a "sound therapy," it is not a traditional clinically available AIT (e.g., Ref. (40, 41)) and differs from these procedures in method and theory. First, LPP is based on the polyvagal theory and reflects a strategic attempt to engage neural regulation of specific structures involved in the social engagement system (28). Second, LPP focuses on auditory hypersensitivities that may be expressed by individuals with and without clinical diagnoses. Third, the effectiveness of LPP can be measured through well defined behavioral and physiological features of the social engagement system. Fourth, LPP was designed with several unique features to engage and to exercise the neural regulation of the middle ear muscles, including an understanding of the transfer function of the middle ear structures and the vulnerability of the fast twitch middle ear muscles to fatigue. Fifth, the duration of LPP is shorter (i.e., less than 5 h) than most forms of AIT. Therefore, the effects of LPP described in this study should not be generalized to any other form of auditory intervention.

There are several problems related to the evaluation of traditional auditory intervention therapies. First, since the interventions have evolved from clinical observations and insights, the neurophysiological theory underlying the interventions is often not well developed or tested. Second, research has been frequently structured to ask questions of efficacy instead of developing protocols to test theoretically relevant components of the treatment in order to understand the mechanisms and to refine the methodology. Third, since auditory interventions are applied within a clinical setting, several experimental design parameters are difficult to control including (1) a constant protocol, (2) limiting concurrent treatments including medication, (3) randomization

of participants into conditions, and (4) the selection of outcome variables that are theoretically relevant to the intervention model. Perhaps the greatest limiting factor is the broad range of domains that auditory interventions are proposed to improve without a description of a causal link through which the intervention would result in functional changes in behavior. Due, in part, to the above limitations, the literature documenting an efficacy for the clinically available forms of AIT has been difficult to interpret.

Some studies evaluating the effectiveness of the AIT report improvements (42, 43) and others do not (36, 44–47). However, some of the above studies that do not support unique positive effects of AIT provide documentation of positive effects. For example, Bettison (36) reports positive effects in both the experimental group (received auditory training) and the control group (listened to the same unmodified music under the same conditions). Bettison suggests, consistent with our findings, that features in the AIT shared with listening to selected unmodified music may have beneficial effects on children with autism. Moreover, as our data suggest, if participants do not have auditory hypersensitivities, then the effects of LPP may be mediated through different biobehavioral pathways with unpredictable (i.e., non-specific) positive outcomes, which are not consistent with the middle ear transfer function model. Perhaps, similar to the outcomes with children without auditory hypersensitivities in the LPP trials, observed positive effects of AIT may be recruiting pathways outside of the middle ear model via the potential therapeutic calming effects of music and social support by clinicians.

Gilmor (48) conducted a meta-analysis based on several studies conducted in the 1980s with the Tomatis method involving 231 children. Gilmor clustered the outcome measures into five behavioral domains and identified small effects for linguistic, psychomotor, personal and social adjustment, and cognitive domains. Interestingly, he found no reliable effect in the auditory domain. These findings should be cautiously interpreted because the studies were limited by small sample sizes, issues related to defining control conditions, and limited use of random assignment. Regardless of these limitations, parents and clinicians of children with ASD have reported that forms of auditory intervention therapy have been helpful.

LIMITATIONS OF THE CURRENT STUDY

The data from the current study need to be cautiously interpreted for the reasons outlined below.

1. The major findings were dependent on the subjective reports of parents.
2. Some of the hypotheses tested were dependent on the small sizes of critical subgroups (e.g., individuals with or without auditory hypersensitivities who did or did not show improvements partitioned by the various treatment conditions).
3. The participants were receiving other treatments during the intervention and assessment period. Several participants were receiving daily interventions using behavioral approaches and other therapies, which may have enhanced or dampened the effects of the LPP.
4. Frequent contact of parents with therapists might bias parental reports and compromise the validity of the parents as objective informants. These factors could obfuscate the real effects of the intervention and inaccurately identify changes. Alternatively, features that might have improved could have been neglected. Possibly the hearing sensitivity domain on the parent questionnaire is less vulnerable to clinician–parent bias. Based on our experience, the therapists and parents appear to be less interested in this dimension, although it was the focal point of our study.
5. Improvements were observed in the groups not receiving the filtered music. Approximately 40% of the parents of children not receiving the filtered music reported improvements on at least one behavioral feature. These positive reactions might be due to non-specific features of the protocol, such as a relaxed intervention environment fostering social engagement and spontaneous play, as well as a positive "expectation" bias and the effects of familiarity with staff and context as the child progressed through the five laboratory sessions. However, the groups receiving filtered music diverged from the control groups when parents reported improvements in hearing sensitivity.
6. Standardized assessments of cognitive function and developmental landmarks were not evaluated. The lack of this information precluded confirmation of matching on these variables, although, based on the sample size, random assignment should have led to a reasonable expectation of matched samples. The randomization of participants, with regard to the evaluated parameters, was effective and there were no group differences in their representation. Standardized assessments of cognitive function and development would provide data to investigate two questions: (1) Are auditory hypersensitivities related to cognitive function and developmental landmarks? (2) Is the effectiveness of the LPP related to individual differences in cognitive function?
7. Our participants were young and on the severe end of the autism spectrum and the findings may not generalize to older or less severe ASD.
8. The studies precluded an opportunity to confirm the specific neural pathways responsible for the observed behavioral improvements. The methods employed could not confirm whether auditory hypersensitivity was due to a compromise in functional neural regulation of the middle ear muscles (as proposed by the polyvagal theory) and remediated through an exercise model.
9. The studies did not provide information necessary to distinguish among alternative pathways leading to or remediating auditory hypersensitivities, such as the potential influence of the intervention on damaged neural pathways (e.g., auditory or facial nerve), on damaged peripheral structures (e.g., middle ear and inner ear), or central structures involved in processing the acoustic signal or in cortical representation.
10. The hypothesized link between the middle ear transfer function and auditory hypersensitivities could be limited. Hypersensitivities, especially to high frequency sounds, might be due, not to the neural regulation of the middle ear muscles, but to the olivary cochlear reflexes. Tests of inner ear function and the degree of auditory hypersensitivity to high frequency sounds need to be evaluated to rule out this possibility.

11. The general improvements in behavior observed following a reduction in hearing sensitivity might not be related to the proposed integrative social engagement system. Rather, the enhanced behavior might be naturally occurring when the sounds are no longer painful and distracting.

FUTURE DIRECTIONS

A measure of the hypothesized intervening mechanism, the middle ear transfer function, has been missing from the formal experiments evaluating effectiveness of LPP. At the time the participants were tested, no commercial clinical or research device was available to monitor the middle ear transfer function. Without a sensitive measure of the middle ear transfer function, the only method to demonstrate efficacy was to quantify physiology, auditory processing, and measures of behavior and to infer that the LPP normalized an atypical middle ear transfer function. Recently we have developed a middle ear sound absorption system (MESAS) to measure the middle ear transfer function (49). MESAS provides an objective measure of the potential mediating role that middle ear muscles play in experiencing auditory hypersensitivities (see Ref. (50)).

By providing an objective measure of the middle ear transfer function, future research with MESAS will enable a selective test of the efficacy of LPP in normalizing the middle ear transfer function. If confirmed, LPP could be applied to individuals with atypical middle ear function including rehabilitation following otitis media. In addition, MESAS will enable future research to evaluate the behavioral and psychological consequences of an atypical middle ear transfer function, provide data to validate a quantitatively scaled measure of auditory hypersensitivities independent of subjective reports, and contribute to the improvement of interventions (e.g., LPP) that may function as efficient neural exercises to normalize the middle ear transfer function.

AUTHOR CONTRIBUTIONS

Stephen W. Porges was involved in all aspects of the research, including conception, design of the intervention stimuli, design of the protocol, analysis, interpretation, and writing the manuscript. Olga V. Bazhenova and Elgiz Bal were involved in the design, acquisition, and preliminary analyses and drafts. Nancy Carlson and Yevgeniya Sorokin were involved in acquisition of the data. Keri J. Heilman was involved in data acquisition, data analyses, and contributing to the final drafts. Edwin H. Cook was involved in developing the final draft and in interpreting the data. Gregory F. Lewis was involved in the development of the stimuli, developing the Middle Ear Sound Absorption System (MESAS), and in collecting and interpreting the preliminary data with MESAS.

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Peer-mediated theatrical engagement for improving reciprocal social interaction in autism spectrum disorder

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The hallmark characteristic of autism spectrum disorder (ASD) is poor reciprocal social communication. Interventions designed to improve this core deficit are critically needed. Social skills interventions such as direct training, peer mediation, and video modeling have contributed to improvements in various social skills in children with ASD. This paper reviews existing social competence interventions available for children with ASD while highlighting hypothesized critical components for advancing, maintaining, and generalizing skills, which include (1) peer mediation, (2) active learning, and (3) implementation in supportive, natural contexts. As a framework for these approaches, this conceptual paper describes SENSE Theatre, a novel intervention that combines trained peers that facilitate the performance-based theatrical treatment delivered in a supportive, community-based environment. A review of previous research shows early feasibility, setting the stage for more rigorous studies to aid in developing a standardized intervention package.

Keywords: autism, peer mediation, social skills, active learning, theatre, context

INTRODUCTION

Children with autism spectrum disorder (ASD) have difficulty with social interaction, communication, and responding in a flexible way to everyday life (1). A recent report suggested that 1 in 68 individuals are now affected by this complex neurodevelopmental disorder (2). Affected individuals display a wide range of ability in language, sociability, and intellectual functioning. However, the defining characteristic across all levels of functioning is impairment in reciprocal social interaction, which can manifest as limitations in many areas of functioning including social-emotional reciprocity, to-and-fro conversation, play behavior, use of non-verbal communication, and in the development of age-appropriate relationships (1). There is significant diversity in symptom presentation, which can range from spontaneous social approaches with poor understanding of social rules to avoidant behavior with little evidence of social interest (3–5). Similarly, there is significant diversity in repetitive and stereotyped patterns of behavior (6) and a range of hyper- and hypo-sensitivity to sensory experiences (7).

Interacting with others relies on the coordinated effort of many complex and integrated skills to include the ability to recognize, identify, integrate, plan, and respond appropriately to dynamic socioemotional information. This information is conveyed, in part, through the interpretation and expression of verbal (e.g., speech prosody) and non-verbal (e.g., facial expressions and gestures) forms of communication (8), which can be difficult for those affected by ASD to interpret. Additionally, many individuals with ASD have difficulty perceiving, understanding, and remembering emotions (9, 10) and human faces (11–17). In addition to understanding how others feel, children with ASD have limitations in empathizing and predicting how others will think and act (18). Understanding other people's thoughts and feelings is a part of the concept of "theory of mind," which is defined as the ability to attribute mental states, such as beliefs, to other people and then use these ideas to understand and predict other peoples' behavior (18–20). Since sociability is a fundamental and encompassing skill, interventions designed to improve the complexity and diversity of social functioning are critically needed.

During social interaction with peers, many children with ASD experience heightened physiological stress as measured by cortisol, a primary stress hormone in human beings (21–24). Moreover, many children and adolescents with ASD show significant anxiety in social contexts especially as they get older and gain insight into their challenges engaging with others (25, 26). Despite showing improvements in social communication through experience and development, many children with ASD display higher levels of stress as measured by increased cortisol during benign social interactions with same age peers (22, 23, 27). It has been shown that behavioral patterns of approach and avoidance, as well as age factors can contribute to distinct patterns of stress in youth with ASD (22). Thus, stress responsivity may be an important moderator of social interaction for children with ASD and should be considered in programs developed to improve social skills (28, 29).

Currently, there are no known pharmaceutical treatments that have been shown to improve the core symptoms of ASD; thus, behavioral-based interventions designed to improve social skills are essential. There are many different types of social skills interventions available including direct skills training, applied behavior analysis, social skills groups, video modeling, and peer training (30–35). Many training programs produce positive outcomes in children with ASD; however, only a few meet strict criteria for

empirical support (e.g., inclusion of control groups and randomization) (33) in order to distinguish observed effects attributed to the intervention from natural improvements over time (36–38).

BACKGROUND

The current paper briefly introduces some existing social skills interventions available for children with ASD that are relevant to the concepts outlined below. Comprehensive reviews of social skills program and treatment options for children with ASD are more thoroughly reviewed elsewhere [e.g., Ref. (38–41)]. Teaching appropriate social skills to children with ASD by direct didactic training in either a group or one-on-one format has received empirical support (33, 42, 43). Qualified psychologists and paraprofessionals often teach these training sessions in clinic settings, which have demonstrated immediate, in-context gains for targeted skills. While participating children with ASD may learn specific social skills such as initiating interaction using priming (35), improving conversation skills through direct instruction with caregivers (44), or enhancing perspective taking from teaching theory of mind and social skills (45), acquired skills often do not generalize across people, contexts, or everyday environments (38, 46). Thus, the strong context-dependent learning in children with ASD is the most common barrier to behavioral change in children and adolescents with ASD (47). Thus, the inclusion of typically developing peers in the training and active practice of social skills can significantly contribute to the acquisition, maintenance, and generalization of sociability. Moreover, as described below, the context in which treatment is delivered also can impact social skill development.

Rather than reviewing or critiquing a broad range of social skills programs, the strengths of the approaches that are relevant to our theatre-based social engagement treatment are highlighted. In the context of the paper, three key components are discussed that we hypothesize to be necessary for advancing, maintaining, and generalizing social interaction abilities in children and youth with ASD, which include (1) peer mediation, (2) active learning, and (3) implementation in supportive, natural contexts. Moreover, these central ideas involve the inclusion of the recipient or target of the treatment in the intervention model to achieve changes in self-efficacy. Additionally, the conceptual paper presents SENSE Theatre, a novel intervention that combines trained peers that facilitate the performance-based theatrical approach delivered in a supportive, community-based environment.

PEER MEDIATION

Peers can have a profound impact on the psychological, social, and physiological functioning of other children, including children with ASD (22–24, 27). Peer-mediated interventions utilize typically developing peers as agents of change, facilitating the development of social behavior and engagement in children with ASD (48–50). Peer mediation programs vary in the degree to which peers are trained in the delivery of social skills. One widely used peer-mediated program for children with ASD in school settings involves a one-on-one assistant who helps the child throughout the school day and is given the task of helping the child integrate socially with others (51). Although well intended, if the peers are untrained, the approach may produce unwelcome effects as it may label the child with social deficits as "different," possibly leading to stigmatization and greater social isolation. Peers' negative expectations of the target children must be managed in order to prevent this effect, and peer training is an effective way to achieve more positive expectations (52). For example, studies utilizing students who participated in trained mediation groups show greater generalization of skills for children with ASD (53). Peers should not simply be a social partner but also an active model of appropriate social behavior (54). In some programs, peers are extensively trained in how to elicit certain target behaviors through modeling, reinforcement, scripts, and other program-specific methods (55, 56). Thus, peers may serve as the optimal agent of change because they are not only the interventionists but they are also the intended recipients of improved socialization.

Since the primary objective for any social skills program is for children to be able to interact more competently with peers in natural settings (52), the inclusion of trained peers in treatment is both logical and beneficial. Moreover, it may be economical; utilizing peers as a resource for learning has the potential for reducing the demands on teachers and parents (57). Utilizing peers increases the number of training models for practice (49), which supplements instruction provided by parents and professionals. Zhang and Wheeler (58) performed a meta-analysis of peer-mediated interventions for children aged 8 years and younger and found them highly effective for increasing social interactive behavior. Additionally, Lang et al. (57) performed an analysis of several studies that utilized recess time to improve social skills and found that use of peer mediation increased social interaction for children with ASD. Further, peers participating as mediators can increase peer acceptance and engagement with others, and this in turn can be a powerful agent of change for children with ASD (51).

Peer-mediated interventions not only benefit the child with ASD but they also provide positive results for the typically developing children delivering the intervention. While it can be argued that school-based peer mediation takes away valuable learning opportunities for those administering the intervention, Hunt and Goetz (59) report that these peers demonstrate positive social-emotional growth and participation does not interfere with academic performance. As an added benefit, participating peers often develop positive and accepting attitudes when they observe the capability of their peers with a disability (60).

Kasari and colleagues (51) performed one of the first randomized controlled studies that compared the effectiveness of one-on-one direct social skills training to a peer-mediated model for high-functioning children with ASD. Peers received extensive instruction (40 min per week for 6 weeks) and training in how to recognize and lend social support to those who may have social difficulties. Results showed that children who received the peer-mediated intervention had increased involvement in classroom social networks as rated by teachers and untrained peers in the classroom, as well as less isolation on the playground. These treatment gains persisted to a 3-month follow-up. Despite these improvements, reciprocal friendships remained low for children with ASD and they still exhibited isolated play on the playground, although to a lesser extent.

While the previous study shows that peer mediation may be an independently superior treatment than simply direct skills

training, using the treatments in combination should also be considered when creating an efficacious social skills intervention. Banda et al. (61) conducted a study that combined direct instruction and peer training for increasing social skills in two elementary students with ASD. They had teachers in a classroom setting model to participants and peers how to initiate questions and answer with appropriate responses, while praising students for properly asking and responding to questions. Results showed an increase in participant initiating and responding. A similar study had an interventionist teach a participant and two peers various phrases and skills to promote cooperative play at recess and provide cues during appropriate times of play. All three participants had higher rates of communicative acts during the intervention sessions than at baseline (62). Schmidt and Stichter (63) had adolescent peer mediators interact with three target students with ASD who had just completed a direct skills training program delivered in a school setting and found that the addition of the peer mediators lead to improved skill generalization for all three students with ASD. As these studies illustrate, adult supervision using direct skills training in concert with peer intervention may be a strategic combination for improving the social engagement of children with ASD.

SOCIAL LEARNING IS ACTIVE *"PRACTICE IS THE KEY TO SOCIAL SUCCESS"*

Whether by direct skills didactic training or peer-mediated interventions, social learning theory posits that much of human behavior is learned by watching and imitating others (64). In fact, four processes are deemed necessary to promote the acquisition of social behavior, namely, attention, retention, production, and motivation. In other words, despite the misleading title, observational learning is not a passive task of merely watching others; it is an active and engaging process. Thus, programs that include techniques to activate learning and engage the participant through the actual performance and practice of social skills can advance learning (65–68). As discussed below, acting is fundamentally active and thereby may provide the opportunity to enhance observational learning and the practice of social skills.

Reciprocal social interaction requires active engagement and practice. While it is helpful to teach social rules and strategies, there are a variety of possible encounters and, therefore, the proficiency, spontaneity, and improvement in socialization is gained through interacting with a variety of partners. In infancy and early childhood, many fundamental social skills are acquired by imitating others; yet, children with autism have difficulty with imitation (69), which includes knowing who to model, what behaviors to imitate, and in what context to apply the behaviors learned (70). Since imitation is one of the ways in which children learn social rules, early deficits in this ability could impair a child's social development (71).

As a key component in social learning (64), imitation is not a passive process, rather, it is a volitional, effortful, and active process (72). Studies show that children with autism are impaired in imitating various actions and emotions compared to peers with other developmental delays or typical development (69). In this study, Rogers and colleagues postulate that imitation deficits in children with autism may lead to a lack of active imitation practice due in part to a diminished interest in socially motivating rewards (69). This suggests that in order to engage children with autism to practice imitating others, they must be motivated to observe and imitate the actions of others. One approach shown to capture the motivation of children with ASD is video modeling (31, 73, 74).

Video modeling is an effective way to work with children on the autism spectrum to capture and transfer the targeted skills into their repertoire. In video modeling, an individual watches specific skills presented on a video, then imitates through active practice of the performed skills often in the form of a role-play that may be initially guided by a trained paraprofessional or parent. Video modeling has consistently gained empirical support across a number of studies for improving various aspects of social functioning and teaching adaptive behaviors, such as reciprocal play and promoting social language verbalizations in children with autism (30, 31, 75–81). The use of video can facilitate observational learning and generalization of behavior because it is an inherently motivating medium (28, 31, 73, 74). Since video modeling involves not only watching the performance of others but also includes role-play with another person, it is complimentary to several acting approaches used in theatre outlined below.

NATURAL AND SUPPORTIVE CONTEXTS

It is well established that the context in which an individual learns can significantly impact the retention and transfer of skills in typically developing children (82, 83) and ASD (37, 57). Motivation is a key component in active learning, and is influenced by external factors, including the learning context (84). An environment that is perceived as naturally stimulating and supportive leads to greater motivation and participation compared to restrictive and controlled settings (85). This is shown in children with ASD as well as other developmental disabilities demonstrated by Gresham and colleagues (86), who state that social skills interventions with poor generalization outcomes usually take place in more restrictive settings that are unlike the child's usual environment. In contrast, the positive impact of social skills training with or without peers can be enhanced when conducted in natural settings, thereby leading to greater generalization and maintenance (46, 87, 88). Providing a least restrictive environment allows children of all achievement and developmental needs to receive an adequate and effective education (89). Peer-assisted learning strategies implemented within an inclusive classroom environment can provide reciprocal learning when delivered in a supportive and structured manner (90). In the process, children can benefit from different instructional procedures that are conducive to individual learning needs while allowing the teacher the opportunity to supervise a variety of instructional methods simultaneously (90).

The context in which typically developing peers and children with developmental needs are brought together is especially critical. For example, an increase in communication and interaction was demonstrated in children with ASD utilizing peer mediation at recess where it is possible to *encourage* appropriate social interactions with peers, although the authors acknowledge that such an approach lacks structure and close adult supervision (62). Kasari and Smith (91) propose that contexts outside of school that mimic the school environment could be an effective compromise between the naturalistic environment of a cafeteria or playground and the structure of a clinic setting. Ideally, a combined supportive

structure may be the best. For example, Sansosti (92) proposes a multitier framework to teach social skills for children with ASD that includes core (e.g., positive behavior support), targeted (social skills groups), and intensive (individualized videos) intervention strategies.

In treatment, the contextual framework plays an integral role in the development and transfer of skills (37, 57, 82). In order for treatment to be successful, interventionists have the responsibility of cultivating applicable learning environments that will lead to successful dissemination within the community (93). Effective learning conditions ought to be natural, motivating, and supportive (46, 84, 85, 87, 88). Moreover, the study of an intervention needs to be applied within the same context and under the same conditions for which it was developed (93). In short, context is not an extraneous variable, it is a critical factor. The SENSE Theatre intervention discussed below fosters these principal conditions while providing a supportive context in which there is clear structure and one-to-one peer supervision, with many adults in close contact with the participants.

SENSE THEATRE, A NOVEL, PEER-MEDIATED, PERFORMANCE-BASED PROGRAM DELIVERED IN A COMMUNITY-BASED INTERVENTION

While there is an inherent drive in science, and intervention research in particular, to reveal the key contributing components or mechanisms that lead to change, the treatment of such complex skills will naturally require several components. Nevertheless, we argue that a few important components are critical for the acquisition, maintenance, and generalization of sociability in ASD. As previously described, peer mediation, active practice, and training conducted in a supportive, natural context can significantly impact the acquisition of important reciprocal social interaction skills. Here, we highlight the importance of these techniques that are integral to a novel program called SENSE Theatre that includes these important elements as well as a unique form of treatment delivery, the use of theatre, and acting techniques.

Acting is an interactive process that involves many aspects of socializing, namely, observing, perceiving, interpreting, and expressing thoughts and ideas. For example, an actor must pay attention to the other actors, listen and react to their cues, and express the thoughts and feelings of their characters. It also encompasses other important elements known to be problematic in individuals with ASD, theory of mind and empathy (18, 19). An actor must learn to take on the perspective of another character, which includes their beliefs and feelings. The process can lead to enhanced awareness and increased understanding of the experiences of other people. Thus, in the process, children with ASD may gain greater insight and ability to attribute mental states and feelings onto oneself and others. In fact the inclusion of various theatrical approaches into earlier intervention programs for individuals with typical development and ASD have shown improvement in relevant areas of social functioning, such as empathy and perspective taking (28, 94–98). In the SENSE Theatre program, a variety of acting techniques are employed to include role-play, scripts, and improvisation that provide an opportunity for the participant with ASD to explore and practice social interaction skills in a safe and supportive environment (28, 29). Participants engage in peer-mediated theatre games, such as mirroring, which involve imitating a partner's actions, thoughts, or feelings. Acting provides the opportunity for the children with ASD to interact with peers and indirectly practice social skills. For example, role-play exercises require verbal and non-verbal turn taking, which can facilitate reciprocal communication and interaction skills. Improvisation is also an active and dynamic process in which scenarios are presented to perform without preparation, which engages the child's imagination and allows more flexible thought and behavior.

PEERS AS AN EXPERT MODEL

The SENSE Theatre intervention utilizes highly interactive peers to facilitate social interaction in children with ASD. While there are different types of models that can be employed in treatment studies, the utilization of an expert model can provide direct instruction from persons that characterize optimal functioning. For example, integrated playgroup models have been used in which *expert players* help instruct the *novice player*s on particular skills (99, 100). In the current theatre intervention, each participant with ASD is paired with a typically developing child with acting skills who, in addition to being a co-actor in a play, serves as the participant's peer model. In the SENSE approach, youth actors are conceptualized as "experts" of reciprocal social interaction skills that include verbal and non-verbal communication, socioemotional perception, and expression, as well as behavioral and affective control. It has been shown that learning from an expert with instructional cues can enhance learning (101). Learning by watching and interacting with the expert models in a to-and-fro social exchange develops the skills in an efficient and precise manner.

In fact, the peers are selected, in part, based on their exceptional skills in key areas of social functioning, which are often found in actors (e.g., empathy, social communication) (102). While testing of peers for inclusion is not required, prior to training, we recently identified some of the underlying characteristics of many of the peers. Based on self-report measures, the majority of peers score highly on empathy (103) and self-efficacy (104). It is likely that these key attributes are fundamental to peer mediation such that strong empathy skills allow the peers to be concerned about the welfare of the child with ASD and that high confidence allows them to serve as an expert model. Although this is currently conjecture, efforts are underway to examine these hypotheses.

Prior to the start of the intervention, peer actors receive comprehensive training in key topics including characteristics of ASD, a variety of established behavioral intervention techniques, and the SENSE Theatre manualized approach. When peer training is included in an intervention, it is important to examine the integrity of the training by testing the acquisition of knowledge [e.g., Ref. (63)]. In the SENSE Theatre program, this is conducted immediately before and after peer training.

Moreover, to ensure that training is implemented appropriately especially when working with peers or students, delivery fidelity is recommended (105, 106). To assess fidelity of implementation of the behavioral techniques and core objectives of the program, raters behaviorally code the peers during semi-structured activities at three time points in the course of the program. For SENSE Theatre, to achieve fidelity, the peer must obtain a minimum

performance of skill implantation (e.g., 80%) on each of the techniques and objectives observed. Research personnel supervise the application of the technique using objective and reliable coding throughout the program. If individual peers have difficulty implementing the techniques or maintaining a high level of skill, then booster sessions and individualized feedback are employed.

PERFORMANCE

Through *in vivo* and video modeling with expert peer models, children with ASD are given the opportunity to practice and perform the skills necessary for acquisition. One of the goals of repeated performance of newly learned skills is to reach the status of automaticity (107) in which domain-specific skills became a natural part of the child's repertoire. Moreover, as part of the SENSE Theatre program, the children perform in a multi-context manner, specifically, in the theatre with their peers and practice at home via video modeling (29), and also perform their skills and roles for the public. Thus, the program is performance-based, enhancing the ability of children to exercise their new and developing social skills for each other, as well as for the public in the form of a play.

CONTEXT

The foundational work of Vygotsky has shown that the context in which learning occurs is critical (83). As part of his theory of the zone of proximal development, a child benefits from training that bridges their actual developmental level with his or her potential developmental level. In SENSE Theatre, one of the core principals is to create an environment that provides the opportunity to advance the child's learning while supporting the child's current ability. As noted above, motivation is an important part of active learning, which is influenced by the learning context (84). Social interaction with peers can be challenging and stressful for children with ASD; however, they can be motivated by the inclusion of peers, modeling of positive social engagement, and playful group activities. SENSE Theatre, with the inclusion of supportive theatre games and peers, is naturally stimulating thereby contributing to enhanced motivation and participation (85).

The SENSE Theatre program has been conducted in natural, community-based environments that are supportive and less restrictive, which likely contributes to greater maintenance and generalization of learned skills (46, 87–90). Training is implemented in a hierarchical manner via supervised large group, small groups, and one-on-one peer-mediated teaching allowing individualized support, as needed (92). Additionally, snack breaks are provided fostering less formal engagement and *in vivo* modeling. Thus, the delivery of treatment in a context outside of the child's school; yet, in the community or school theatre merges the feel of a natural setting with the oversight of a clinical setting (91). Video modeling approaches allow participants to view and practice daily with videotaped peers from home via a password protected website to extend the learning practice (29). Finally, the program includes multiple trained peers that engage directly or indirectly with the participants; as such, the variability and diversity of modeled behavior is more representative of natural social interactions (108).

Thus, acting and the theatrical techniques used in the SENSE Theatre program help to teach social skills through the use of expert peers models who provide multiple opportunities for socialization in real and imagined supportive contexts. Children with ASD require clear and repetitive exemplars, which are delivered in the role-plays and exercises. Simultaneously, the use of improvisation and theatre games stimulates imagination and fosters more flexible thinking and behavior. Moreover, the positive reinforcement received from the peers can enhance social motivation to engage with others. Since ASD has been conceptualized as a disorder of social motivation (3, 4), treatments that show the benefits of socialization set the stage for targeting a core deficit. Acting is interactive, a dynamic process that can fundamentally enhance the attention to, practice of, and motivation to engage in reciprocal social interaction.

RESEARCH FINDINGS

The SENSE Theatre intervention has been offered to dozens of children with ASD and formally examined in two published studies that included a pre-test, post-test design in which measures were assessed immediately before and following the intervention (28, 29). Briefly, the first study was conducted in a community theatre model including approximately 40 intervention hours delivered over a 2 1/2-month duration. Practices were held approximately one to two nights per week including several days of rehearsal the week before the public performance. Participants included eight children with ASD (seven boys and one girl) ranging in age from 7 to 18 years that were paired with typically developing peers. Dependent measures included neuropsychological, biological (cortisol and oxytocin), and behavioral scales to assess social perception, hormone levels, and adaptive behaviors. Primary dependent neuropsychological measures included subtests from the NEPSY (109) to measure memory for faces, affect recognition, and theory of mind, all hypothesized to show skill improvement following the intervention. Cortisol, a measure of stress responsivity, was hypothesized to show initial rise followed by gradual decline as the participants acclimated to the social intervention. The participants with ASD showed moderate improvement in face identification and theory of mind skills following the intervention. Additionally, they demonstrated a reduction in cortisol levels over time (28). The findings suggested changes in social perception and adaptation to the social environment.

Recently, we reported findings on the implementation of a summer camp model that included a comparable dose of 10 sessions. However, it was conducted over a shorter duration and more concentrated dose intensity. Specifically, the treatment was delivered over 2 weeks for 3 1/2 h per day in a summer camp model. Sixteen youth with ASD between 8 and 17 years completed the treatment and 12 participated in the research. A multilevel approach was again employed in which examination of neuropsychological, biological, and behavioral variables was measured. Significant differences in social perception on the NEPSY (109) (memory for faces), social function on the Social Responsiveness Scale (SRS), (110) (awareness, cognition), and adaptive skills on the Adaptive Behavior Assessment System (ABAS (111)) (home living, self-care) were observed. Stress reduction as measured by salivary cortisol was also observed (29).

Since this initial model was implemented, subsequent camp interventions have been delivered using a pre-test, post-test

design. In a combined cohort of participants ($N = 20$) from the 2012 and 2013 SENSE Theatre summer camps, several findings were replicated using paired samples t-tests. Specifically, social perception in the form of memory for faces immediate [$t(18) = -2.612$, $p = 0.018$] and memory for faces delayed [$t(18) = -4.194$, $p = 0.001$] showed significant change upon post testing. In regard to generalized social functioning based on parental report using the SRS (110), improvement was reported on total social responsiveness [$t(19) = 2.665$, $p = 0.015$], and social cognition [$t(19) = 3.523$, $p = 0.002$] in particular. These results further support the findings that the SENSE Theatre intervention produces improvement in core areas of functioning for many children with ASD, namely, gains in social cognition, memory, and behavior. Moreover, in the most recent data, increases in adaptive skills were reported for functional academics [$t(18) = -2.617$, $p = 0.017$] and self-direction [$t(18) = -2.179$, $p = 0.043$] suggesting that the treatment has positive impact beyond the targeted social communication skills.

Improvements in social functioning have likewise been reported in other peer-mediated social skills interventions previously mentioned (51, 57, 58). In addition, the randomized social skills program given by Solomon and colleagues (33), which targeted emotional awareness, theory of mind, and problem solving, led to improvement in social perception. Also, another program that used theatre techniques to improve social skills (96) similarly found increases in social perception, social assertiveness, and reduction in social problems.

The observed gains in children with ASD in social perception, social functioning, and adaptive skills, which are important building blocks of reciprocal social interaction, provide support for the utilization of theatre-based approaches. While randomized experimental studies are warranted, these preliminary studies collectively provide strong support for the SENSE Theatre model (28, 29).

DISCUSSION

The hallmark characteristic of ASD is poor reciprocal social communication and a variety of interventions have been employed to improve functioning in these critical skills. Promising approaches, such as peer mediation have contributed to improvements in various specific social skills in children with ASD. The inclusion of typically developing peers in social interaction programs can improve social development and engagement in children with ASD (48–50). Peers can be trained to elicit target behaviors using behavioral approaches such as modeling, reinforcement, and scripting that can enhance generalization (53, 55, 56). In addition to working *in vivo*, video modeling has been shown to be a promising approach to teach a variety of skills to children with ASD (30, 31, 75, 76, 80, 112, 113).

The aforementioned efficacious behavioral approaches have been combined with performance-based theatrical techniques in SENSE Theatre to address barriers to behavioral change and explicitly advance the learning and application of social functioning in youth with ASD. While theatrical techniques such as role playing, improvisation, and character development are rarely used in the treatment of ASD, they have been shown to target core deficits in reciprocal social communication (28, 29, 96, 114). Acting is explicitly active, requiring the performance of thoughts, ideas, and actions thereby providing the child with ASD the opportunity to practice social skills in a supportive albeit dynamic setting. As in acting, interacting with others relies on the ability to recognize, identify, integrate, plan, and respond appropriately to dynamic socioemotional information; therefore, utilizing theatre techniques provides the opportunity to learn and practice these social skills. The trained typically developing peer actors are the primary agents of change and in the process serve as both teacher and recipient of reciprocal social exchange. The program is also enhanced by treatment delivery in a community setting, demonstrating that promising interventions can be delivered in natural contexts while maintaining the rigor of a clinic setting.

The SENSE Theatre model suggests that the inclusion of these approaches in combination with the core theatre methods enhances the training, social experience, and motivation of the participants to engage with peers (28, 29). Combining treatments that have been independently effective has been shown to demonstrate even greater gains when delivered in combination (115). While previous efforts lend preliminary support for SENSE Theatre for improving social interaction skills in ASD, we are conducting a randomized, waitlist control group study with a large sample of participants enrolled in a 10-week model of the SENSE Theatre treatment. Additionally, a manual is in final development, which will allow the treatment to be delivered systematically by other interventionists in various settings to increase reliability and determine efficacy. Some may insist on trying to discover the turnkey ingredient that contributes to the promising results for this or any other treatment. For a complex set of behaviors, such as reciprocal social skills, it is highly unlikely that a single basic factor will be identified to target or explain the benefits when remediated. Indeed, the aforementioned theatrical treatment framework provides evidence that synthesizing peer mediation, active learning, and a supportive, natural context can promote the advancement, maintenance, and generalization of skills for youth with ASD.

CONCLUDING REMARKS

Interventions aimed at improving core impairment in reciprocal social communication in children with ASD are critically needed. Evidence is presented highlighting important components for advancing, maintaining, and generalizing social competence, which include peer mediation, active learning, and the implementation of interventions in supportive, natural contexts. Moreover, acting specifically targets the core ASD impairment in reciprocal social communication and rigid, inflexible thought and behavior by having the child actively engage in activities such as role-playing and improvisation to develop these skills. SENSE Theatre, a promising treatment for youth with ASD, serves as a model for utilizing these elements by combining trained peers that facilitate the performance-based theatrical treatment delivered in a supportive, community-based environment.

AUTHOR CONTRIBUTIONS

Blythe A. Corbett conceptualized the paper, developed the SENSE Theatre program, wrote the initial draft of the paper, and revised the final manuscript. Lydia R. Qualls researched peer mediation and active practice, assisted with writing the first full draft, and revised and copyedited the final version of the manuscript.

Blythe Valencia researched the importance of context and natural environments in learning, as well as the section on peers as expert models, and helped draft those sections of the manuscript. Stéphanie-M. Fecteau contributed background information regarding autism spectrum disorders and peer mediation, and helped with the revision of the manuscript. Deanna M. Swain contributed to early ideas regarding peer mediation and video modeling strategies for ASD and contributed to the original draft of the paper. All authors have read the final work and agree to be accountable for its accuracy and integrity.

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GABAergic signaling as therapeutic target for autism spectrum disorders

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γ-Aminobutyric acid (GABA), the main inhibitory neurotransmitter in the adult brain, early in postnatal life exerts a depolarizing and excitatory action. This depends on accumulation of chloride inside the cell via the cation–chloride importer NKCC1, being the expression of the chloride exporter KCC2 very low at birth. The developmentally regulated expression of KCC2 results in extrusion of chloride with age and a shift of GABA from the depolarizing to the hyperpolarizing direction. The depolarizing action of GABA leads to intracellular calcium rise through voltage-dependent calcium channels and/or *N*-methyl-D-aspartate receptors. GABA-mediated calcium signals regulate a variety of developmental processes from cell proliferation migration, differentiation, synapse maturation, and neuronal wiring. Therefore, it is not surprising that some forms of neuro-developmental disorders such as autism spectrum disorders (ASDs) are associated with alterations of GABAergic signaling and impairment of the excitatory/inhibitory balance in selective neuronal circuits. In this review, we will discuss how changes of $GABA_A$-mediated neurotransmission affect several forms of ASDs including the Fragile X, the Angelman, and Rett syndromes. Then, we will describe various animal models of ASDs with GABAergic dysfunctions, highlighting their behavioral deficits and the possibility to rescue them by targeting selective components of the GABAergic synapse. In particular, we will discuss how in some cases, reverting the polarity of GABA responses from the depolarizing to the hyperpolarizing direction with the diuretic bumetanide, a selective blocker of NKCC1, may have beneficial effects on ASDs, thus opening new therapeutic perspectives for the treatment of these devastating disorders.

Keywords: autism spectrum disorders, GABA receptors, bumetanide, neuro-developmental disorders, excitatory inhibitory balance

Autism comprises a heterogeneous group of neuro-developmental disorders known as autism spectrum disorders (ASDs) characterized by deficits in verbal and non-verbal communication, social interaction, restricted interests, and stereotyped behavior (1). The incidence of ASDs (20–60/10000 children) has dramatically increased over the past decades because of the improvement of diagnostic criteria and increased attention of medical community (2). Complications arising from later age pregnancies and from excessive exposure of fetuses with a genetic vulnerable background to environmental factors (i.e., toxic agents) may also contribute to the higher incidence of these disorders in recent years.

In spite different etiologies, ASDs share overlapping symptoms, indicating common deficits in some neuro-developmental pathways. One of these involves the γ-aminobutyric acid $(GABA)_A$-mediated neurotransmission, known to play a crucial role in synaptic tuning and neuronal wiring in late pre and early postnatal days (3). Studies from animal models of ASDs indicate that a dysfunction in GABAergic signaling within particular neuronal circuits may account for most of the clinical symptoms found in autistic patients. The high co-morbidity of ASDs with epilepsy (30% of cases) further confirms this issue (4).

γ-Aminobutyric acid is the main inhibitory neurotransmitter in the adult mammalian brain. It inhibits neuronal firing by activating two different classes of receptors, $GABA_A$ and $GABA_B$. $GABA_A$ receptors are integral ion channels while $GABA_B$ receptors are coupled to ion channels via guanine nucleotide-binding proteins and second messengers. The opening of $GABA_A$ receptors causes a net influx of chloride with consequent membrane hyperpolarization and reduction of cell firing. However, in particular conditions and during brain maturation the intracellular chloride concentration $[Cl^-]_i$ rises in such a way that the opening of anion channels by GABA produces a chloride efflux and a membrane depolarization that through the activation of a persistent non-inactivating sodium conductance (5) may reach the threshold for action potential generation (6, 7). Generally, low $[Cl^-]_i$ facilitates GABA-mediated inhibition, whereas high $[Cl^-]_i$ facilitates GABA-mediated excitation. The mechanisms underlying chloride accumulation inside immature neurons, start to be unveiled with a different efficacy of chloride co-transporters such as NKCC1 and KCC2, which import and export chloride, respectively. Before and immediately after birth, chloride accumulates inside the cell due to a reduced expression of the cation–chloride exporter KCC2. Later in development, the intracellular chloride concentration decreases thanks to the up-regulation of KCC2 (8–10). GABA-induced membrane depolarization facilitates calcium entry via voltage-dependent calcium channels and *N*-methyl-D-aspartate

(NMDA) receptors. Calcium rise leads to the activation of second messengers involved in a variety of developmental processes from cell migration and differentiation to synaptogenesis and circuit formation (11).

How GABA orchestrates these processes has been extensively reported (3, 12–14). GABAergic signals operate with multiple modalities at different developmental stages before glutamatergic ones (15). At the beginning, GABA works as a trophic factor, modulating neuronal migration and maturation (16). GABA receptors are expressed in neuronal progenitors before the establishment of synaptic contacts (17). At this stage, the receptors work as sensors for GABA present in the extracellular space after its release in a calcium- and SNARE-independent way from growth cones and astrocytes (18). The absence of an efficient uptake system enables this neurotransmitter to accumulate in the extracellular space and to reach a concentration sufficient to exert its depolarizing and excitatory effects on distal neurons. Blocking the depolarizing action *in utero* heavily affects migration and circuit formation (11, 19, 20).

At later developmental stages, when synapses are formed, the release of GABA and glutamate, generate a primitive form of network-driven oscillatory events known as giant depolarizing potentials (GDPs). GDPs are characterized by recurrent membrane depolarizations (lasting several hundred of milliseconds) that give rise to bursts of action potentials, separated by quiescent periods. This network activity thought to be the *in vitro* counterpart of "sharp waves" recorded in pups during immobility periods, sleep, and feeding (21), is reminiscent of the "trace discontinue" first described by Dreyfus-Brisac in the electroencephalogram of immature babies and characterized by intermittent bursts separated by periods of virtually complete suppression of activity (22).

In analogy with the synchronized activity generated in the disinhibited hippocampus by $GABA_A$ receptor antagonists (23), GDPs emerge when a sufficient number of cells fire and the excitability of the network attains a certain threshold within a restricted temporal window (24). Although the entire hippocampus possesses the capacity to generate GDPs, for its extensive glutamatergic connections via recurrent collaterals, the CA3 area is particularly well equipped to generate synchronized activity. Furthermore, this area is able to initiate, upon membrane depolarization, intrinsic bursts which, by virtue of their spontaneous discharges and large spike output can drive other neurons to fire (25, 26). Burst firing is facilitated by a persistent slow sodium current (27) and by a tonic $GABA_A$-mediated conductance generated by the activation of extrasynaptic $GABA_A$ receptors by "ambient" GABA whose depolarizing action would bring the membrane to the voltage window for activation of voltage-dependent sodium and calcium channels (28). Intrinsic bursting activity is boosted by the low expression of Kv7.2 and Kv7.3 channels responsible for the non-inactivating, low-threshold M current (I_M), which in adulthood controls spike after-depolarization and burst generation (29). The low density of I_M at birth contributes to produce intrinsic bursts that, in comparison with those observed in adults, are more robust, last longer and recur more regularly (25). GDPs-associated calcium transients act as coincident detector signals for enhancing synaptic efficacy at emerging GABAergic (30) and glutamatergic synapses (31). Therefore, this early synchronized activity is fundamental for synaptic wiring and refinement of local neuronal circuits according to the Hebbian rule that "neurons that fire together wire together."

γ-Aminobutyric acid is released from GABAergic interneurons that constitute a very heterogeneous group of cells, differentially classified according to their morphology, biophysical properties, molecular expression profile, and connectivity (32). These cells, mainly derived from the medial and caudal ganglionic eminences, undergo their final mitosis in these regions prior to their tangential migration into the cortical plate. The migration process, supposed to be calcium dependent, is regulated by a sequence of well-orchestrated processes involving guidance cues, neurotransmitter receptors (NMDA, $GABA_A$ receptors) and voltage-dependent calcium channels (33, 34).

GABAergic interneurons not only exert a powerful control on network excitability but, in spite of their relatively low number (10–15% of the entire neuronal population), are able to synchronize a large number of principal cells giving rise to coherent oscillations, which support different behavioral states of the animals and high cognitive tasks (35).

Altogether, these observations point to GABA as one of the major players in the early assembly and formation of neuronal circuits in the developing brain. Therefore, it is not surprising that dysfunctions of GABAergic circuits have been implicated in various neuro-developmental and psychiatric disorders such as schizophrenia, autism, and epilepsy.

GABAergic DYSFUNCTIONS IN THE BRAIN OF ASD PATIENTS

The high frequency of epileptiform activity and the altered brain rhythms detected in the EEG of ASDs patients suggest a dysfunction of GABAergic transmission and an imbalance between excitation and inhibition (E/I) in local circuits involved in sensory, mnemonic, social, and emotional processes. However, as summarized in **Table 1**, more direct evidence in favor of a GABAergic dysfunction in ASDs derives from:

a. Genetic observations
b. *In vitro* analysis of post-mortem brain tissues
c. *In vivo* studies on patients affected mainly by idiopathic forms of ASDs.

It is clear from the **Table 1** that most of cases are from juvenile and adult patients. This can be attributed to difficulties in obtained post-mortem material from young children and to perform complex *in vivo* examinations such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) in children of pediatric age. However, we cannot exclude that the same alterations are already present at early stages of development.

GENETIC OBSERVATIONS

The involvement of $GABA_A$ receptors in ASDs was provided by genetic studies that have revealed submicroscopic abnormalities known as "copy-number variations" in chromosomal loci 15q11–q13, which contains a number of genes encoding for $GABA_A$ receptor subunits (54). These loci can be affected either directly by

Table 1 | Alterations of GABAergic signaling in patients with idiopathic forms of autism and Rett syndrome.

Clinical phenotype	Alterations of GABAergic signaling	Age (years)	Reference
GENETIC OBSERVATIONS			
Idiopathic autism	Linkage disequilibrium in *GABRB3*	7.6 ± 6.2	(36, 37)
Idiopathic autism	Altered gene expression in interneurons	Not specified	(38)
***IN VITRO* ANALYSIS OF POST-MORTEM BRAIN TISSUES**			
Idiopathic autism	Reduction in the density of $GABA_A$, $GABA_B$ receptors and benzodiazepine binding sites in the anterior cingulate cortex	19/43	(39)
Idiopathic autism	Reduction in the density of $GABA_B$ receptors cingulate cortex and fusiform gyrus	19/43	(40)
Idiopathic autism	Reduction in 3[H]flunitrazepam labeled benzodiazepines binding sites in the hippocampus	16/22	(41)
Idiopathic autism	Decreased number of GABAergic Purkinje cells in cerebellum	13/54	(42)
Idiopathic autism	Reduced level of GAD65 and GAD67 in Purkinje cells of cerebellar and parietal cortices	19/30	(43)
Idiopathic autism	Decreased GAD67 mRNA levels in cerebellar Purkinje cells	16/30	(44)
Idiopathic autism	Decreased GAD65 mRNA levels in cerebellar dentate nuclei	16/30	(45)
Idiopathic autism	Increased expression of GABAergic interneurons expressing calcium-binding proteins in the hippocampus	13/63	(46)
Rett syndrome	Disruption in the inhibitory architecture of the cell mini-columns	14.4 ± 4	(47)
***IN VIVO* STUDIES**			
Idiopathic autism	Reduction of GABA concentration in the frontal lobe	2/12	(48)
Idiopathic autism	Reduced GABA levels in the perisylvian region of the left hemisphere	12.4 ± 5.2	(49)
Idiopathic autism	Reduced expression of $GABA_A$ receptors in the superior and medial frontal cortex	7.3 ± 3.5	(50)
Rett syndrome	Reduced $GABA_A$ receptor density in fronto-temporal cortex	27/41	(51)
Idiopathic autism	Significant reduction of α5 $GABA_A$ receptor subunits in limbic areas	34/43	(52)
Rett syndrome	Reduced KCC2/NKCC1 ratio in the cerebrospinal fluid	0/19	(53)

Numbers refer to mean (usually ± SEM) or range values.

single point mutations or indirectly by epigenetic factors. Potential gene targets include *GABRB3*, *GABRA5*, and *GABRG3*, encoding for β3, α5, and γ3 subunits containing $GABA_A$ receptors, respectively (36, 37). Systematic changes in $GABA_A$ receptor subunit expression were found in the superior frontal cortex, parietal cortex, and cerebellum of autistic subjects. In addition, autism-related genes have been found to be expressed mainly in GABAergic interneurons (38).

IN VITRO ANALYSIS OF POST-MORTEM BRAIN TISSUES

Post-mortem analysis on brain tissues from ASD patients as well as genetic and *in vivo* studies have largely contributed to unveil the impact of GABAergic signaling in these disorders. Thus, as compared to controls, a significant reduction in the density of $GABA_A$, $GABA_B$ receptors, and benzodiazepine binding sites was detected in the supra and infragranular layers of the anterior cingulate cortex (known to participate in a variety of processes including socio-emotional behavior and other associative functions via prefrontal cortex connectivity) in brain samples from autistic subjects (39, 40). A reduction of ^{3}[H]muscimol labeled $GABA_A$ receptors and 3[H]flunitrazepam labeled benzodiazepines binding sites was found also in the hippocampus (41, 55). Neuropathological studies from the cerebellum of individuals with ASDs have demonstrated that GABAergic Purkinje cells are particularly vulnerable since their number appears considerably reduced respect to controls (42, 56, 57). This effect was found to be associated with reduced levels of mRNA encoding for glutamic acid decarboxylase (GAD) 65 and 67, rate limiting enzymes responsible for the conversion of glutamate to GABA (43–45, 58). In contrast with Purkinje cells, enhanced levels of mRNA for GAD67 were found in stellate cells, a subtype of interneuron innervating Purkinje cells (43). Interestingly, pathological studies of brains of individual affected by autism demonstrated an increased expression of GABAergic interneurons expressing calcium-binding proteins such as calbindin-, calretinin-, and parvalbumin in the hippocampal formation (46). Since these interneurons are capable of buffering calcium, the intracellular messenger that controls several transduction pathways, an alteration of calcium signaling may have dramatic consequences on neuronal functions and dynamics.

Abnormalities in micro-columnar organization of prefrontal cortex in brain tissues from autistic individuals, including two with Angelman syndrome, have been also detected (59, 60). The narrower size of mini-columns in autistic patients respect to controls may reflect defects in GABAergic fibers within and

between cortical mini-columns due to reductions in the neuropil, which separates adjacent mini-columns (47). This may alter local connectivity and lateral inhibition.

IN VIVO STUDIES ON PATIENTS AFFECTED MAINLY BY IDIOPATHIC FORMS OF ASDs

In vivo studies from ASD patients are rather limited due to the difficulty of measuring GABAergic function *in vivo*. Using proton magnetic resonance spectroscopy, Harada et al. (48) reported a reduction of GABA concentration in the frontal lobe of children with ASDs respect to controls. However, in this study ASD patients were sedated with triclofos a compound that, by potentiating GABA action, may have biased the results. Using the same technique, Rojas et al. (49) have reported reduced GABA levels in the perisylvian region of the left hemisphere of autistic patients, further supporting the involvement of GABAergic neurotransmission in ASDs. Using SPECT and ^{123}I-iomazenil, a selective $GABA_A$-benzodiazepine ligand, a reduced expression of $GABA_A$ receptors in the superior and medial frontal cortex of autistic patients was detected (50). Using the same technique, a reduced $GABA_A$ receptor density was observed in three adult females affected by the Rett syndrome (51). In addition, a pilot study using PET and [^{11}C]Ro15-4513 to measure the expression levels of α5 $GABA_A$ receptor subunits (localized mainly to extrasynaptic regions where they mediate tonic inhibition), has demonstrated a significant reduction of these subunits in limbic areas of ASD patients respect to controls (52).

Indirect evidence for deficits of GABAergic transmission in ASDs was provided by a recent magneto-encephalographic study that revealed impaired gamma-band activity in selective brain areas of adults with autism during perceptual visual processing (61). Gamma-band oscillations known to critically depend on negative feedback inhibition of principal cells by GABAergic interneurons, including parvalbumin-positive ones (62), are crucial for integrating multisensory information into a coherent representation (63). Although no data from young ASD patients are available, the possibility that such impairment may result from aberrant pre and perinatal development cannot be excluded.

Although informative, these studies referred mainly to idiopathic forms of ASDs and failed to address whether particular types of autistic syndromes, including monogenic ones were similarly affected. In this perspective, it is worth noting that a significant reduction of the cation–chloride importer KCC2 in the cerebrospinal fluid of Rett syndrome patients as compared to controls, with a consequent reduction in the KCC2/NKCC1 ratio was found (53). In accord with the notion that the methyl-CpG-binding protein 2 (MeCP2), encoded by the X-linked *Mecp2* gene, is highly expressed in GABAergic interneurons where it regulates their function these data suggest that a GABA dysfunction underlies the pathophysiology of the Rett syndrome.

Altogether, these findings indicate that alterations of GABAergic signaling in selective microcircuits of well-defined brain areas exert a fundamental role in the pathogenesis of ASDs.

GABAergic DYSFUNCTIONS IN ANIMAL MODELS OF ASDs

Over the last decade several animal models of ASDs have been generated, in order to identify the molecular and cellular mechanisms underlying these disorders and to develop new therapeutic tools. To this aim, genetic defects detected in patients with ASDs have been introduced in the genome of mice. Animal models have been produced not only by manipulating candidate genes but also environmental factors or drugs such as valproic acid (VPA), an antiepileptic known to be a risk factor for autism in offspring of mothers treated during pregnancy with this drug (64). In accordance with human observations, animal studies have revealed important dysfunctions in GABAergic signaling occurring at different locations of the GABAergic synapse (**Figure 1**).

Table 2 summarizes alterations of GABAergic transmission observed in the most commonly used animal models of ASDs.

The *Fragile X* syndrome is a monogenetic disorder caused by mutations of the *FMR1* gene, located in chromosome X (Xq27.3) and encoding for the *Fragile X* mental retardation protein (FMRP), which is involved in the translation of a variety of mRNAs. Gene mutations do not allow the expression of the FMRP protein, determining a phenotype characterized by intellectual disabilities associated with language deficits, hyperactivity, autistic behavior, and seizures (85). Mice lacking the *FMR1* gene (Fmr1 KO mice) show an increased network excitability associated with an E/I imbalance in cortical circuits involving specific types of interneurons (65, 67, 86, 87). Thus, in the somatosensory cortex, the reduced activity of parvalbumin-positive cells (66) may contribute to enhance cell excitability and to affect gamma frequency oscillations, thought to be involved in high cognitive functions (86). The enhanced coupling of group I metabotropic glutamate receptors (mGluR) signaling and cannabinoid receptors mobilization may further enhance suppression of inhibition (88). Other factors contributing to enhance cell excitability include: (i) a reduced expression of $GABA_A$ receptors subunits in the cortex. This may represent an evolutionary conserved hallmark of the *Fragile X* syndrome since similar reduction has been detected in the *Fragile X* fruit fly model (89). (ii) A down regulation of mRNA encoding for GAD65 and GAD67 in presynaptic terminals of the cortex and cerebellum; (iii) a reduction of mRNA encoding for gephyrin, the scaffold molecule responsible for glycine and $GABA_A$ receptor clustering and stabilization at post-synaptic sites; (iv) reduced phasic and tonic $GABA_A$-mediated conductances in the amygdale associated with a down regulation of $GABA_A$ receptor subunits (65, 68, 89); (v) a down regulation of $GABA_A$-mediated tonic inhibition associated with a reduced expression of α5 and δ $GABA_A$ receptors subunits in subicular neurons (69). In contrast, electrophysiological recordings from the striatum of Fmr1 KO mice have revealed a selective increase in basal inhibitory neurotransmission, caused by the enhanced probability of GABA release (90). Overall these data suggest that in this animal model modifications of the GABAergic function are region-specific. Interestingly, a recent study from the hippocampus of mice carrying the *Fragile X* mutation, has revealed the loss of oxytocin-mediated $GABA_A$-mediated inhibition during the delivery process (70). In rats, oxytocin, the maternal hormone involved in delivery, has been shown to cause an abrupt shift of GABA from the depolarizing to the hyperpolarizing direction, thus exerting a neuroprotective and analgesic action on newborns (91). Unlike wild-type animals, the depolarizing action of GABA, apparently caused by the reduced expression of KCC2 with consequent high $[Cl^-]_i$, persists in juvenile life. The

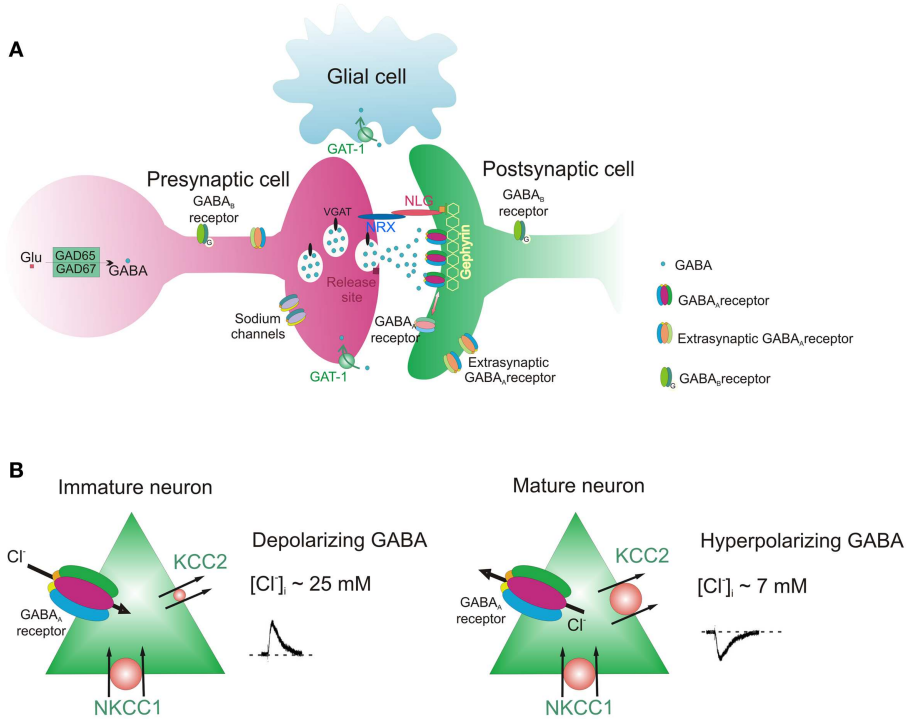

FIGURE 1 | GABAergic synapse. **(A)** Pre and post-synaptic sites of the synapse with different components whose functions can be altered in ASDs. **(B)** Direction of GABA action (depolarizing and hyperpolarizing) in immature and mature neurons.

increased excitatory drive to principal cells boosts network activity in the hippocampus, suggesting that an altered E/I balance may be caused not only by a decreased $GABA_A$-mediated inhibition but also by an enhanced $GABA_A$-mediated excitation (70).

Another neuro-developmental disorder showing high co-morbidity with autism is the *Rett syndrome*. This is caused by mutations in the X-linked *Mecp2* gene that codes for the transcriptional factor MeCP2, highly expressed in GABAergic neurons (92). This disorder affects mainly girls who develop normally until the age of 6/18 months. Later, they develop cognitive deficits, loss of speech, motor abnormalities, respiratory dysrhythmias, stereotyped behavior, and seizures leading sometimes to premature death (93). Mice lacking MeCP2 or engineered to express an allele mimicking some mutation present in the *Rett syndrome* exhibit neurological symptoms reminiscent of those found in patients affected by the *Rett syndrome*. Similarly to Fmr1 KO mice, these animals exhibit alterations of the E/I balance (71), associated with a decrease in somatic GABA content and low levels of mRNA encoding for GAD65 and GAD67 in cortical and striatal neurons (75). While in the thalamus, the *Mecp2* gene appears to differentially regulate the development of GABAergic synapses in excitatory and inhibitory neurons (94), in the brain stem, the E/I imbalance is due to the depression of GABAergic transmission originating at both pre- and post-synaptic levels (72). A reduced GABAergic inhibition is present also in the locus coeruleus (73). In the hippocampus of *Mecp2* KO mice, a significant reduction in the quantal size of miniature inhibitory post-synaptic currents (mIPSCs) has been detected. This would account for the impairment of long-term potentiation (LTP) induced at CA3–CA1 synapses by theta burst stimulation (74).

An E/I imbalance has been found also in individuals with *Tuberous sclerosis*, a genetic multisystem disorder characterized by wide spread hamartomas in several organs, including brain, heart, skin, eyes, kidney, lung, and liver (95). Tuberous sclerosis patients exhibit a variety of neurological disorders including mental retardation, autism-like disorders, and epilepsy. The affected genes are *Tsc1* and *Tsc2* encoding hamartin and tuberin, respectively. The hamartin–tuberin complex inhibits the mammalian target of rapamycin pathway that controls cell growth and proliferation (95). Immunocytochemical and western blots experiments have demonstrated that this disorder is associated with a decrease of α1 $GABA_A$ receptor subunits and reduced and enhanced levels of KCC2 and NKCC1, respectively, in tubers. Changes in the expression of KCC2 and NKCC1 account for the excitatory action of GABA revealed with patch clamp in slices from *Tuberous sclerosis* tubers (96). However, electrophysiological data are still preliminary and should be taken with caution since they refer only to experiments from cortical slices obtained from tubers of a single patient. The *Angelman* or the closely related *Prader–Willi* syndromes (depending from which parent the deletion has been inherited), characterized by mental retardation, autistic behavior, and seizures, are determined by the loss of function of the *Ube3a* gene encoding for the a ubiquitin E3 ligase or *Gabrb3*, *Gabra5*, and *Gabrg3* genes, encoding, respectively for β3, α5, and γ3 $GABA_A$ receptor subunits localized in the same chromosome region 15q11–q13. Ube3a-deficient mice exhibit a reduced tonic

Table 2 | Alterations of GABAergic signaling in animal models of ASDs (E, embryonic day; P, postnatal day).

Animal models	Alterations of GABAergic signaling	Age	Reference
Fmr1 KO *(X Fragile)*	Reduced GABA release in the amygdala	P20–30	(65)
	Reduced number of parvalbumin-positive interneurons	Adult	(66)
	Altered E/I balance	P14–P30	(67)
	Decreased expression of GAD67 and $GABA_A$ receptor subunits	Adult	(68)
	Down regulation of $GABA_A$-mediated tonic inhibition	Adult	(69)
	Persistent depolarizing effect of GABA in juvenile animals	E20–P30	(70)
MECP2 KO (Rett syndrome)	Altered E/I balance	P14–P35	(71)
	Depressed GABAergic synaptic transmission	P7	(72)
		P14–P28	(73)
		Adult	(74)
	Reduced expression of GAD65 and GAD 67	Adult	(75)
Ube3a-deficient mouse (Angelman syndrome)	Reduced $GABA_A$-mediated tonic inhibition	P25–P28 or adult	(76)
GABRB3 KO (Angelman syndrome)	Decreased expression of $GABA_A$ receptors	Adult	(77)
Scn1a$^{+/-}$ mice (Dravet's syndrome)	Altered E/I balance	P21–P30	(78)
$NL3^{R451C}KI$	Increased GABAergic neurotransmission; increased VGAT and gephyrin expression	P13–P16	(79)
	Increased frequency of GDPs	P4–P35	(14)
	Circuit specific changes in GABAergic signaling in the hippocampus and in the cortex	P21–P35	(80)
		P9–P15	(81)
	Decreased number of PV^+ interneurons	P21–P35	(82)
En2 KO	Reduction of GABAergic markers during development; reduced number of GABAergic interneurons	Adult	(83)
VPA model	Decreased $GABA_A$-mediated neurotransmission	P23–P45	(84)
	Persistent depolarizing effect of GABA in juvenile animals	E20–P30	(70)

GABA-mediated inhibition in the cerebellum, caused by a GAT-1 dependent decrease of GABA concentration in the extracellular space (76). Moreover, β3-deficient mice, which have a phenotype similar to some forms of *Angelman* syndrome, exhibit a reduced expression of $GABA_A$ receptors in selective brain regions as determined by quantitative autoradiography (77).

The *Dravet's* syndrome is caused by a haploinsufficiency of the *SCN1A* gene encoding for voltage-gated sodium channel $Na_V1.1$. Children affected by this disorder show intractable seizures, cognitive deficit, and autism spectrum behaviors. GABA plays a pivotal role also in this disorder as demonstrated by the reduced activity of $Na_V1.1$ channels in forebrain GABAergic interneurons of *Scn1a*$^{+/-}$ mice. These animals exhibited an E/I imbalance resulting from a decreased frequency of spontaneous inhibitory post-synaptic currents and an increased frequency of spontaneous excitatory post-synaptic currents in the hippocampus and prefrontal cortex. The impairment of GABAergic neurotransmission is associated with behavioral and cognitive deficits similar to those found in patients affected by the *Dravet's* syndrome (78).

Although rare, single mutations of genes encoding for adhesion molecules of the Neuroligin (NL) family found in individuals with autism have been introduced in mice. One of these, the R451C mutation of the *Nlgn3* gene encoding for NL3, has been found in a family with children affected by ASDs (97). NLs are adhesion post-synaptic proteins that, by binding to their presynaptic partners, neurexins, functionally couple the post-synaptic densities with the transmitter release machinery, thus contributing to synapses stabilization (98). Mice carrying the NL3 R451C mutation ($NL3^{R451C}$ knock-in mice) show modifications of GABAergic signaling associated with behavioral deficits reminiscent of those found in autistic children (79, 99). These mice exhibit an increased frequency of mIPSCs in the somatosensory cortex (79) and in the CA3 region of the hippocampus (100) where they contribute to boost GDPs activity. A more detailed analysis of GABAergic microcircuits in the hippocampus has unveiled an increased GABA release at synapses between cholecystokinin (CCK)-positive endocannabinoids-sensitive interneurons and pyramidal cells and a decreased GABA release at synapses between parvalbumin-positive basket cells and principal cells (80). The similar phenotype found at CCK–pyramidal cell synapses in $NL3^{R451C}$ knock in mice and in NL3 KO mice suggests a loss of function consisting in the loss of tonic endocannabinoid signaling at these connections. It is worth noting that $NL3^{R451C}$ knock-in mice present an asymmetric reduction of parvalbumin-positive basket cells across the two hemispheres (82).

Similarly to Földy et al. (80), a reduced probability of GABA release has been found in parvalbumin-positive basket cell–spiny

neuron synapses in layer IV somatosensory barrel cortex of juvenile $NL3^{R451C}$ knock in mice. Such deficit determines an alteration of the E/I balance in this cortical layer together with a modification of the temporal window for integration of sensory inputs in principal cells (81). The altered sensory representations may underline deficits in coherent percepts of autistic children.

Mutations in the *En2* gene, coding for the homeobox-containing transcription factor engrailed-2 (EN2), involved in patterning and neuronal differentiation of the midbrain/hindbrain region, have been associated with ASDs. *En2* KO mice have been proposed as a model for ASDs due to their behavioral abnormalities similar to those observed in individuals with ASDs (101). The *En2* gene is also involved in the development or maintenance of GABAergic interneurons as demonstrated by the selective loss of parvalbumin, somatostatin, and neuropeptide Y positive interneurons in the cortex and hippocampus of *En2* KO mice (83). This effect appears to be region-specific since different subpopulations of interneurons are affected in posterior brain areas (102). Deficits in GABAergic neurotransmission account for the higher susceptibility to seizures of En2 KO mice respect to controls (103).

Valproic acid is a histone deacetylase inhibitor, widely used to cure epilepsy and bipolar disorders. VPA is also a potent teratogen since children exposed *in utero* to this drug have a much higher risk of developing an autistic-type behavior than normal children (64). Pups exposed to VPA *in utero* show neuro-developmental abnormalities and behavioral deficits similar to ASDs (104). As in other animal models of ASDs, the autistic phenotype is associated to an altered E/I balance due to a decreased GABAergic signaling, which affects both pre- and post-synaptic sites, leaving the extrasynaptic transmission unaffected (84). Interestingly, like the NL3 model, the VPA model exhibits an asymmetric reduction of parvalbumin-positive cells across the two hemispheres (82). Similarly to *FRM1* KO mice, also in this animal model a loss of oxytocin-mediated $GABA_A$-mediated inhibition occurs during the transition from fetal to postnatal life (70). Also here as in Fmr1 KO mice, the depolarizing action of GABA in the hippocampus persists in juvenile life. The excitatory GABAergic drive to principal cells leads to an increased network activity in a broad spectrum of frequencies including gamma oscillations.

The different animal models mentioned here have in common dysfunctions of GABAergic signaling leading to alterations of the E/I balance in selective brain circuits. These alterations can be rescued by selective tools that regulate $GABA_A$-mediated synaptic transmission.

THERAPEUTIC INTERVENTIONS TO RESCUE GABAergic DYSFUNCTIONS IN ASDs

Figure 1 shows a GABAergic synapse with its different constituents.

As summarized in **Figure 1A**, GABA released from a presynaptic terminal binds to post-synaptic $GABA_A$ receptors localized on precise apposition to presynaptic release sites. It binds also to presynaptic and post-synaptic $GABA_A$ and $GABA_B$ receptors [for a review see Ref. (105)]. Post-synaptic $GABA_B$ receptors are localized mainly at perisynaptic sites. Post-synaptic $GABA_A$ receptors are maintained in the right place by gephyrin, a scaffold protein which, by interacting with cytoskeletal anchoring elements, contributes to regulate receptor trafficking in and out of the synapses (106). Before being released in a calcium-dependent way, GABA is synthesized by GAD65 and GAD67 and it is stored in presynaptic vesicles by the vesicular transporter VGAT, which uses the electrochemical gradient for H^+ to shuffle and pack GABA into synaptic vesicles (107). The probability of GABA release is under control of presynaptic receptors including $GABA_A$ and $GABA_B$ receptors. Adhesion molecules of the neuroligin–neurexin families ensure the cross-talk between post and presynaptic elements of the synapses (98). The figure also shows that the presynaptic terminal contains sodium channels, since, as already mentioned, a selective mutation of these channels in GABAergic interneurons of the forebrain is responsible for the Dravet's syndrome. After being released, GABA is taken up into nerve terminals and astrocytes by GABA transporters (GATs) localized on presynaptic nerve terminals and astrocytes.

Figure 1B shows the direction of chloride fluxes through post-synaptic $GABA_A$ receptors in immature and mature neurons. The influx or efflux of chloride in and out of the cells is mainly dictated by the activity of two developmentally regulated cation–chloride co-transporters, the NKCC1 and KCC2, which pump chloride inside and outside of neurons, respectively (8). Therefore, different components of the synapses can be selectively targeted by agents that can rescue their functions.

Due to the co-morbidity of ASDs with epilepsy, anticonvulsants are widely used for the symptomatic treatment of these disorders (108). Some of these drugs have also an anti-anxiety effect and may indirectly increase $GABA_A$ neurotransmission by promoting the synthesis of GABA or by inhibiting their reuptake or breakdown. Interestingly, treatment with vigabatrin, which blocks GABA catabolism by inhibiting GABA transaminase, is able to control seizures and to improve the autistic behavior of children affected by tuberous sclerosis (109).

γ-Aminobutyric acid agonists have proved to be effective in normalizing the E/I imbalance in animal models of Autism: they can either directly enhance inhibition or indirectly reduce excitation. Thus, in BTBR mice [an animal model of idiopathic autism, Ref. (110)] or in $Scn1a^{+/-}$ mice [a monogenic model of ASDs, Ref. (78)], in which a reduced $GABA_A$-mediated inhibition occurs, the treatment with low non-sedative, non-anxiolytic doses of benzodiazepines, or clonazepam, known to enhance GABAergic signaling *via* allosteric modulation of post-synaptic $GABA_A$ receptors, leads to an improvement of social and cognitive deficits. Interestingly, in both BTBR and $Scn1a^{+/-}$ mice, the up-regulation of α2 and/or α3 containing $GABA_A$ receptors subunits by L-838,417, a selective positive allosteric modulator of these subunits, which does not induce tolerance, is able to mimic the effects of benzodiazepines, thus providing an attractive tool to enhance GABAergic neurotransmission and to improve behavioral deficits in ASDs (110). Consistent with this view, clinical trials using α2/α3 selective positive allosteric modulators of $GABA_A$ receptors have been developed with AstraZeneca and the National Institutes of Health (http://clinicaltrials.gov/show/NCT01966679).

Animal models of the Rett syndrome are currently used in translational studies for the preclinical evaluation of new therapeutic trials (111). Genetic studies have provided evidence that neuronal dysfunctions and autistic symptoms can be reversed

upon restoration of MeCP2 protein in mice in which the *Mecp2* gene has been silenced using a *lox-Stop-lox* cassette (112, 113), suggesting that in this animal model, the neuronal connectivity is not altered and neurons and glia are not permanently damaged by the *Mecp2* loss. Pharmacological studies have demonstrated amelioration of autistic symptoms with IGF-1 (114) or BDNF (115). However, since most of autistic features of the Rett syndrome can be recapitulated by deleting the *Mecp2* gene in GABA releasing neurons (75), these are expected to be rescued by drugs that enhance GABAergic neurotransmission.

Treatment of Fmr1 KO mice (an animal model of *Fragile X* syndrome), exhibiting an hyper-excitability and a GABAergic dysfunction in the basolateral nucleus of the amygdala, with gaboxadol (THIP), a selective agonist at δ subunits containing perisynaptic or extrasynaptic $GABA_A$ receptors, results in an increased $GABA_A$-mediated tonic conductance and in beneficial effects on learning deficits and behavioral disturbances linked to this disorder. This supports the hypothesis that tonic inhibition is a putative target for the treatment of *Fragile X* syndrome (65, 116). In addition, consistent with the notion that mGluR are important regulators of protein synthesis, which is translationally repressed by the FMR protein, partial inhibition of mGluR5 or inhibition of excessive glutamate release by the $GABA_B$ receptor agonist arbaclofen in *Fmr1* KO mice have led to promising results (117, 118). Although the selective activation of $GABA_B$ receptors with arbaclofen has the potential to improve social function and behavior in patients with Fragile X syndrome (119), its use in clinical trials is still under debate (120).

It is worth mentioning that in some children affected by ASDs, allosteric modulators of $GABA_A$ receptors such as benzodiazepines have a paradoxical effect, increasing anxiety, and aggression (121). Therefore, restoring low $[Cl^-]_i$ and the inhibitory action of GABA, using the selective blocker of the NKCC1 chloride importer bumetanide, may have beneficial effects. In an elegant study, Tyzio et al. (70) have convincingly demonstrated that, treating pregnant VPA rats and *Fragile X* mice shortly before delivery with bumetanide, suppresses in both animal models the excitatory action of GABA and prevents the autistic-like behavior in off springs. Bumetanide may not only reverse GABA action from the depolarizing to the hyperpolarizing direction, but it may also reduce cell excitability by an ephaptic type of mechanism involving regulation of the cell volume and the extracellular space (122). Animal data validate previous findings by the same group, showing amelioration of autistic symptoms in children treated for 3 months with bumetanide (123, 124). Although these studies should be expanded to larger multicenter trials with more restricted inclusion and exclusion criteria and more extended investigations on the dose/response action of the diuretic as well as persistence of action after drug's withdrawal, the significant improvement of the autistic behavior associated with a statistically significant amelioration of childhood autism rating scale (CARS) and clinical global impressions (CGI) scores in the absence of clear side effects make this diuretic a very promising drug to cure ASDs. In a parallel study, bumetanide was shown to improve accuracy in facial emotional labeling and to increase brain activation in areas involved in social and emotional perception (125).

CONCLUSION

Several lines of evidences suggest that ASDs are neurodevelopmental disorders characterized by a clear E/I imbalance in selective neuronal circuits. Such disequilibrium appears to be mainly related to heterogeneous defects of GABAergic signaling in different brain structures. This has paved the way toward the development of new drugs for the cure of these devastating disorders. In particular, several studies have shown that drugs acting on GABAergic synapses are able to rescue behavioral deficits in animal models of autism and to ameliorate at least some of the symptoms observed in ASD patients.

In order to successfully translate therapeutic approaches from animal to humans, it is necessary to develop animal models of ASDs that faithfully trace the behavioral alterations detected in ASD patients. In addition, the effects of drugs should be validated in humans in large scale clinical trials with accurate controls.

Since ASDs are developmental disorders, the early pharmacological intervention, guaranteed by an early diagnosis, is essential. This means that very young children will receive drugs, whose side effects have to be carefully considered. For instance, many drugs acting on GABAergic synapses can generate addiction or give rise to paradoxical reactions, as brain circuits are still immature and GABA may still exert a depolarizing and excitatory action which can be prolonged at late stages of development. Only accurate studies and the use of well suited animal models can help to design pharmacological tools with minimal risks.

AUTHOR CONTRIBUTIONS

Giada Cellot and Enrico Cherubini wrote the paper.

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Involvement of synaptic genes in the pathogenesis of autism spectrum disorders: the case of synapsins

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Autism spectrum disorders (ASDs) are heterogeneous neurodevelopmental disorders characterized by deficits in social interaction and social communication, restricted interests, and repetitive behaviors. Many synaptic protein genes are linked to the pathogenesis of ASDs, making them prototypical synaptopathies. An array of mutations in the synapsin (Syn) genes in humans has been recently associated with ASD and epilepsy, diseases that display a frequent comorbidity. Syns are pre-synaptic proteins regulating synaptic vesicle traffic, neurotransmitter release, and short-term synaptic plasticity. In doing so, Syn isoforms control the tone of activity of neural circuits and the balance between excitation and inhibition. As ASD pathogenesis is believed to result from dysfunctions in the balance between excitatory and inhibitory transmissions in neocortical areas, Syns are novel ASD candidate genes. Accordingly, deletion of single Syn genes in mice, in addition to epilepsy, causes core symptoms of ASD by affecting social behavior, social communication, and repetitive behaviors. Thus, Syn knockout mice represent a good experimental model to define synaptic alterations involved in the pathogenesis of ASD and epilepsy.

Keywords: autism, synaptopathies, synaptic vesicles, synaptic transmission, social behavior, human mutations, knockout mice

Autism spectrum disorders (ASDs) represent a wide array of neurodevelopmental disorders characterized by restricted interest, defective social interactions, repetitive behaviors, and deficit in language and verbal communication that manifest within the first 3 years of life (1, 2). The complexity of ASD is evident both at the levels of symptoms variability and of causative factors. An important genetic contribution has been observed for ASD, however, the mechanism of inheritance remains largely unknown (1). More than 500 genes have been associated with different forms of autism, but each of them account only for the minority of ASD cases and indeed environmental contributions and other modulating factors, as environment-genetic interplay and epigenetic modifications, are emerging as potential risk factors for ASDs (3).

INVOLVEMENT OF SYNAPTIC PROTEINS IN THE PATHOGENESIS OF ASDs

Autism spectrum disorders may result from mutations in a large array of genes having roles in various physiological processes such as chromatin remodeling, translation, metabolism, and synaptic functions. In experimental models of ASD, a common breakdown appears to occur at the level of synapse formation and stabilization, as well as of the ability of synapses to be modified by experience through plasticity mechanisms. Synapse dysfunctions are also at the convergence between ASD and other neuropsychiatric disorders with unknown etiology, such as schizophrenia and intellectual disabilities (4). In addition, ASDs frequently occur together with epilepsy and there may be common underlying mechanisms as well as common genetic and environmental risk factors. Synapse maturation and function rely on a vast array of compartmentalized protein–protein interactions that allow fidelity in neurotransmitter release and synaptic vesicle (SV) cycling at the pre-synaptic site and in neurotransmitter receptor localization and signaling at the post-synaptic site. Moreover, synaptic adhesion molecules link and stabilize pre- and post-synaptic sites and control synaptic modification induced by plasticity.

The synaptic theory for autism originally stems from the identification of ASD mutations in the Neuroligin genes [NLGN3 and NLGN4X; (5, 6)] coding for synaptic adhesion molecules expressed at the post-synaptic site. Since then, a growing repertoire of synaptic genes, coding for both pre- and post-synaptic proteins, have been implicated in non-syndromic ASDs: synaptic adhesion molecules [neuroligins, neurexins, cadherins, contactins and contactin-associated protein-like 2, or CNTNAP2; (7–9)], synaptic scaffold proteins [PROSAP/SHANK gene family; (10)], ion channels, and neurotransmitter receptors (11–13). Moreover, mutations in additional synaptic genes, such as the pre-synaptic RIMS3/NIM3 and the post-synaptic IL1RAPL1 and SynGAP1, involved in either SV organization or synapse formation, have been recently associated with ASD cases [(14–17); **Figure 1** and **Table 1**]. The identification of synaptic genes implicated in ASDs is expanding together with the characterization of the respective animal models bearing mutations or deletions in these genes. These models allow a more systematic analysis to study the role of those genes in ASD etiology, to discover the biological mechanisms underlying autistic behaviors and evaluate the efficacy of new potential treatments (18–21).

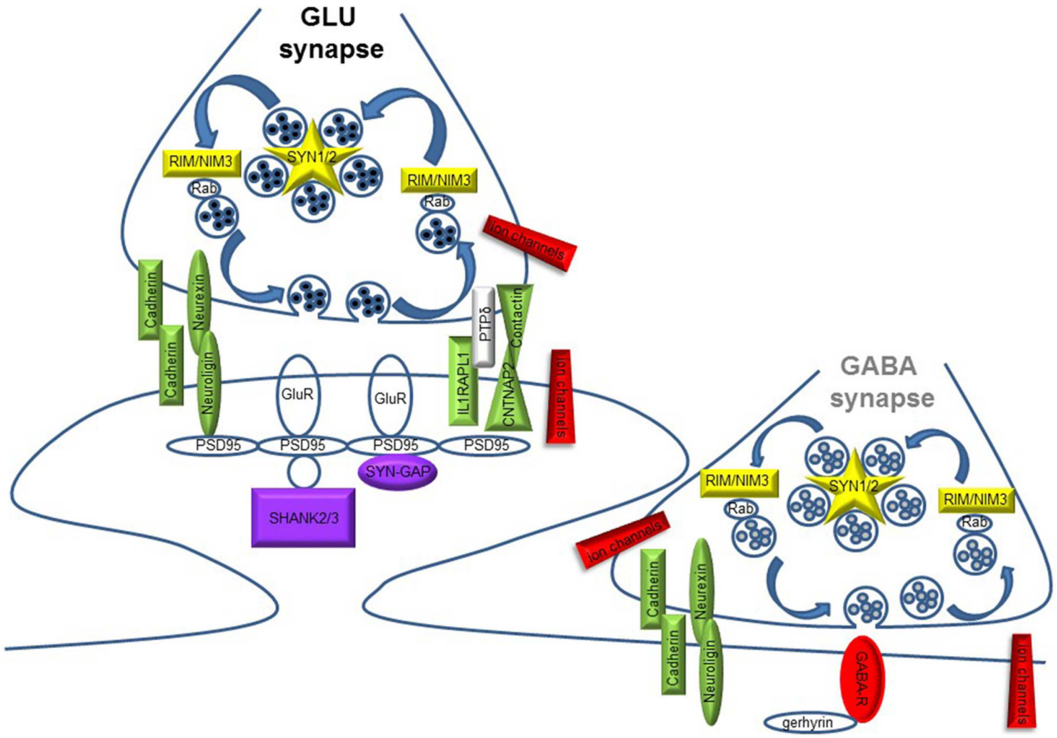

FIGURE 1 | Schematic diagram illustrating pre- and post-synaptic gene products implicated in ASD. Glutamate (GLU) and GABA synapses are shown. Different colors code for synaptic function: yellow, synaptic vesicle cycling; green, synapse formation and maintenance; red, neuronal excitability and neurotransmission; violet, glutamate receptors (GluR) signaling/trafficking.

ASD IS ASSOCIATED WITH DYSFUNCTIONS IN CORTICAL CIRCUITS THAT ALSO PREDISPOSE TO EPILEPSY

Autism spectrum disorder-like phenotypes are observed in a wide variety of neurological and neurodevelopmental disorders, including epilepsy, Rett syndrome, Fragile X Syndrome, Tuberous Sclerosis, or Fetal Anticonvulsant Syndrome, which are characterized by an imbalance of the excitatory/inhibitory tone. This aspect is consistent with the very high prevalence of epilepsy in autistic patients (about 25%) with respect to the average prevalence of 1% in the general population. Characteristic ASD phenotypes are associated with impairments or gains of GABAergic transmission (22–25).

Decreases in the GABA synthesizing enzyme GAD and a reduction in quantal size have been reported in the experimental model of Rett syndrome, the Mecp2 knockout (KO) mice (26). Various impairments in GABAergic function, including deficient GABAergic circuitry, decreased expression of $GABA_A$ receptor subunits (particularly α5 and γ subunits) and of the tonic GABA current, have been observed in mice lacking FMRP, a recognized experimental model of Fragile X Syndrome (27). The chromosomal region 15q11–q13, which is deleted or duplicated in 1–2% of idiopatic ASD patients, contains a cluster of genes encoding GABA receptor subunits [Gabra5, Gabrb3, and Gabrg3; (11)] and deletion of the Gabrb3 gene in mice, encoding for the $GABA_A$ β3 receptor subunit, leads to an ASD-like phenotype (28). Moreover, Reeler mice, lacking the protein reelin that is expressed in cortical interneurons, display an ASD phenotype that is associated with a decrease in GABA turnover (29, 30). Mice lacking synapsins (Syns) also display a primary impairment in GABA release dynamics that is associated with an ASD-like phenotype [see below; (31–35)]. Taken together, all these experimental data indicate that GABA systems are major actors in the development and functioning of cortical networks, and that their dysfunction can lead to altered development and/or function of cortical circuits resulting in epilepsy, ASD, or both. Moreover, the role of GABAergic dysfunctions in ASD pathogenesis is complex given the switch between excitatory and inhibitory GABA transmission that occurs during development and the multiple sites of action of GABA in mature neuronal networks, where it acts on predominantly post-synaptic $GABA_A$ receptors, extrasynaptic $GABA_A$ receptors regulating excitability and predominantly pre-synaptic $GABA_B$ receptors modulating glutamate release and short-term plasticity properties of excitatory synapses. Thus, disruption or dysfunction of GABAergic systems may delay critical periods in specific brain regions and perturb γ-oscillations implicated in high cognitive functions. Given the importance of the excitation/inhibition balance in the activity-dependent formation and plasticity of neocortical networks, we here review on the role of the Syns, a family of presynaptic proteins regulating release and plasticity in inhibitory and excitatory synapses, in the etiology of ASDs and the use of Syn KO animals as a model for these complex neuropsychiatric disorders.

Table 1 | Synaptic genes associated with ASD.

Gene	Name	Chromosomal locus	Phenotype	Function
SYN1	Synapsin1	Xp11.23	ASD, epilepsy	Synaptic vesicle cycling
SYN2	Synapsin2	3p25	ASD, epilepsy	Synaptic vesicle cycling
RIMS3	Regulating synaptic membrane exocytosis 3	1p34.2	ASD	Synaptic vesicle cycling
CACNA1E	Calcium channel, voltage-dependent R type, alpha 1E subunit	1q25.3	ASD	Neurotransmission
CACNB2	Calcium channel, accessory beta2 subunit	10p12	ASD	Neurotransmission
SCN1A	Voltage-regulated sodium channel type 1	2q24.3	Dravet syndrome, ASD	Neuronal excitability
SCN2A	Voltage-regulated sodium channel type 2	2q24.3	ASD, epilepsy	Neuronal excitability
SCN3A	Voltage-regulated sodium channel type 3	2q24	ASD, epilepsy	Neuronal excitability
KCNMA1	Potassium calcium-activated channel, subfamily M, alpha member 1	10q22.3	ASD	Neuronal excitability
KCNMB4	Potassium calcium-activated channel, subfamily M, beta member 4	12q	ASD	Neuronal excitability
KCNQ3	Potassium voltage-gated channel	8q24	ASD, epilepsy	Neuronal excitability
KCNQ5	Potassium voltage-gated channel	6q14	ASD, epilepsy	Neuronal excitability
KCND2	Potassium voltage-gated channel	7q31	ASD, epilepsy	Neuronal excitability
NRXN1	Neurexin1	2p16.3	ASD, schizophrenia	Cell-adhesion
NLGN3	Neuroligin3	Xq13.1	ASD	Cell-adhesion
NLGN4X	Neuroligin4	Xp22.32–p22.31	ASD, intellectual disability	Cell-adhesion
CNTNAP2	Contactin-associated protein-like 2	7q35	ASD, intellectual disability, epilepsy schizophrenia	Cell-adhesion
CDH5	Cadherin 5	16q22.1	ASD	Cell-adhesion
CDH8	Cadherin 8	16q22.1	ASD	Cell-adhesion
CDH9	Cadherin 9	5p14	ASD	Cell-adhesion
CDH10	Cadherin 10	5p14.2	ASD	Cell-adhesion
CDH11	Cadherin 11	16q21	ASD	Cell-adhesion
CDH13	Cadherin 13	16q23.3	ASD	Cell-adhesion
CDH15	Cadherin 15	16q24.3	ASD, intellectual disability	Cell-adhesion
PCDHB4	Protocadherin beta4	5q31	ASD	Cell-adhesion
PCDH10	Protocadherin delta 10	4q28.3	ASD	Cell-adhesion
PCDH19	Protocadherin delta 19	Xq22.1	ASD, intellectual disability	Cell-adhesion
CNTN4	Contactin 4	3p26.3	ASD, intellectual disability	Cell-adhesion
CNTN5	Contactin 5	11q22.1	ASD	Cell-adhesion
CNTN6	Contactin 6	3p26–p25	ASD	Cell-adhesion
IL1RAPL1	Interleukin 1 receptor accessory protein-like 1	Xp22.121.3	ASD, intellectual disability	Cell-adhesion
SHANK1	SH3 and multiple ankyrin domain protein 1	19q13.3	ASD	Glutamate receptor signaling
SHANK2	SH3 and multiple ankyrin domain protein 2	11q13.3	ASD	Glutamate receptor signaling
SHANK3	SH3 and multiple ankyrin domain protein 3	22q13.3	ASD	Glutamate receptor signaling
SYNGAP1	Synaptic Ras GTPase activating protein 1	6p21.3	ASD	Glutamate receptor signaling
GABRG3	Gamma3 subunit of GABA-A receptor	15q12	ASD	Neurotransmission

SYNAPSINS

The Syns are a family of abundant neuronal phosphoproteins that participate as regulators in synaptic transmission and plasticity, as well as in neuronal development [see Ref. (36, 37), for review]. The family is composed of 10 homologous proteins: Syn Ia–b, Syn IIa–b, and Syn IIIa–f (38–40), encoded in mammals by alternative splicing of three distinct genes (*SYN1*, *SYN2*, and *SYN3*) mapping on distinct chromosomes (chromosome X, 3, and 22, respectively) in human and mouse. Notably, Syn III is the most precociously expressed isoform that has a role in the early phases of neural

development and is downregulated in mature neurons (40). On the other hand, Syn I and Syn II are expressed at low levels at birth and their expression progressively increases along synaptogenesis to reach a stable plateau at 1–2 months of life, approximately the time window epilepsy appears (41).

All Syn isoforms display a domain structure with the NH_2-terminal region, highly conserved across isoforms and species, divided in domains A, B, and C, and the COOH-terminal portion, more divergent, composed of different spliced domains [D–I; (38)]. Many of these isoforms share consensus sequences for phosphorylation by several protein kinases, which all contribute to the modulation of Syn function (36). Domain A contains the phosphorylation site for PKA and CaMKI/IV that modulates the reversible association of Syn with SVs. Domain B, less conserved and considered as a link region, contains phosphorylation sites for MAPK/Erk, which also causes the redistribution of Syn from SVs to the cytosol. Domain C, a large central region of about 300 amino acids, mediates the interaction with actin filaments and SVs and promotes SV clustering by inducing Syn homo/hetero-dimerization (42, 43). This domain is phosphorylated by the tyrosine kinase Src (44) and contains residues mediating the binding to ATP (45). The sequences of Syn isoforms at the COOH-terminal region diverge (domain D in Syn Ia and Ib, domain G in Syn IIa and IIb, domain H in Syn IIa, and domain J in Syn IIIa), although they all bear proline-rich regions binding to several SH3-containing proteins (46, 47), and additional phosphorylation sites for CaMKII, MAPK/Erk, and cdk1/5, which affect the biochemical properties of Syn I resulting in a drastic reduction of its binding to both actin and SVs (48, 49). Finally domain E, highly conserved and common to all "*a*" isoforms, modulates Syn targeting to the pre-synaptic terminals and SV trafficking (50–53).

The best-characterized function of Syns is to control SV trafficking and modulate neurotransmitter release at the pre-synaptic terminal. The fine regulation of the balance between the reserve and the readily releasable pool of SVs is strictly controlled by Syn site-specific phosphorylation in response to stimulation, which modulates Syn association with SVs, actin cytoskeleton, and other synaptic proteins, and leaves SVs free to move close to the active zone and undergo fusion. Besides the function of Syns in these pre-docking stages of neurotransmission, recent data, supported by the fact that at least part of Syn do not dissociate from SVs upon fusion, strongly indicate that Syns play a role in the final post-docking stages of exocytosis, including SV priming, fusion, and recycling of the synaptic membrane in the area surrounding the active zone [see Ref. (36), for review].

Beyond the role in synaptic transmission, the various Syn isoforms play an important role in neuronal growth and synaptogenesis. Lack of Syn I or Syn II was shown to impair neurite outgrowth during the first days *in vitro* (54), while downregulation or ablation of Syn III caused an impairment in the development of axons at early stages in culture (55). Moreover, clear-cut structural and physiological defects were observed in the pre-synaptic terminals of Syn KO neurons (56–58), confirming the role of Syn isoforms in the modulation of synapse formation, maintenance, and rearrangement [see Ref. (37), for review].

SYNAPSIN KO MICE ARE EPILEPTIC

Knockout mice for either Syn gene are viable and fertile, have a normal life expectancy and brains of normal size and gross structure. As Syns are involved in the regulation of the excitability of neuronal networks, it is not surprising that the impairment of Syn function can result in epilepsy. Syn I KO, Syn II KO, Syn I/II double KO, and Syn I/II/III triple KO are all prone to epileptic seizures that appear approximately at 2–3 months of age, and progressively aggravate with aging and the number of Syn genes ablated [(59, 60); see Ref. (61) for review]. The fact that epilepsy does not appear at birth after ablation of Syn I or Syn II genes and that Syn III KO mice are not epileptic can be explained by the specific expression profile of the three Syn genes during development (55, 62). It is therefore likely that mature synapses require physiological levels of both Syn I and Syn II to achieve a stable excitation/inhibition balance during activity, while Syn III seems to be dispensable in this respect. In general, the loss of Syns disrupts the reserve pool of SV and alters release dynamics. However, the pre- and post-docking effects of the Syns differentially affect excitatory and inhibitory neurons. This, together with the selective distribution of the various Syn isoforms in distinct neuronal populations and the non-overlapping functions of Syn isoforms on neurotransmitter release, can result in an imbalance between excitatory and inhibitory synaptic transmission, both under conditions of basal activity and of high-frequency stimulation, potentially leading to epileptogenesis (31, 33, 34).

SYNAPSIN KO MICE DISPLAY AN ASD-LIKE PHENOTYPE

Although it is not an easy task to translate the complex symptoms of human ASD into mouse behaviors, the study of the phenotype of mice bearing deletions in the genes found to be mutated in ASD patients is fundamental for the understanding of how dysfunction of single components of the synaptic protein network may result in a general functional impairment that generates the disease. Such mouse models of ASDs should display decreased interest toward the environment, impaired sociability, and social interactions/communication, as well as repetitive behaviors.

Synapsin KO mice have generally preserved cognitive functions. A prospective study performed on Syn I and Syn II KO mice revealed that cognitive and emotional performances are not altered before the onset of epilepsy both in terms of spatial memory, object recognition, and emotional memory. Only later on, during aging and in the presence of an overt epileptic phenotype, behavioral deficits in emotional memory in both genotypes and spatial memory in Syn II KO mice emerged with respect to wild type controls, and were associated with neuronal loss and gliosis in the cortex and hippocampus (63). On the other hand, Syn III KO mice that are not epileptic, exhibit only minor alterations in spatial memory, object recognition, fear conditioning, and fear-potentiated startle (40, 64).

When an array of socially directed behaviors (social interaction and novelty, social recognition and social dominance, social transmission of food preference, and social memory) were investigated in Syn I, Syn II, and Syn III KO mice before (2-months old) and after (6-months old) the appearance of epilepsy (in Syn I and Syn II KO mice), it was immediately clear that mice presented

various impairments in social behaviors and repetitive behaviors well before the appearance of the epileptic phenotype (65).

Synapsin III mice had the mildest phenotype and showed impairments only in social interactions with an intruder, a decreased social dominance and a decreased social transmission of food preference. Syn I KO mice had an intermediate phenotype and had deficits in social and environmental exploration, social transmission, and an increased social dominance. Finally, Syn II KO had the more severe behavioral phenotype and exhibited significant deficits in virtually all social behaviors tested (with the exception of the social transmission of food preference) together with an increased social dominance and repetitive self-grooming behaviors (**Figure 2**). These data indicate that Syn KO mice represent an interesting animal model for ASDs. The physiopathological importance of this model for the understanding of ASD pathogenesis is underlined by the occurrence of ASD-linked loss-of-function mutations in human SYN genes, as described in the following section.

MUTATIONS IN THE SYN GENES ARE ASSOCIATED WITH ASD IN HUMANS

Autism spectrum disorder-associated mutations were found in both human SYN1 and SYN2 genes. Mutations in SYN1 were mainly identified in patients affected by both epilepsy and autism, whereas, mutations in SYN2 were observed in cases of ASD without association with epilepsy. The identified mutations are schematized in **Figure 3**. Two SYN1 nonsense mutations, causing truncations at protein level (W356X and Q555X), were identified in two large families with epilepsy with recessive X-linked transmission. In addition to epilepsy, few males carrying the mutations in SYNI also presented learning difficulties, low average IQ and three of them meet criteria for ASD (66, 67). The SYN1 missense mutation A550T was isolated in four patients: two with epilepsy, one with autism, and one with both, whereas, the missense mutation T567A was isolated in two individuals with ASD only. A frameshift (A94fs199X) and two missense (Y236S and G464R) mutations were identified in the SYN2 gene in three males affected by ASD (68). The mutation was transmitted by the non-affected mother. Although this phenomenon was observed only in a limited number of individuals, it is consistent with a recent report on the autosomal SHANK1 gene deletions associated with ASD in males but not females (69). Autosomal sex-limited expression, in addition to the mutation in X-linked genes, may contribute to the increased prevalence of ASD in males with respect to females. The exact mechanism at the basis of the higher penetrance in males remains to be determined.

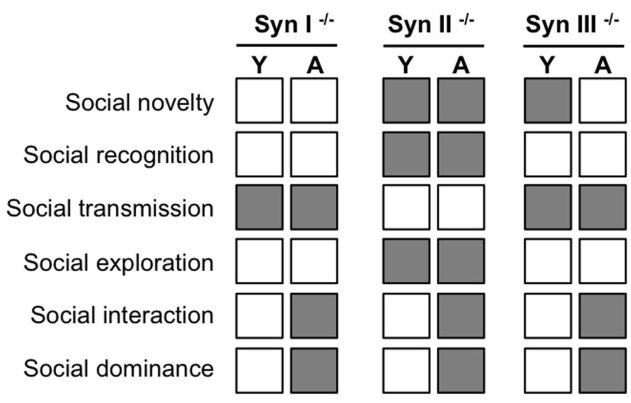

FIGURE 2 | Summary of the abnormalities in social behavior (gray squares) observed in young (Y, 2-month old) and adult (A, 6-month old) Syn I, Syn II, and Syn III KO mice with respect to the behavior of age-matched controls sharing the same genetic background [data from Ref. (65)]. Adults, but not young, Syn I and Syn II KO mice are epileptic.

EFFECTS OF HUMAN SYN MUTANTS EXPRESSED IN SYN KO NEURONS

To get insight into the molecular mechanisms of the pathogenesis of these diseases, the physiological effects of the SYN genetic variants associated with ASDs or epilepsy (or both) were analyzed *in vitro* by expressing the mutants in primary hippocampal neurons from Syn I KO or Syn II KO mice and their effects on neuronal development, nerve terminal targeting, dynamics of exo-endocytosis, and synaptic transmission were studied and compared with wild type Syn.

For the W356X mutation in Syn I, the presence of a premature stop codon in the human SYNI transcript leads to nonsense-mediated mRNA decay (NMD). The few transcripts escaping NMD process give rise to a mislocalized and non-functional protein (70). On the contrary, the second nonsense mutation (Q555X) in Syn I does not lead to NMD and a truncated form of Syn I is expressed that lacks about a half of the D domain and the COOH-terminal domains E/F. The lack of D domain impairs its binding to SVs, its phosphorylation by CaMKII and MAPK/Erk and its interactions with SH3 domain-containing proteins such as PI3K, Src, endophilin, and intersectin (67). Neurons expressing the Q555X-Syn I showed a transient impairment of axonal outgrowth, but normal dendritic arborization, nerve terminal targeting, and synaptic density (67). However, the exocytosis was impaired and the size of the readily releasable and recycling pools of SVs decreased. Electrophysiological recordings from neurons expressing Q555X-Syn I mutant in Syn I KO background showed that, while the basal

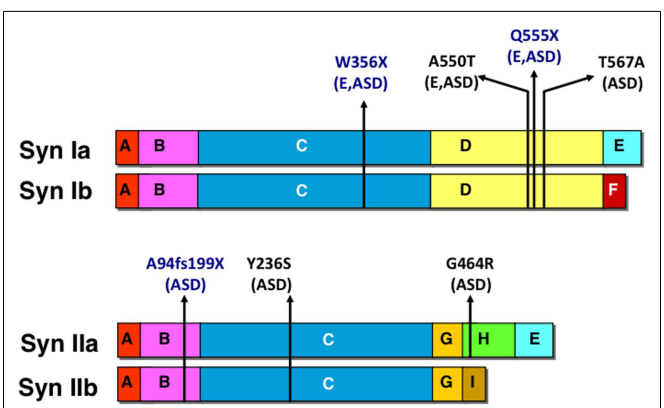

FIGURE 3 | Human mutations in SYN1 and SYN2 associated with epilepsy and/or ASD. Nonsense and missense mutations are indicated in blue and black, respectively together with the associated pathology (E, epilepsy; ASD, autism spectrum disorder).

excitatory and inhibitory transmissions were equally depressed by the mutant, a clear imbalance in short-term plasticity was present with excitatory synapses showing markedly increased paired-pulse facilitation and post-tetanic potentiation as well as faster recovery from depression, whereas, inhibitory synapses displaying an enhanced post-tetanic depression and synaptic depression during sustained high-frequency stimulation, as well as a marked slowed-down recovery from depression. This excitatory/inhibitory balance in the temporal domain of short-term synaptic plasticity produced a marked hyperexcitability and enhanced network bursting behavior at network level as demonstrated by multi-electrode array recordings, also underlining the key role of short-term plasticity at excitatory and inhibitory synapses in the regulation of network excitability (71). Two further missense mutations in Syn I (A550T and T567A) located in domain D did not significantly affected phosphorylation, molecular interactions of Syn I, or neuronal development. However, the mutants were not correctly targeted to nerve terminals and, in addition, the dynamics and sizes of the readily releasable and recycling pools of SVs at synaptic terminals were impaired (67).

The Syn II mutants had distinct physiological effects. The A94fs199X-Syn II mutant was not expressed in neurons, probably because fast degradation of the aberrant protein. Both missense mutants Y236S- and G464R-Syn II were correctly expressed in Syn II KO neurons and targeted to nerve terminals. However, both mutants impaired the size of the recycling pool of SVs, leaving the readily releasable pool unaffected. Moreover, the G464R-Syn II mutant also caused an impaired axonal growth and dendritic development of neurons (68). Similar defects in neuronal development and dendritic arborization were found in neurons silenced for the ASD-associated gene CNTNAP2, with a resulting impairment of neural circuit assembly and changes in network activity, possible causes of ASD pathogenesis (72).

Synapsin KO neurons expressing the genetic variants of Syns share common defects in SV pool dynamics. Syn I and Syn II are known to control the density of SVs at the nerve terminal and regulate their availability for release differentially, with Syn I affecting both the readily releasable and recycling pools of SVs, and Syn II only affecting the latter pool. The analysis of the dynamics of exo-endocytosis in neurons expressing genetic variants of Syns reflects these distinct effects. ASD manifestations begin in the second/third year of life, a period of intense refinement, remodeling, and experience-dependent plasticity of synapses. This periods overlaps with developmental expression pattern of Syns. Impairments in SV pool dynamics, associated with defects in short-term plasticity and/or neuronal development, may thereby destabilize the key processes of assembly of neuronal networks and the balance between excitation and inhibition.

CONCLUDING REMARKS

Synapsins are not essential for synaptic transmission, but play a key role in synaptic homeostasis and plasticity with direct consequences in network activity and excitatory/inhibitory balance. Based on the findings in human and mice, Syn genes may represent a common genetic basis for epilepsy and ASD and, accordingly, Syn KO mice can be considered a potentially interesting animal model for ASD. Similar to what occurs in children with ASDs and epilepsy, ASD-related behaviors in Syn I KO and Syn II KO mice precede the onset of seizures and epilepsy does not significantly affect the expression of the behavioral alterations in adult mice. Moreover, the non-epileptic Syn III KO mice also display some traits of social deficits. These observations lead to the idea that epilepsy and ASD follow distinct and independent pathogenic pathways, although the genetic basis appears to be largely shared by the two diseases. Although the mutations in the Syn genes found thus far account only for a limited number of ASD cases, they map into a "synaptic autism pathway" in which dysfunctions of any of the genes essential for the regulation of synapse formation, excitation/inhibition balance and activity-dependent plasticity can result in a similar ASD phenotype. Despite the inherent redundancy and robustness of mammalian biological systems, a focused dysfunction in one synaptic gene can induce secondary changes in the synaptic machinery impacting on synaptic plasticity, leading to complex dysfunctions at the circuit level associated with the appearance of the pathological phenotype. An example of this potential derangement of a complex machinery by genetic dysfunction of a single component is provided by the numerous and diverse genes implicated in phototransduction whose mutation converges toward the common clinical phenotype of *Retinitis pigmentosa* (73). In addition, it has been proposed that gene alterations and secondary dysfunctions may accumulate non-linearly in complex gene networks implicated in neural computation and higher brain functions, such as those constituting the synaptome (74, 75). In conclusion, although our map of ASD vulnerability genes is rapidly progressing, many challenges remain for the future, particularly concerning the interactions between genetic, epigenetic, and environmental factors to produce the complex ASD clinical manifestations.

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GH dysfunction in Engrailed-2 knockout mice, a model for autism spectrum disorders

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Insulin-like growth factor 1 (IGF-1) signaling promotes brain development and plasticity. Altered IGF-1 expression has been associated to autism spectrum disorders (ASD). IGF-1 levels were found increased in the blood and decreased in the cerebrospinal fluid of ASD children. Accordingly, IGF-1 treatment can rescue behavioral deficits in mouse models of ASD, and IGF-1 trials have been proposed for ASD children. IGF-1 is mainly synthesized in the liver, and its synthesis is dependent on growth hormone (GH) produced in the pituitary gland. GH also modulates cognitive functions, and altered levels of GH have been detected in ASD patients. Here, we analyzed the expression of GH, IGF-1, their receptors, and regulatory hormones in the neuroendocrine system of adult male mice lacking the homeobox transcription factor Engrailed-2 (*En2*$^{-/-}$ mice). *En2*$^{-/-}$ mice display ASD-like behaviors (social interactions, defective spatial learning, increased seizure susceptibility) accompanied by relevant neuropathological changes (loss of cerebellar and forebrain inhibitory neurons). Recent studies showed that *En2* modulates IGF-1 activity during postnatal cerebellar development. We found that GH mRNA expression was markedly deregulated throughout the neuroendocrine axis in *En2*$^{-/-}$ mice, as compared to wild-type controls. In mutant mice, GH mRNA levels were significantly increased in the pituitary gland, blood, and liver, whereas decreased levels were detected in the hippocampus. These changes were paralleled by decreased levels of GH protein in the hippocampus but not other tissues of *En2*$^{-/-}$ mice. IGF-1 mRNA was significantly up-regulated in the liver and down-regulated in the *En2*$^{-/-}$ hippocampus, but no differences were detected in the levels of IGF-1 protein between the two genotypes. Our data strengthen the notion that altered GH levels in the hippocampus may be involved in learning disabilities associated to ASD.

Keywords: autism spectrum disorders, hippocampus, pituitary gland, liver, growth hormone, insulin-like growth factor, neuroendocrine axis, mouse model

INTRODUCTION

Insulin-like growth factor 1 (IGF-1) is a hormone primarily produced by the liver, which exerts an endocrine action on multiple target tissues. It is also locally produced in tissues, including brain, where it acts in a paracrine/autocrine fashion. Several studies demonstrate that IGF-1 profoundly modulates brain function, both during development and adult life. During development, IGF-1 promotes neuronal survival [reviewed in Ref. (1)] and maturation of cortical and retinal function (2–4), whereas in adult life, it exerts multiple actions ranging from the control of synaptic plasticity to neuroprotection [reviewed in Ref. (1, 5)]. During adulthood, IGF-1 is crucial for both basal and exercise-induced hippocampal neurogenesis (6, 7) and markedly regulates learning and cognition (8).

A link between IGF-1 pathway and autism spectrum disorders (ASD) has been proposed. IGF-1 levels have been found increased in the blood (9) and decreased in the cerebrospinal fluid (10, 11) of ASD children, as compared to healthy individuals. In addition, deregulation of IGF-1 signaling pathway, which involves downstream effectors such as PI3 kinase (PI3K), protein kinase B (AKT), and mammalian target of rapamycin (mTOR), has been shown in syndromic forms of ASD [reviewed in Ref. (12)]. This led to propose that IGF-1 treatment might be beneficial for ASD, and clinical trials have been approved[1]. Indeed, IGF-1 administration is able to rescue ASD-related molecular changes in neurons derived from ASD patients (13) as well as ASD-like behavioral deficits in mouse models (14, 15). Altered levels of other hormones of the IGF-1 pathway have been associated to ASD (9). IGF-1 synthesis in the liver depends on levels of circulating growth hormone (GH) produced in the pituitary gland. GH also modulates cognitive functions (16), and altered GH levels have been detected in ASD patients (17).

Genome-wide association studies identified the transcription factor Engrailed-2 (*En2*) as a candidate gene for ASD (18), and two recent studies confirmed that *En2* expression is altered in the

[1] http://clinicaltrials.gov

cerebellum of ASD patients (19, 20). Mice lacking the homeobox domain of *En2* [*En2*$^{hd/hd}$ mice; (21); here referred to as *En2*$^{-/-}$] are considered a suitable animal model to study the neurodevelopmental basis of ASD. *En2*$^{-/-}$ mice display neuropathological and behavioral changes relevant to ASD. Reduced social interactions, defective spatial learning (22–24), and increased seizure susceptibility (25) accompanied by neuropathological changes relevant to ASD [loss of cerebellar and forebrain inhibitory neurons; (26, 27)] were indeed described in *En2*$^{-/-}$ mice. In addition, we recently showed that several genes related to ASD are markedly deregulated in the cerebellum and hippocampus of *En2*$^{-/-}$ mice (28), thus indicating that *En2* mutants are a reliable model to investigate gene expression changes relevant to ASD.

A recent study established an important link between *En2* and the modulation of IGF-1 pathway (29). During postnatal development, *En2* controls proliferation and differentiation of cerebellar granule neuron precursors (GNPs). Notably, in postnatal *En2*$^{-/-}$ cerebellum, the activity of downstream effectors of IGF-1 is increased, and IGF-1 has a stronger mitogenic effect on GNPs, as compared to WT (29). These results indicate that *En2* negatively regulates IGF-1 signaling during postnatal cerebellar development.

Here, we investigated the relationship between *En2* and GH/IGF-1 pathway in the neuroendocrine system. We analyzed the expression of GH, IGF-1, their receptors, and regulatory hormones in the brain–pituitary–liver axis of adult wild-type (WT) and *En2*$^{-/-}$ mice. We show that GH levels are lowered in the hippocampus of *En2*$^{-/-}$ mice, suggesting that this alteration might contribute to learning disabilities in this ASD mouse model.

MATERIALS AND METHODS

ANIMALS

Experiments were conducted in conformity with the European Community Directive 2010/63/EU and were approved by the Italian Ministry of Health. Animals were housed in a 12-h light/dark cycle with food and water available *ad libitum*, and all efforts were made to minimize animal suffering during the experiments. The generation of *En2* mutants were originally generated on a mixed 129Sv × C57BL/6 genetic background (21) and then backcrossed at least five times into a C57BL/6 background (27). *En2*$^{+/+}$ (WT) and *En2*$^{-/-}$ mice used in this study were obtained by heterozygous mating (*En2*$^{\pm}$ × *En2*$^{\pm}$) and genotyped by PCR as previously described (27). A total of 34 adult (3–5 months old) male mice were used: 6 mice per genotype for quantitative RT-PCR, 5 mice per genotype for enzyme-linked immuno-sorbent assay (ELISA) tests, 3 mice per genotype for immunohistochemistry, and 3 mice per genotype for *in situ* hybridization.

QUANTITATIVE RT-PCR

Tissues from WT and *En2*$^{-/-}$ mice ($n = 6$ per genotype) were dissected and frozen in dry ice. Blood samples were centrifuged in an Eppendorf benchtop centrifuge for 15 min at 3,000 rpm to separate serum from cell fraction, and then frozen in dry ice. All samples were stored at −80°C until use. Total RNAs were extracted by Trizol reagent (Invitrogen), treated with DNAse, purified by the RNAeasy Kit (Qiagen), and pooled. cDNAs were synthesized from pooled RNAs (3 μg) by SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative reverse-transcription PCR (RT-PCR) was performed in a C1000 thermal cycler (BioRad) with real-time detection of fluorescence, using the KAPA SYBR FAST master mix reagent (Resnova). Mouse mitochondrial ribosomal protein L41 (Mrpl41) was used as a standard for quantification. Primers (Sigma Genosys, UK) sequences are reported in **Table 1**. Ratios of comparative concentrations of each mRNA with respect to L41 mRNA were then calculated and plotted as the average of three to four independent reactions with technical replicates obtained from each RNA pool. Expression analyses were performed using the CFX3 Manager (BioRad) software.

BIOINFORMATIC ANALYSIS

Analysis of *En2* binding sites on GH, GHR, mGRF, SST, IGF-1, and IGF-1R gene promoters was performed using the Matinspector web-based search algorithm available from Genomatix Software[2]. The algorithm calculated the similarity between the core motif and overall sequence of the *En2* consensus binding site (matrix ID: V$EN2.01) and the gene promoter sequences of interest. The *En2* matrix was determined based on the Genomatix Matrix Library Version 9.1.

IMMUNOHISTOCHEMISTRY

Brains from adult WT and *En2*$^{-/-}$ mice ($n = 3$ per genotype) were used for immunohistochemical characterization of

[2]http://www.Genomatix.de

Table 1 | Primers used for quantitative RT-PCR experiments.

Gene	GenBank no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
En2	NM_010134.3	ACTGCACGCGCTATTCTG	ACCTGTTGGTCTGAAACTCAG
GH	NM_008117.3	GCAATGGCTACAGACTCT	AAACAGACTGGACAAGGG
GHR	NM_001286370.1	CGTTCCCCTGAACTGGAGAC	CAGCTTGTCGTTGGCTTTCC
IGF-1 class 1	NM_001111275.1	AGCGATGGGGAAAATCAGCA	CAGAGCGCCAGGTAGAAGAG
IGF-1 class 2	NM_001111276.1	CTGATGTCTGGTCCTTCGGG	CACCCTCCATGACGAAACGA
IGF-1R	NM_010513.2	CTGATGTCTGGTCCTTCGGG	CACCCTCCATGACGAAACGA
mGRF	NM_010285.2	AGGATCCAGGAACAAAGGGC	GCAAGATGCTCTCCAGGGTC
SST	NM_009215	AGGACGAGATGAGGCTGG	CAGGAGTTAAGGAAGAGATATGGG

Abbreviations are as in the text.

hypothalamic SST. Brains were fixed by transcardial perfusion with 4% paraformaldehyde followed by 1 h post-fixation, and coronal sections (40 μm thickness) were cut by a vibratome (Leica). Serial sections at level of the hypothalamus were incubated overnight with and anti-SST rabbit polyclonal (Peninsula-Bachem; 1:2000 dilution). Signals were revealed using appropriate secondary antibodies and fluorophores as described (27). Hypothalamic nuclei were identified according to the Allen Brain Atlas[3]. Images were acquired at 10× objective magnification using a Zeiss AxioImager M2 microscope.

IN SITU HYBRIDIZATION

Brains from WT and *En2*$^{-/-}$ mice ($n = 3$ per genotype) were rapidly removed and frozen on dry ice. Coronal cryostat sections (20 μm thick) were fixed in 4% paraformaldehyde. Non-radioactive *in situ* hybridization was performed as previously described (27) using digoxigenin-labeled riboprobes specific for GH [GenBank ID: X02891; (30)], mGRF (31), and IGF-1 (GenBank ID: NM_010512). The IGF-1 cDNA was cloned by PCR from hippocampal cDNA and recognizes both class 1 and class 2 IGF-1 mRNAs. Signal was detected by alkaline phosphatase-conjugated anti-digoxigenin antibody followed by alkaline phosphatase staining. The specificity of the results was confirmed by the use of sense riboprobes (not shown).

ENZYME-LINKED IMMUNO-SORBENT ASSAY

Blood samples ($n = 5$ per genotype) were collected at the time of animal sacrifice, kept in ice for 30 min, and then centrifuged in an Eppendorf benchtop centrifuge for 15 min at 3,000 rpm to separate serum. Dissected liver and hippocampal tissues ($n = 5$ per genotype) were homogenized in lysis buffer (50 mM Tris–HCl pH 7.5, 1% NP-40, 1% Triton-100, 1 mM PMSF, 10% glycerol, and protease inhibitor cocktail; Sigma-Aldrich). Homogenates were incubated in ice for 30 min, centrifuged at 12,000 rpm for 5 min at 4°C, and supernatants were recovered and stored at −80°C. Protein concentration in serum, liver, and hippocampal samples was determined by BCA method (Pierce). Samples were processed for GH (EZRMGH-45K, Millipore) and IGF-1 (ELM-IGFI; RayBiotech) ELISA according to manufacturers' protocol. All samples were analyzed in duplicate.

STATISTICAL ANALYSIS

Statistical analysis was performed with SigmaPlot 11.0 and Prism 6 (GraphPad) softwares. Values were expressed as mean ± SEM and quantitative gene expression differences between WT and *En2*$^{-/-}$ mice were assessed by Student's *t*-test, with the level of statistical significance set at $p < 0.05$.

RESULTS

EXPRESSION OF *En2* AND IGF-1 SIGNALING GENES IN THE MOUSE BRAIN–PITUITARY–LIVER AXIS

By using quantitative RT-PCR, we first investigated mRNA expression of *En2*, GH, GH receptor (GHR), mGRF, SST, IGF-1, and IGF-1 receptor (IGF-1R) in the neuroendocrine axis of WT adult male mice from our colony. mRNA expression was studied in the hypothalamus, pituitary gland, and liver (the crucial tissues involved in GH and IGF-1 synthesis), as well as hippocampus and blood cell fraction. In agreement with previous findings, we confirmed that *En2* mRNA is expressed in the hypothalamus and hippocampus (23, 25, 27). *En2* mRNA was also expressed at detectable levels in blood, pituitary gland, and liver (**Figure 1A**). As expected, GH mRNA was mainly detected in the pituitary gland, while much lower levels were present in the hippocampus, hypothalamus, liver, and blood (**Figure 1B**). Consistent with the notion that liver is the main target of GH action, we found GHR mRNA predominantly expressed in the liver, and at lower levels in the other tissues analyzed (**Figure 1C**). mRNA expression of mGRF and SST, the two hypothalamic hormones controlling GH synthesis, was mainly detected in the hypothalamus (**Figures 1D,E**). High levels of SST mRNA were also present in the hippocampus, as previously described (27). Two major different transcripts have been described for IGF-1 [class 1 and class 2; (32, 33)]. Both IGF-1 class 1 and class 2 mRNAs were predominantly expressed in the liver (**Figures 1F,G**), as expected (32). Finally, IGF-1R mRNA was mainly expressed in the pituitary gland (the target for IGF-1 negative feedback for GH production) (**Figure 1H**), but also throughout the neuroendocrine axis, consistent with the widespread action of IGF-1 on multiple tissues. These results clearly indicate that our RT-PCR protocol can detect the expression of genes belonging to the GH/IGF-1 pathway in the appropriate tissues throughout the brain–pituitary–liver axis.

ALTERED GH mRNA EXPRESSION IN THE BRAIN–PITUITARY–LIVER AXIS OF *En2*$^{-/-}$ MICE

Before investigating GH, GHR, mGRF, SST, IGF-1, and IGF-1R mRNA expression in the neuroendocrine axis of *En2*$^{-/-}$ mice, we first verified whether *En2* might directly regulate their transcription. Indeed, bioinformatic analysis revealed that *En2* binding sites are present in the promoters of all these genes (**Table 2**). We next studied GH and GHR mRNA expression in the neuroendocrine axis of WT and *En2*$^{-/-}$ adult mice. We found that GH mRNA expression was markedly deregulated throughout the neuroendocrine axis in *En2*$^{-/-}$ mice. A statistically significant increase of GH mRNA levels was detected in the pituitary gland (+72%, $p < 0.001$), liver (+376%, $p < 0.001$), and blood (+87%, $p < 0.05$) of *En2*$^{-/-}$ mice, as compared to WT littermates (**Figure 2A**). GH mRNA levels were instead significantly lower in the *En2*$^{-/-}$ hypothalamus (−97%, $p < 0.001$) and hippocampus (−98%, $p < 0.001$), as compared to WT (**Figure 2A**). *In situ* hybridization confirmed GH mRNA decrease in the *En2*$^{-/-}$ hippocampus, mainly in the CA3 subfield (**Figure 2C**). No significant differences in GHR mRNA levels were detected between genotypes in the analyzed tissues, with the exception of blood, where a marked increase was detected in mutant mice compared to controls (+312%, $p < 0.05$) (**Figure 2B**).

ALTERED EXPRESSION OF mGRF AND SST IN THE HYPOTHALAMUS OF *En2*$^{-/-}$ MICE

We then investigated the expression of mGRF (also known as growth hormone releasing hormone, GHRH) and SST, the two hypothalamic hormones regulating GH synthesis, in the

[3]http://www.brain-map.org/

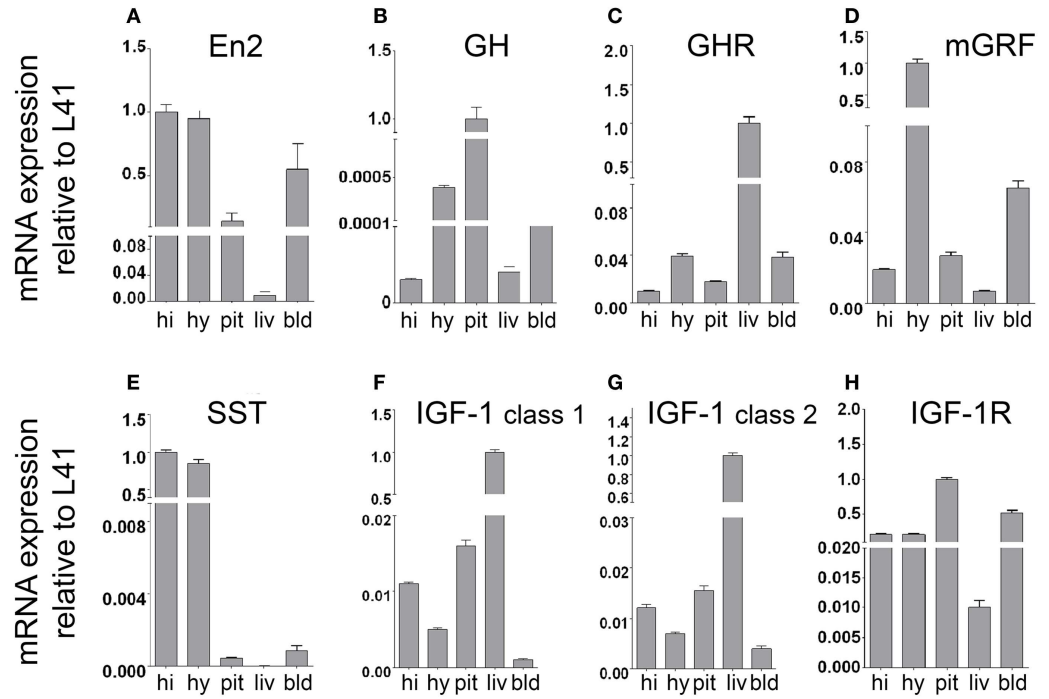

FIGURE 1 | mRNA expression of *En2* and genes involved in the GH/IGF-1 pathway in the neuroendocrine axis of WT mice. *En2* **(A)**, GH **(B)**, GHR **(C)**, mGRF **(D)**, SST **(E)**, IGF-1 **(F,G)**, and IGF-1R **(H)** mRNA expression levels in the hippocampus, hypothalamus, pituitary gland, liver, and blood, obtained by quantitative RT-PCR. For each mRNA, relative expression levels (normalized to L41) are reported on a log scale. Two different transcripts (class 1 and class 2) were analyzed for IGF-1. Values are plotted as mean ± SEM of three independent experiments. Abbreviations: hi, hippocampus; hy, hypothalamus; pit, pituitary gland; liv, liver; bld, blood (cell fraction). Other abbreviations are as in the text.

Table 2 | Presence of *En2* binding sites onto GH, GHR, mGRF, SST, IGF-1, and IGF-1R gene promoters.

Gene	Accession no.	Start	End	Strand	Core	Matrix	Sequence
GH	GXP_219787	534	552	+	0.752	0.73	agccatgAATAaatgtata
GHR	GXP_889069	584	602	–	1	0.863	ccccataAATTaataatcc
IGF-1	GXP_4345839	966	984	–	1	0.858	ttttatgAATTaagccctc
IGF-1R	GXP_183481	512	530	–	1	0.854	aacattgAATTagttcttg
mGRF	GXP_4357410	266	284	–	1	0.797	ggaaacaAATTgaacaaat
SST	GXP_82140	62	80	–	1	0.774	cagaatgAATTtgcaatta

The promoter regions of the six genes belonging to the GH/IGF-1 pathway were analyzed for the presence of En2 binding site using the Matinspector program. Core similarity (Core) indicates the match against the nucleotides representing the core motif of the En2 binding site (AATT), where 1 is 100% identity. Matrix similarity (Matrix) is the overall similarity to the En2 binding motif, where 1 is 100% identity. For each sequence, the start/end position respect to ATG is indicated, together with the strand on which they are present. For each promoter, several regions containing the En2 binding site were found; the table reports only those with highest values for core and matrix similarity. Genomatix Promoter database (GXP) accession numbers are given for each gene. Abbreviations are as in the text.

brain–pituitary–liver axis of WT and *En2*$^{-/-}$ adult mice. As compared to WT controls, a marked increase of mGRF mRNA (+53%, $p < 0.05$; **Figure 3A**) and a significant decrease of SST mRNA (−13%, $p < 0.01$; **Figure 3B**) was found in the *En2*$^{-/-}$ hypothalamus. Significantly higher mRNA levels for the two hormones were also found in blood (mGRF: +125%, $p < 0.05$) and liver (mGRF: +129%, $p < 0.01$; SST: +147%, $p < 0.001$) from *En2*$^{-/-}$ mice (**Figures 3A,B**). According to our previous study (27), lower levels of SST mRNA were found in the *En2*$^{-/-}$ hippocampus, as compared to WT (−10%, $p < 0.05$; **Figure 3B**). *In situ* hybridization and immunohistochemistry experiments, respectively, confirmed the increased expression of mGRF mRNA and decreased levels of SST protein in the dorsomedial/ventromedial paraventricular nuclei of the *En2*$^{-/-}$ hypothalamus, as compared to WT (**Figure 3C**).

ALTERED IGF-1 mRNA EXPRESSION IN THE BRAIN–PITUITARY–LIVER AXIS OF *En2*$^{-/-}$ MICE

We next analyzed mRNA expression of IGF-1 and its receptor in the brain–pituitary–liver axis of WT and *En2*$^{-/-}$ adult mice.

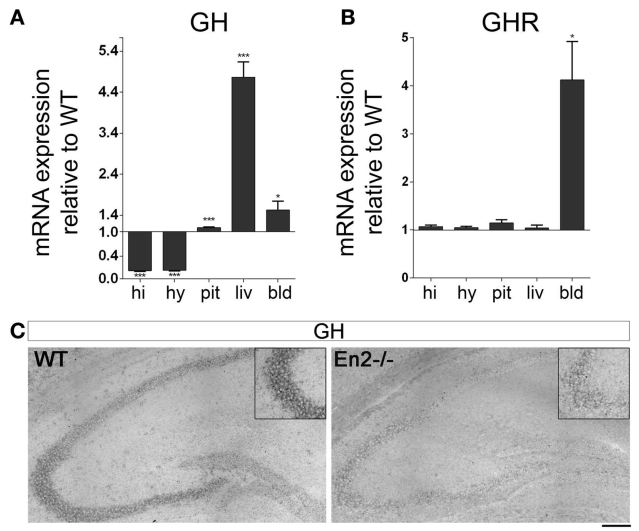

FIGURE 2 | Expression of GH and GH receptor mRNAs in the neuroendocrine axis of WT and *En2*$^{-/-}$ mice. **(A)** GH mRNA quantitative RT-PCR. **(B)** GHR quantitative RT-PCR. Values are plotted as each gene/L41 comparative quantitation ratios normalized on the expression of WT (mean ± SEM of three replicates from pools of six animals per genotype; $*p < 0.05$, $***p < 0.001$; Student's *t*-test, *En2*$^{-/-}$ vs. WT). **(C)** Representative pictures of GH mRNA *in situ* hybridization on the dorsal hippocampus from WT and *En2*$^{-/-}$ mice. Insets show the CA3 subfield. Scale bar: 200 μm (whole hippocampi) and 125 μm (insets). Abbreviations are as in **Figure 1**.

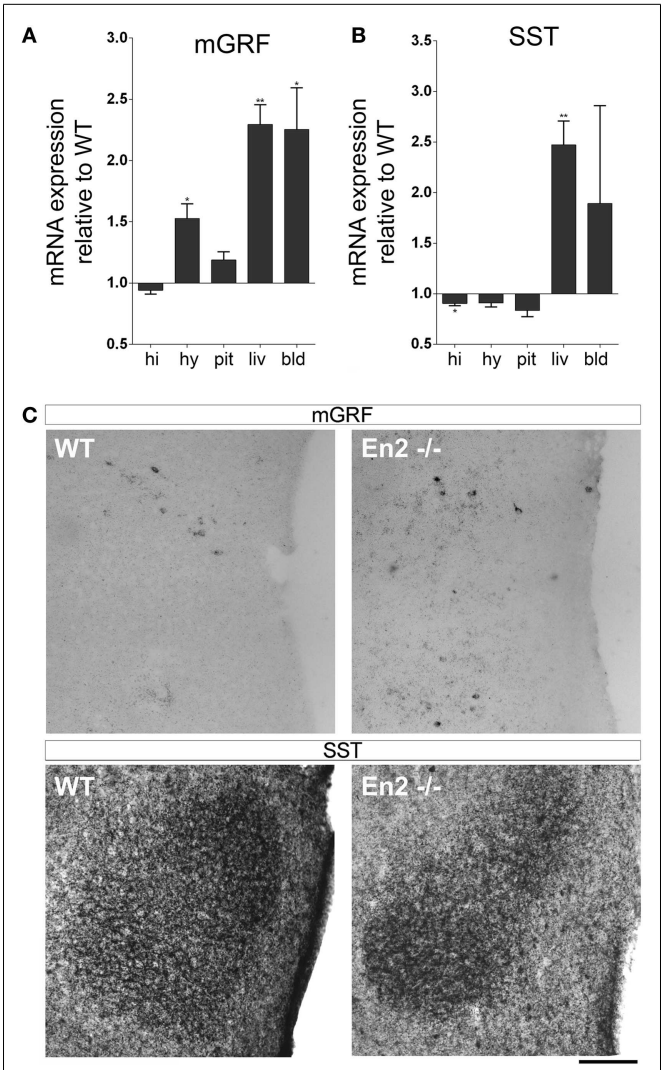

FIGURE 3 | Expression of mGRF and SST mRNAs in the neuroendocrine axis of WT and *En2*$^{-/-}$ mice. **(A,B)** mRNA expression levels of mGRF **(A)** and SST **(B)**, obtained by quantitative RT-PCR. Values are plotted as each gene/L41 comparative quantitation ratios normalized on the expression of WT (mean ± SEM of three replicates from pools of six animals per genotype; $*p < 0.05$, $**p < 0.01$; Student's *t*-test, *En2*$^{-/-}$ vs. WT). **(C)** mGRF *in situ* hybridization and SST immunohistochemistry. Representative pictures show mGRF mRNA and SST protein staining in the dorsomedial and ventromedial paraventricular nuclei of the hypothalamus. Scale bar: 200 μm. Abbreviations are as in **Figure 1**.

IGF-1 mRNA exists in two major forms (class 1 and class 2), class 2 mRNA transcription being directly regulated by GH (33, 34). We found that levels of IGF-1 class 1 mRNA were significantly reduced in the hippocampus but not other tissues of *En2*$^{-/-}$ mice, as compared to WT (−18%, $p < 0.05$ for all comparisons) (**Figure 4A**). In keeping with GH mRNA expression data (**Figure 2**), IGF-1 class 2 mRNA was significantly up-regulated in the liver (+43%, $p < 0.001$) and down-regulated in the hypothalamus (−19%, $p < 0.001$) of *En2*$^{-/-}$ mice, as compared to WT littermates; increased levels of IGF-1 class 2 mRNA were also detected in *En2*$^{-/-}$ pituitary gland (+16%, $p < 0.01$) and blood (+312%, $p < 0.001$) (**Figure 4B**). *In situ* hybridization with a riboprobe specific for both class 1 and class 2 IGF-1 mRNAs confirmed that IGF-1 mRNA levels are decreased in the *En2*$^{-/-}$ hippocampus (**Figure 4D**). Finally, IGF-1R mRNA levels did not differ between genotypes in hypothalamus, hippocampus, and pituitary gland, while a significant increase was found in blood (+84%, $p < 0.01$) and liver (+52%, $p < 0.05$) from *En2*$^{-/-}$ mice compared to WT controls (**Figure 4C**). **Table 3** summarizes mRNA expression data for all the analyzed genes in the brain–pituitary–liver axis of WT and *En2*$^{-/-}$ adult mice.

REDUCED LEVELS OF GH PROTEIN IN THE *En2*$^{-/-}$ HIPPOCAMPUS

The altered levels of GH and IGF-1 mRNA expression detected in the *En2*$^{-/-}$ neuroendocrine axis prompted us to investigate the levels of these hormones in the hippocampus, serum, and liver of both genotypes. ELISA assays revealed a significant reduction (−54%, $p < 0.05$) of GH protein levels in the hippocampus of *En2*$^{-/-}$ mice, as compared to WT littermates, while no difference was detected in serum samples (**Figure 5A**). Hippocampal, liver, and serum IGF-1 protein levels did not significantly differ between the two genotypes (**Figure 5B**).

DISCUSSION

BRIEF SUMMARY OF RESULTS

In this study, we analyzed the expression of GH, IGF-1, their receptors, and regulatory hormones in the brain–pituitary–liver axis of adult *En2*$^{-/-}$ mice, a mouse model for ASD. We found that in mutant mice, GH and IGF-1 mRNA levels were significantly

higher in the pituitary gland and liver, respectively, but this increase was not paralleled by higher levels of circulating hormones. In *En2* mutants, GH and IGF-1 mRNA levels were instead significantly down-regulated in the hippocampus, and this reduction was accompanied by a significant decrease of GH but not IGF-1 protein levels.

En2 INTERACTS WITH IGF-1 SIGNALING IN THE MOUSE NEUROENDOCRINE AXIS

Our results, schematically summarized in **Figure 6**, indicate that regulatory mechanisms controlling GH and IGF-1 mRNA expression in the neuroendocrine system are altered in *En2*$^{-/-}$ mice. The presence of *En2* mRNA in the hypothalamus–pituitary–liver axis (**Figure 1**) and that of an *En2* binding site in the promoters of GH, IGF-1, and other genes of the pathway (**Table 2**) suggest that *En2* might directly contribute to their transcriptional control. Indeed, recent studies revealed an unprecedented interaction between *En2* and IGF-1 signaling. In the absence of *En2*, IGF-1 has a stronger mitogenic effect on cerebellar GNPs, due the increased activity of downstream effectors of IGF-1 signaling, such as S6 kinase (29). Thus, *En2* appears to negatively regulate IGF-1 signaling during postnatal cerebellar development. Our results strengthen this link between *En2* and IGF-1 signaling, and suggest that a direct transcriptional control of *En2* onto genes belonging to the IGF-1 pathway takes place also in the brain–pituitary–liver axis.

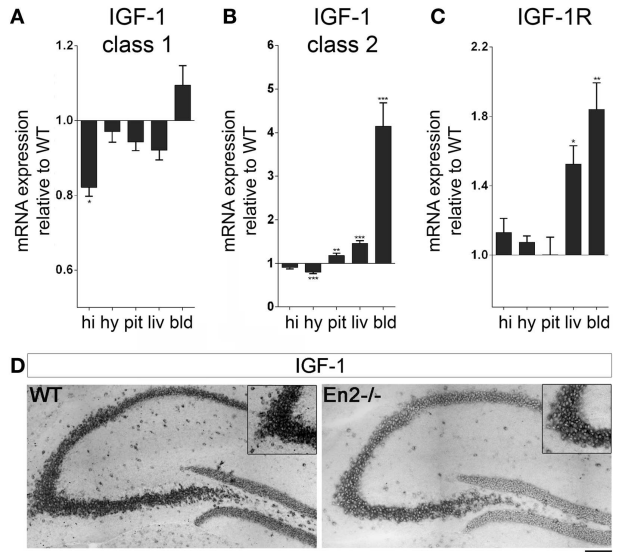

FIGURE 4 | Expression of IGF-1 and IGF-1R mRNAs in the neuroendocrine axis of WT and *En2*$^{-/-}$ mice. **(A,B)** Quantitative RT-PCR for IGF-1 class 1 **(A)** and class 2 **(B)** transcripts. **(C)** IGF-1R quantitative RT-PCR. Values are plotted as each gene/L41 comparative quantitation ratios normalized on the expression of WT (mean ± SEM of three replicates from pools of six animals per genotype; $*p < 0.05$, $**p < 0.01$; $***p < 0.001$; Student's *t*-test, *En2*$^{-/-}$ vs. WT). **(D)** Representative pictures of *in situ* hybridization for IGF-1 mRNA (both transcripts) on the dorsal hippocampus from WT and *En2*$^{-/-}$ mice. Insets show the CA3 subfield. Scale bar: 200 μm (whole hippocampi) and 125 μm (insets). Abbreviations are as in **Figure 1**.

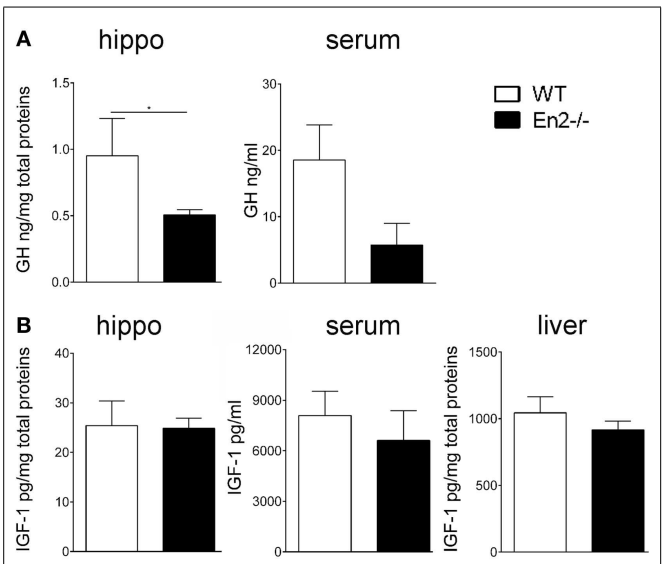

FIGURE 5 | Levels of GH and IGF-1 hormones in WT and *En2*$^{-/-}$ mice. **(A,B)** ELISA quantification of GH **(A)** and IGF-1 **(B)** levels in serum and hippocampal (hippo) and liver protein extracts, as indicated. Values are plotted as mean ± SEM (five animals per genotype, in duplicate; $*p < 0.05$, Student's *t*-test, *En2*$^{-/-}$ vs. WT). Genotypes are as indicated.

Table 3 | Summary of the expression of *En2*, GH, GHR, mGRF, SST, IGF-1, and IGF-1R mRNAs in the neuroendocrine axis of *En2*$^{-/-}$ mice, as compared to WT.

Gene	Hippocampus	Hypothalamus	Pituitary	Liver	Blood
GH	−98%***	−97%***	+72%***	+376%***	+87%*
GHR	No difference	No difference	No difference	No difference	+312%*
mGRF	No difference	+53%*	No difference	+129%**	+125%*
SST	−10%*	−13%**	No difference	+147%**	No difference
IGF-1 Class 1	−18%*	No difference	No difference	No difference	No difference
IGF-1 Class 2	No difference	−19%***	+16%**	+43%***	+312%***
IGF-1R	No difference	No difference	No difference	+52%*	+84%**

Summary of quantitative RT-PCR data. Data are presented as the mean percentage of the decrease/increase of each mRNA in En2$^{-/-}$ *mice, as compared to WT littermates.*

Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (En2$^{-/-}$ *vs. WT). Abbreviations are as in the text.*

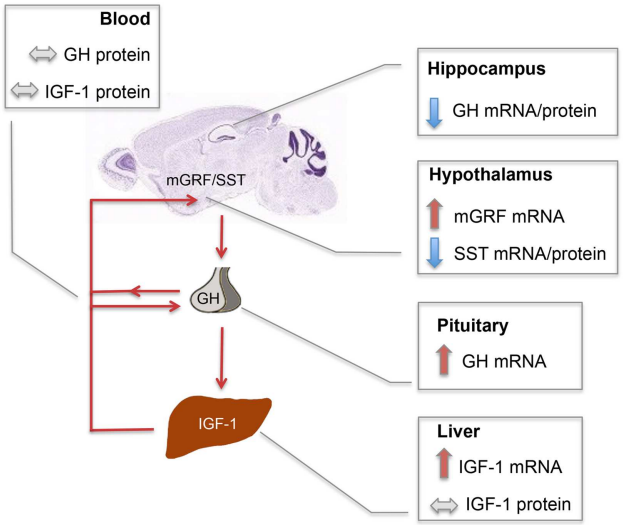

FIGURE 6 | Schematic summary of SST, mGRF, GH, and IGF-1 expression in the *En2*$^{-/-}$ neuroendocrine axis. Red and blue arrows, respectively, indicate up- and down-regulations observed in *En2*$^{-/-}$ mice as compared to WT controls. Double-arrowed gray lines indicate comparable levels between WT and *En2*$^{-/-}$ mice. Increased levels of GH and IGF-1 mRNA, respectively observed in *En2*$^{-/-}$ pituitary and liver, are not paralleled by higher levels of circulating hormones, suggesting that a complex post-translational control of GH and IGF-1 synthesis takes place in mutant mice. GH mRNA and protein levels are instead significantly down-regulated in *En2*$^{-/-}$ hippocampus. Arrowed red lines connecting different organs indicate the action of circulating hormones onto their target tissues. The mouse brain sagittal section is a Nissl stain taken from the Allen Mouse Brain Atlas (see text footnote 2). Abbreviations are as in the text.

DIFFERENTIAL EXPRESSION OF GH AND IGF-1 mRNAs IN THE BRAIN AND PERIPHERAL TISSUES OF *En2*$^{-/-}$ MICE

IGF-1 mRNA exists in two main isoforms, derived from alternative splicing of the two first exons contained in the 5′ untranslated region (5′ UTR) of the mRNA precursor. Class 1 transcripts contain exon 1, whereas class 2 transcripts contain exon 2 (33). Inclusion of exon 1 or exon 2 is mutually exclusive, resulting in different 5′ UTRs of the IGF-1 mRNA containing different leader sequences of the pre-pro-IGF-I peptide. In addition, transcription of class 1 and class 2 mRNAs is differently controlled; class 2 mRNA being directly regulated by GH. It is, however, important to note that both class 1 and class 2 transcripts code for the same IGF-1 mature peptide (33).

In *En2* mutants, we described a differential expression of IGF-1 mRNAs in different tissues of the brain–pituitary–liver axis. A significant increase of IGF-1 class 2 mRNA was detected in liver and blood, whereas IGF-1 class 1 mRNA was significantly decreased in the *En2*$^{-/-}$ hippocampus (**Figure 4**). The negative regulation exerted by *En2* onto IGF-1 signaling in the cerebellum (29), therefore, appears to occur also at a transcriptional level in the hippocampus.

Interestingly, *En2*$^{-/-}$ mice showed increased hypothalamic levels of mGRF and decreased levels of somatostatin (SST), the two hormones controlling GH synthesis. Previous studies from our laboratory already showed that loss of *En2* results in lower levels of SST mRNA and protein in the hippocampus and cerebral cortex. Here, we extended this observation, demonstrating that lower levels of SST are also present in the *En2*$^{-/-}$ hypothalamus; hypothalamic levels of mGRF mRNA were instead increased in mutant mice (**Figure 3**). Deregulation of hypothalamic hormones controlling GH synthesis might result in increased levels of circulating GH and IGF-1 in mutant mice. However, we showed that serum levels of both hormones, as well as IGF-1 levels in liver did not differ between WT and *En2*$^{-/-}$ mice (**Figure 5**). Indeed, behavioral studies did not reveal gross weight and growth abnormalities in *En2* mutants (23), suggesting that differences in GH and IGF-1 mRNA levels might be blunted at the protein level, via multiple post-transcriptional control mechanisms. Indeed, a tight dependence of IGF-1 translation via several miRNA binding site in the 3′ UTR of the transcripts has been described (33, 35).

FUNCTIONAL CONSEQUENCES OF REDUCED GH LEVELS IN THE *En2*$^{-/-}$ HIPPOCAMPUS

The decreased expression of GH mRNA and protein observed in the *En2*$^{-/-}$ hippocampus might contribute to learning deficits observed in *En2* mutants. Indeed, *En2*$^{-/-}$ mice show impaired learning in hippocampal-dependent tasks, such as the Morris water maze and contextual fear conditioning (22, 23). Profound effects on cognitive function have been demonstrated for GH. GH-deficient spontaneous dwarf rats display marked deficits in hippocampus-dependent spatial learning and memory, accompanied by an imbalance in hippocampal glutamatergic/GABAergic synapses and neurogenesis (36). Chronic stress, which is known to affect hippocampal function, also reduces hippocampal GH levels (37). Restoration of normal GH levels in the hippocampus (obtained via viral-mediated gene transfer) is able to reverse stress-dependent behavioral impairment, as tested by hippocampus-dependent tasks (contextual fear conditioning) (37). Taken together, these results indicate that reduced levels of hippocampal GH detected in *En2*$^{-/-}$ mice might contribute to hippocampal dysfunction observed in these mutants.

CONCLUSION

To our knowledge, this is the first demonstration that brain GH levels are reduced in a mouse model of ASD. Considering the important role of GH on cognitive functions, our data strengthen the notion that reduced expression of GH in the hippocampus may be implicated in learning disabilities associated to ASD.

AUTHOR CONTRIBUTIONS

Giovanni Provenzano and Elena Clementi equally contributed to this study. Giovanni Provenzano designed and performed experiments, analyzed data and wrote the paper. Elena Clementi designed and performed experiments, analyzed data. Sacha Genovesi, Manuela Scali, Prem Prakash Tripathi, and Paola Sgadò performed experiments. Yuri Bozzi provided funding, conceived the study, analyzed data and wrote the paper.

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Region specific up-regulation of oxytocin receptors in the opioid *Oprm1*$^{-/-}$ mouse model of autism

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Autism spectrum disorders (ASDs) are characterized by impaired communication, social impairments, and restricted and repetitive behaviors and interests. Recently, altered motivation and reward processes have been suggested to participate in the physiopathology of ASDs, and μ-opioid receptors (MORs) have been investigated in relation to social reward due to their involvement in the neural circuitry of reward. Mice lacking a functional MOR gene (*Oprm1*$^{-/-}$ mice) display abnormal social behavior and major autistic-like core symptoms, making them an animal model of autism. The oxytocin (OXT) system is a key regulator of social behavior and co-operates with the opioidergic system in the modulation of social behavior. To better understand the opioid-OXT interplay in the central nervous system, we first determined the expression of the oxytocin receptor (OXTR) in the brain of WT C57BL6/J mice by quantitative autoradiography; we then evaluated OXTR regional alterations in *Oprm1*$^{-/-}$ mice. Moreover, we tested these mice in a paradigm of social behavior, the male–female social interaction test, and analyzed the effects of acute intranasal OXT treatment on their performance. In autoradiography, *Oprm1*$^{-/-}$ mice selectively displayed increased OXTR expression in the Medial Anterior Olfactory Nucleus, the Central and Medial Amygdaloid nuclei, and the Nucleus Accumbens. Our behavioral results confirmed that *Oprm1*$^{-/-}$ male mice displayed social impairments, as indicated by reduced ultrasonic calls, and that these were rescued by a single intranasal administration of OXT. Taken together, our results provide evidence of an interaction between OXT and opioids in socially relevant brain areas and in the modulation of social behavior. Moreover, they suggest that the oxytocinergic system may act as a compensative mechanism to bypass and/or restore alterations in circuits linked to impaired social behavior.

Keywords: oxytocin receptor, μ-opioid receptor, brain autoradiography, social behavior, autism, *Oprm1*$^{-/-}$ mice, ultrasonic vocalizations

INTRODUCTION

Autism spectrum disorders (ASDs) are characterized by a triad of symptoms that includes impaired communication, social impairments, and restricted and repetitive behaviors and interests (1). The prevailing hypothesis regarding the physiopathology of ASDs identifies the area of social cognition as a primary deficit. In particular, it focuses on the impaired capabilities of affected people to attribute mental states to others (and oneself) in order to explain and predict behavior (i.e., the theory of mind hypothesis proposed by Baron-Cohen and colleagues (2, 3). However, altered affectivity is evident in autistic children (4) and, as recently proposed by Chevallier and others (5), motivation and reward processes might also participate to the physiopathology of ASDs. Reward circuitry dysfunctions might lead to deficits in social seeking and maintenance, resulting in reduced social capabilities and interests, and if appearing early in life, in social learning. Deficit in social cognition will thus be a consequence, rather than a cause, of impaired social behavior. Excessive brain opiate activity has been proposed in the past as a neurochemical feature in autism (6) and due to their involvement in the neural circuitry of reward, μ-opioid receptors (MORs) have also been investigated in relation to social reward, emotion, and social behavior (7–9), and represent a key target to understand the neurobiological basis of social reward dysfunction in humans and animals.

In humans, MOR activation in specific brain regions such as the amygdala, the periaqueductal gray, and the subgenual cingulated cortex is believed to be protective or adaptive in relation to social rejection. In fact, positron emission tomography (PET) scanning showed that social rejection, exemplified by a paradigm in which the test subject shows interest toward another individual who does not return it, could increase the binding of endogenous opioids to MOR in these areas, where greater binding also seemed to correlate to better resiliency. In a contest of social acceptance, MOR activation in the ventral striatum was shown to correlate with desire for social interaction (10). In a similar study, the G variant of the A118G polymorphism of the MOR-encoding gene (OPRM1)

correlated with a greater sensitivity to social rejection, reflected in a higher activation of specific brain regions, such as the dorsal anterior cingulate cortex and the anterior insula. Genetic variations in the OPRM1 gene can also influence individuals' ability to engage in social interactions. People carrying the G allele of the A118G polymorphism of the OPRM1 gene seem to experience a greater pleasure during social situations and they tend to engage in affectionate relationships more easily in comparison with people carrying the more common A variant of the gene (11). A crucial role of MOR in partner preference has also been established by studies in prairie and mountain voles. In the monogamous prairie vole, dorsal striatal MOR has been proved fundamental for the development of partner preference, which leads to the establishment of pair bonding, although pharmacological disruption of MOR signaling did not consistently alter the pattern of mating behaviors (9, 12).

In species where individuals develop selective affective bonds during their life, such as sheep or primates, MOR signaling has been implicated in the modulation of the mother–infant attachment: in infant rhesus macaques the genetic variant C77G of the MOR gene, which increases its affinity for β-endorphin (13), has been associated with increased attachment to the mother and stronger protest response and distress during separation (14). At the same time, the maternal attachment toward the offspring seems to be subjected to MOR effects. Higham and colleagues (15) evidenced that free-ranging macaque females carrying at least one copy of the minor allele (G) of the OPRM1 gene were more possessive toward their infants than mothers homozygous for the C allele. Mouse pups where the MOR gene (*Oprm1)* has been permanently disrupted (*Oprm1*$^{-/-}$ mice) produce fewer ultrasound vocalizations (USVs) in response to isolation from the mother when compared to wild type mice, to indicate that lack of MOR may induce resilience to isolation (16). Moreover, it is possible that the lack of MOR prevents the establishment of an association between maternal stimuli and feelings of reward, as transgenic mice do not show a marked preference for a familiar environment over an unfamiliar one (16). Later on in their life, these mice display reduced interest for interaction with other mice of the same sex and age (17) and they appear indifferent to ultrasounds emitted by mice of the opposite sex (18). A recent extensive behavioral characterization of *Oprm1*$^{-/-}$ mice confirmed that these animals display major autistic-like core symptoms and elegantly provided key neuroanatomical and neurofunctional correlates (19). In particular, *Oprm1*$^{-/-}$ mice display abnormal social behavior, as evidenced by a decreased time in close social contact and increased self-grooming in the direct social interaction test, reduced sociability, and social novelty recognition in the three chamber test, accompanied by increased aggression, and impaired ability in building a nest. These mice also display increased perseverative and stereotyped behaviors such as increased rearing, grooming, circling, and head shaking associated with behavioral inflexibility, as evidenced by deficient pattern of exploration in a Y-maze test. Concerning the neurobiological substrates of such phenotype, of particular interest is the observation of changes in oxytocin (OXT) gene expression in specific areas of the brain: OXT transcripts were found to be reduced in the Nucleus accumbens (NAcc) but not in the Caudate–putamen (CPu) and Central amygdala (CeA) (19).

Together with the opioid system, the OXT system is a key regulator of all the aspects of social behavior, including those involved in reproduction and care of the offspring (20). In humans, OXT facilitates the processing of social information, improves cognitive emphatic abilities and increases interpersonal trust (21). As originally put forward by Modahl (22), a deficit in the OXT system linked to an altered opioid regulation may underline the social deficits in autism. Evidence of opioid–OXT interactions is indeed well established. Endogenous opioids are involved in the modulation of OXT release into the brain and the periphery via mu- and kappa-receptors expressed on OXT-secreting neurons (23–26). Even though the opioidergic modulation of OXT release may account for many shared effects, the interplay of these two systems does not probably end with that. A social motivation circuitry in which OXT, vasopressin, endogenous opioids, and catecholamines were hypothesized to participate in a wide variety of affiliative behaviors was proposed more than 15 years ago (27) and has been more recently integrated into a network of neurobiological mechanisms, which include neuronal, neurotransmitters, and hormone systems whose alterations could underline the social impairment observed in autism (28).

To contribute to unravel the critical interactions between the opioid and OXT systems in the brain, we first reviewed the literature on oxytocin receptor (OXTR) and MOR distributions in the mouse brain. As shown in **Table 1**, we found an overlapped receptors' expression in several regions involved in social behavior.

This observation suggests that OXTR and MOR may reciprocally modulate each other even at the cellular and/or molecular level. To investigate, if alterations in MOR expression might induce changes in the OXTergic system we decided to evaluate the expression and distribution of OXTRs in the brain of *Oprm1*$^{-/-}$ mice.

Oxytocin receptors represent the pharmacological target of OXT, and OXT administration has been proposed as a potential treatment of social deficits in autistic patients (35). In particular, intranasal OXT administration is believed to circumvent the poor blood–brain barrier (BBB) permeability of this peptide. Even if the direct passage of intranasal OXT into the brain is still matter of debate (36, 37) acute and chronic intranasal OXT administration have been shown to exert behavioral effects in rodents (34, 38, 39). Even if it cannot be excluded that some of the behavioral effects of OXT are mediated via peripheral mechanisms, intranasal OXT administration in awake animals represents at present the most convenient and reproducible method to assess the therapeutic effects of this peptide on social behavior. We thus tested *Oprm1*$^{-/-}$ mice in a paradigm of social behavior and analyzed the effect of intranasal OXT treatment on their behavioral performances.

MATERIALS AND METHODS

ANIMALS AND HOUSING CONDITIONS

Oprm1$^{+/+}$ (WT) and *Oprm1*$^{-/-}$ mice were used in this study. *Oprm1*$^{-/-}$ mice were generated by disruption of exon 2 in the *Oprm1* gene as described elsewhere (40). The homozygotic parents (*Oprm1*$^{+/+}$ and *Oprm1*$^{-/-}$) were derived from heterozygous breeding pairs that were fully backcrossed on a C57BL6/J genetic background. The two homozygous lines were maintained separately. Animals were weaned when 28-day-old and maintained

Table 1 | MOR and OXTR expression levels in mouse brain as reported in the literature.

Brain region	MOR	Reference	OXTR	Reference
Olfactory bulb	+	(29–31)	+++	(32)
Anterior olfactory nucleus	++	(31)	+++	(32–34)
Lateral Septum	+/++	(29–31)	+++	(32, 34)
Bed nucleus of the stria terminalis	++	(29–31)	+/+++	(32)
Amygdala				
Basolateral (comprising BLA and BLP)	++/+	(29–31)	++	(33)
Medial	++++	(29–31)	++	(32)
Central	++++	(31)	++/+++	(32, 33)
Cortical amygdaloid area	++	(31)	+++/++++	(32, 33)
Amygdalohippocampal area	+	(31)	++/+++	(33)
Hippocampus	+	(29–31)	++	(32–34)
Caudate–putamen	++/+++	(29–31)	+	(32)
Nucleus accumbens	++++/++	(29–31)	+/++++	(32–34)
Paraventricular thalamic nucleus	+++	(31)	++	(32, 33)
Habenula	++++	(29–31)	N.D.	

in same sex/genotype groups of four to five subjects in transparent high-temperature polysulfone cages (27 cm × 21 cm × 14 cm) with water and food available *ad libitum* (2018 Teklad Global 18% Protein Rodent Diet, Harlan, Lyon, France). Room temperature (21 ± 1°C) and a 12:12 h light–dark cycle (lights on at 1900 h) were kept constant.

Two different groups of adult WT and *Oprm1*$^{-/-}$ male mice (3–4 months old) were used: the first group (4 WT and 3 *Oprm1*$^{-/-}$ mice, one subject per litter) was used for autoradiography and histological examination; the second group of males (18 WT from 6 litters and 17 *Oprm1*$^{-/-}$ mice from 7 litters) underwent intranasal OXT treatment; USVs and behavior during exposure to a female partner were observed shortly after. The genotype of all animals used in this study was controlled by PCR at the end of the experiment, according to already described procedures (40). Every animal procedure used was in strict accordance with standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 2010/63/EU) and the Italian legislation on animal experimentation (D.Lvo 116/92).

OXTR AUTORADIOGRAPHY

Naïve WT and *Oprm1*$^{-/-}$ mice were sacrificed by cervical dislocation, the brains quickly removed and immediately frozen by immersion in cold isopentane at −25°C and subsequent storage at −80° C.

Coronal brain sections (14 μm) were sliced with a cryostat, thaw-mounted on microscope slides pre-coated with chrome-alum–gelatin and kept at −80°C until further use.

Oxytocin receptor autoradiography was performed as described in Huang et al. (34). Briefly, sections were fixed with 0.2% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) and rinsed twice with 0.1% bovine serum albumin in 50 mM Tris-HCl buffer (pH 7.4). OXT binding sites were detected by incubation (1 h at room temperature in a humid chamber) with the radioiodinated OXTR antagonist ornithine vasotocin analog ([^{125}I]-OVTA, specific activity 2200 Ci/mmol; Perkin Elmer, MA, USA) at 0.02 nM in a medium containing 50 mM Tris-HCl, 0.025% bacitracin, 5 mM $MgCl_2$, and 0.1% bovine serum albumin. Sections immediately adjacent to the ones used for [^{125}I]-OVTA binding were used to determine non-specific binding by addition of 2 μM OXT to the incubation solution.

At the end of binding, the unbound excess of ligand was washed out by two rinses in ice-cold incubation medium and a final rinse in cold distilled water. The slides were quickly dried under a stream of cool air and exposed to Biomax MR Films (Kodak) in an autoradiographic cassette for 72 h. The final autoradiograms were digitalized by grayscale high-resolution scanning (600 × 600 dpi) and analysis of the optical binding density of the brain regions of interest (ROIs) was carried out using the ImageJ 1.47v software (NIH, USA). ROIs were identified by comparison with a reference mouse brain atlas (41) and manually delineated with the ROI manager tool of the software. Specific densitometric gray intensity was calculated by subtraction of the gray level of the respective section treated for non-specific binding. For each animal, the final gray intensities of each brain region were calculated by averaging two [for anterior olfactory nucleus (AON), LS, AHiPM, BLP, and PMCo] or three (for OB, NAcc, CPu, Hipp CA3, PV, Hb, BLA, MeA, and CeA) sections at different coronal planes from bregma. The regions of a limited rostro-caudal extension [medial AON (AONm) and BNST] were analyzed on a single coronal plane from bregma. Brain regions were selected on the basis of co-expression of OXTR and MOR receptors at medium-high level as resulting from a review of the data currently available in the literature and summarized in **Table 1**. Even though in literature neither OXTR nor MOR expressions in the hippocampus are reported to be high, we also included the CA3 field of this region in our analysis because it appeared intensely labeled in WT mice. Binding specificity was ensured by comparison with the adjacent sections incubated with an excess of OXT in order to displace any OVTA specifically bound to the OXTRs.

Autoradiographic ^{125}I microscales (Amersham International, UK) also were exposed for 72 h and a reference standard curve was generated. Levels of gray intensity were then converted to

nanocurie per milligram tissue equivalent by interpolation with the standard curve.

In order to increase accuracy in identifying the brain regions within each brain section, the slides labeled for non-specific binding and the slides labeled for total binding were further colored with Nissl staining and acetylcholinesterase (AChE) staining, respectively (see below).

HISTOLOGICAL STAINING

Nissl staining

The non-specific binding labeled slides were treated for Nissl staining. They were defatted by immersion in deionized water (2 min) followed by subsequent immersions in increasing concentrations of ethanol (EtOH 70% v/v, 95% v/v, and 100%, 2 min each). Sections were then rehydrated by 30 s immersions in decreasing concentrations of ethanol (EtOH 100%, 95% v/v, and 70% v/v) and after a final dip in deionized water they were left for 6 h in cresyl violet solution (0.1% cresyl violet, 0.65% sodium acetate trihydrate in 0.5% acetic acid, pH 3.3). Differentiation was obtained by two consecutive changes (3 s each) of deionized water, EtOH 70% (v/v) and EtOH 95% (v/v), and final dehydration was achieved by two changes (10 s each) in EtOH 100%. Sections were finally cleared by immersion in xylene and coverslips were mounted onto the slides with permanent mounting medium (Entellan®, Merck-Millipore, Germany) and left to dry overnight under a fume hood.

Acetylcholinesterase staining

The slides labeled for total binding in autoradiography were processed for AChE staining following the protocol described by Franklin and Paxinos (41). All the reagents used in this procedure were obtained from Sigma Aldrich (Italy) with the exception of the mounting medium. Sections were immersed over night at room temperature in an incubation solution (50 mM sodium acetate, 4 mM copper sulfate, 16 mM glycine, 4 mM S-acetylthiocholine iodide, 10 nM ethopropazine, and pH 5.0 with HCl 1 N) and developed the following day by incubation for 10 min at room temperature in a solution containing 1% sodium sulfide (pH 7.5 with glacial acetic acid). The colored precipitate from the reaction was fixed to the sections by an overnight incubation with formalin 10%. Finally, the slides were left to dry under a fume hood, dehydrated by subsequent immersions in ethanol 100% and xylene 100%, coverslipped with permanent mounting medium (Entellan®, Merck-Millipore, Germany) and left to dry overnight under a fume hood.

For both histological protocols, dried slides were digitalized at high resolution and the obtained images were used as guidance for the identification of brain regions within the sections.

BEHAVIORAL EFFECTS OF OXT INTRANASAL ADMINISTRATION

Oxytocin intranasal administration

Adult males were gently handled during the 4 days before testing to progressively habituate to the intranasal administration protocol. The first-day they were simply handled, the second-day they were firmly kept, the third-day they were firmly kept in supine position with their back supported by the palm of the manipulator's hand, and the fourth-day a drop of saline was introduced in each nostril. After the last manipulation, they were isolated in clean cages for 24 h, treated with OXT or saline and their vocalizations and behavior were recorded.

Oxytocin (Sigma Aldrich, Italy) was dissolved in saline (0.9% NaCl) to a concentration of 0.6 mg/10 ml. A total volume of 5 μl of the OXT solution was administered intranasally by gently placing drops in each nostril, that were taken in when the mice reflexively inhaled (600 ng OXT/mouse). The dosage of OXT was based on data from the literature (34, 38, 39). Control mice received an equal volume of saline (Veh). A 20-μl Eppendorf pipette with gel-loading tips was used for administration. Administration was rapid (<15 s) and handling was consistent across treatment groups.

Male–female social interaction test

Adult WT and *Oprm1*$^{-/-}$ males were isolated for 24 h in clean cages. A total of 17 *Oprm1*$^{-/-}$ (8 OXT and 9 Veh) and 18 WT (9 OXT and 9 Veh) males was used as subjects and an equivalent number of females of the two genotypes were used as partners. Each animal was used only once and administered with OXT or saline. The estrous cycle of the female partners was not considered, as irrelevant on USV quantitative performance during the first minutes of interaction in sexual naïve males facing unfamiliar females (42–44). Five minutes after the male's intranasal drug/saline administration, a female of the same genotype was introduced into the male's home cage and left for 5 min. The delay of only 5 min was chosen according to previous studies analyzing the behavioral effects of OXT after intranasal administration (34, 39). *Oprm1*$^{-/-}$ males were exposed to *Oprm1*$^{-/-}$ females and WT males to WT females. The cage was placed in front of a video-camera and under a microphone for the recording and subsequent analysis of behavior and ultrasonic vocalizations. The videos were analyzed with the Observer software (Noldus Technology, The Netherlands) and the following males' behaviors were considered for the statistical analysis: locomotion, exploration (sniffing, rearing, digging, and climbing), social investigation (following, sniffing nose, body, and ano-genital region of the partner), and self-grooming. Ultrasonic vocalizations were recorded using an UltraSoundGate Condenser Microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) lowered 5 cm above the top of the cage, and analyzed by SasLab Pro (version 4.40; Avisoft Bioacoustics). Details concerning USVs recording and analysis can be found in previous papers (17, 45). According to previous literature, USVs recorded during the first minutes of male–female social interaction, are considered to be mainly uttered by the male (46).

STATISTICAL ANALYSIS

In autoradiography, we quantified the intensity of the binding signal in *Oprm1*$^{-/-}$ mice and compared it with the binding showed by WT mice. Average nanocurie per milligram of tissue equivalents were compared by Student's *t*-test with GraphPad Prism 5.0 (GraphPad Software, USA). Results were deemed statistically significant when $p < 0.05$. The effect of the genotype and treatment on behavioral measures was first analyzed by multiple analysis of variance (MANOVA) followed by univariate ANOVAs and, in cases of significance ($p < 0.05$), Tukey HSD *post hoc* tests.

RESULTS

QUANTITATIVE ANALYSIS OF OXTR DISTRIBUTION IN WT MICE BRAIN

To analyze the pattern of OXTR distribution in *Oprm1*$^{-/-}$ mice, we first analyzed [^{125}I]-OVTA binding data in WT mice on which *Oprm1*$^{-/-}$ animals are backcrossed. **Figure 1** shows representative autoradiographic sections for the selected area and associated Nissl staining of adjacent slices; the reported distances from bregma of the different coronal planes are deduced from a reference mouse brain atlas (41).

The use of a commercial ^{125}I microscales standard allowed us to quantify the levels of [^{125}I]-OVTA binding in the brain of our mice. Quantification of OXTR expression in several areas of the brain in WT mice is reported in **Figure 2**. In WT mice, we found the highest levels of OXTR (here in the text reported as mean nanocurie per milligram tissue equivalent ± SD) in the olfactory bulb (OB, 0.91 ± 0.25), the AON (0.88 ± 0.22), and in some posterior nuclei of the amygdala, specifically the amygdalohippocampal area (AHiPM, 0.94 ± 0.16), the posteromedial cortical amygdaloid area (PMCo, 1.06 ± 0.16), and the posterior part of the basolateral amygdaloid nucleus (BLP, 0.87 ± 0.16). Medium level of OVTA binding was detected in lateral septum (LS, 0.41 ± 0.06), the bed nucleus of the stria terminalis (BNST, 0.34 ± 0.06), the CA3 field of the hippocampus (Hipp CA3, 0.30 ± 0.06), the paraventricular thalamic nucleus (PV, 0.32 ± 0.06), and in the medial amygdaloid nucleus (MeA, 0.26 ± 0.04). In the other anterior nuclei of the amygdala analyzed, the CeA nucleus (0.16 ± 0.05) and the anterior part of the basolateral amygdaloid nucleus (BLA, 0.18 ± 0.04), and in the NAcc (0.10 ± 0.04) OXTR expression was rather low. Interestingly, Dölen et al. (33) report the basolateral nucleus of the amygdala as a whole to display "average" OXTR expression, however, from observation during analysis we noticed a clear difference in OXTR expression between the anterior and the posterior parts of this nucleus, therefore, we deemed it more appropriate to analyze the two subregions separately, distinguishing them into BLA and BLP. Finally, we found minimal expression of OXTR in the CPu (0.02 ± 0.02) and the habenula (Hb, 0.04 ± 0.02) of WT mice. Overall, the expression and the distribution of OXTR in the brains of WT mice, at least in relation to the regions analyzed, is consistent with what has been previously reported (see **Table 1**).

The regions with the lowest OXTR binding signal (CPu and Hb) presented a 20-fold lower signal compared to the regions with the highest OXTR expression such as the PMCo. The NAcc also showed a very low level of OXTR expression, being it around a tenth of the PMCo's OXTR expression. All the remaining regions, such as the anterior nuclei of the amygdala, the LS, the BNST, and

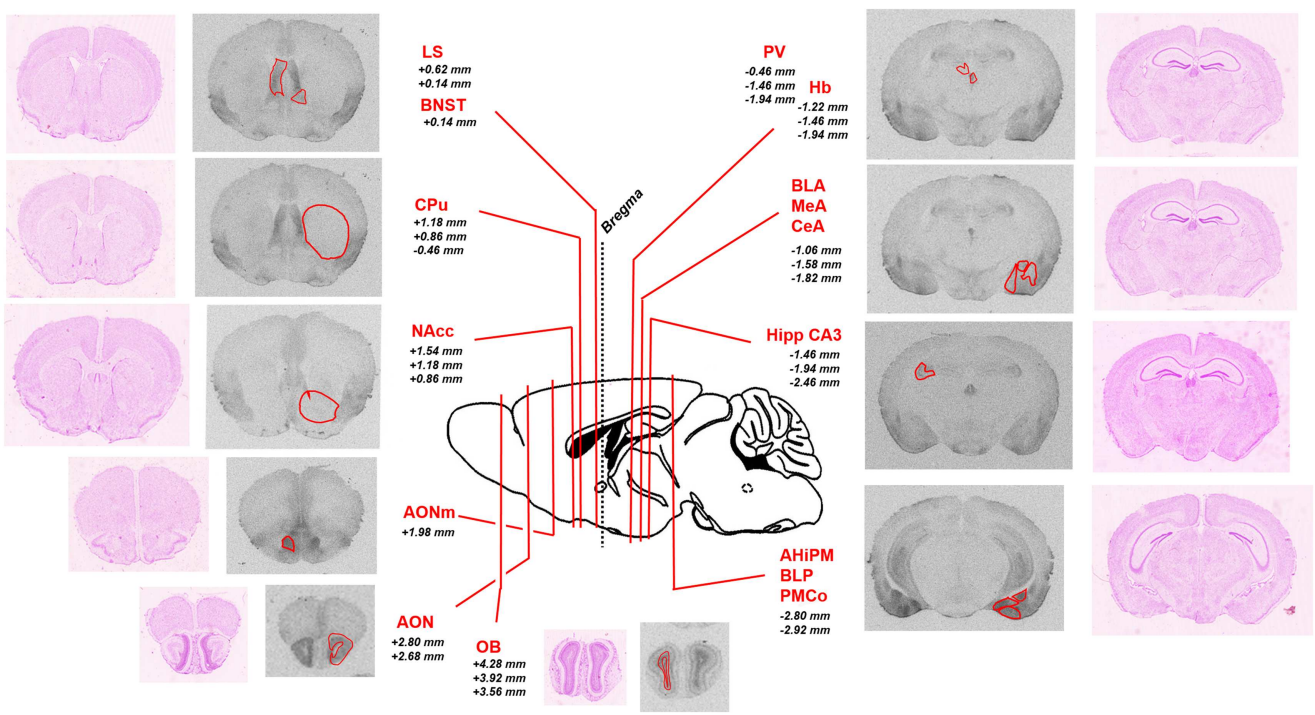

FIGURE 1 | Schematic representation of the brain regions of interest analyzed in the study. Representative sections of autoradiographic labeling of OXTR with [^{125}I]-OVTA 20 pM are displayed in grayscale, flanked by corresponding Nissl stained sections. Red contours outline the regions of interest analyzed. A sagittal schematic representation of a mouse brain is reported in the central panel. Lines indicate the antero-posterior localization of the coronal sections taken into account, defined by the reported distances (millimeter) from bregma (according to the Franklin and Paxinos (41) mouse brain atlas). AHiPM, amygdalohippocampal area, posteromedial part; AON, anterior olfactory nucleus; AONm, anterior olfactory nucleus, medial part; BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; BNST, bed nucleus of the stria terminalis; CeA, central amygdaloid nucleus; CPu, caudate–putamen; Hb, habenular nucleus; Hipp CA3, hippocampus, field CA3; LS, lateral septum; MeA, medial amygdaloid nucleus; NAcc, nucleus accumbens; OB, olfactory bulb; PMCo, posteromedial cortical amygdaloid area.

the PV, showed instead an average OXTR expression, comprised between one-fifth and one-half of the PMCo's levels.

ABSENCE OF MOR INDUCES SPECIFIC INCREASES OF OXTR EXPRESSION IN THE AONm, IN THE ANTERIOR AMYGDALOID NUCLEI AND IN NACCS

In order to reveal possible interaction(s) between MOR and OXTR, we looked for alterations in oxytocinergic binding levels in the brains of *Oprm1*$^{-/-}$ mice.

Our data indicate that the expression of OXTR did not differ significantly between WT and *Oprm1*$^{-/-}$ mice in the OB {t-test; $[t(4) = 0.139,\ p = 0.897,$ n.s.]}, AON {coronal planes corresponding to its central part; t-test; $[t(5) = 0.577,\ p = 0.589,$ n.s.]}, CPu {t-test; $[t(4) = 1.238,\ p = 0.284,$ n.s.]}, LS {t-test; $[t(5) = 0.046,\ p = 0.965,$ n.s.]}, BNST {t-test; $[t(5) = 0.315,\ p = 0.766,$ n.s.]}, Hipp CA3 {t-test; $[t(5) = 0.160,\ p = 0.880,$ n.s.]}, PV {t-test; $[t(5) = 1.269,\ p = 0.260,$ n.s.]}, and Hb {t-test; $[t(5) = 0.219, p = 0.835,$ n.s.]} (**Figure 3A**). Similarly, the posterior nuclei of the amygdala (AHiPM, BLP, and PMCo), did not present alterations in OXTR expression linked to the lack of MOR {t-test; $[t(5) = 1.891,\ p = 0.117,$ n.s.], $[t(5) = 1.442,\ p = 0.209,$ n.s.], and $[t(5) = 1.087,\ p = 0.327,$ n.s.], respectively} (**Figure 3B**).

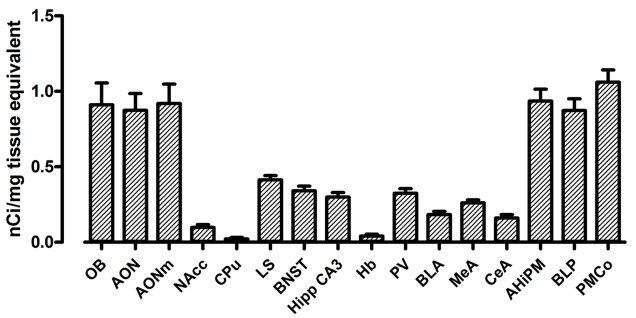

FIGURE 2 | Regional distribution and quantification of OXTR in WT mice. Average regional expression of OXTR defined by [^{125}I]-OVTA binding in WT mice. For each animal, final levels of OXTR expression were calculated averaging data obtained from the different coronal planes analyzed within the same region of interest. Each bar represents data expressed as mean and SEM of four animals.

On the contrary, in the anterior nuclei of the amygdala, and in particularly in the CeA and MeA, the absence of MOR led to an up-regulation of OXTR {t-test; $[t(5) = 3.102,\ p < 0.05]$ and $[t(5) = 4.299,\ p < 0.01]$, respectively, **Figure 3C**}. A trend ($p = 0.07$) toward an increase in OXTR was also found in the anterior basolateral amygdala (**Figure 3C**).

Another region in which we found an up-regulation of OXTR is the AONm in the posterior part of AON {t-test; $[t(7) = 3.590,\ p < 0.01]$} (**Figure 4A**). Because discriminating between the AON and NAcc is particularly tricky in this area of the brain, we performed AChE staining on the same slices processed for autoradiography. AChE selectively labels the NAcc and the olfactory tubercle but not the AON, thus allowing unambiguous identification of the two structures. **Figures 4C,D** show examples of brain sections labeled with [^{125}I]-OVTA and AChE. In the blow-ups of the merged images it is evident that the strong autoradiographic signal observed in this area does not overlap with the AChE staining, but it is adjacent to it. This allowed us to assign the strong specific [^{125}I]-OVTA labeling of this region to the AONm. Given

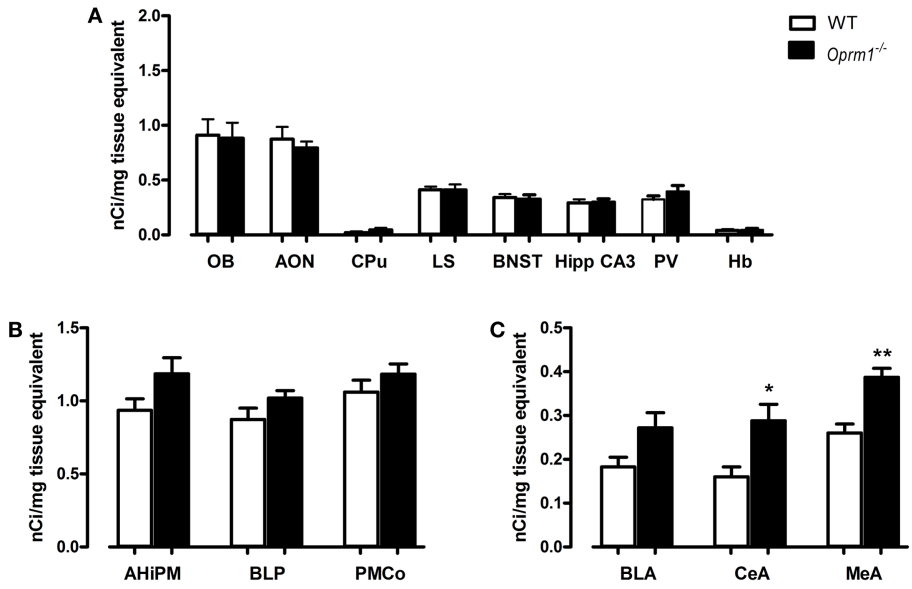

FIGURE 3 | *Oprm1*$^{-/-}$ mice display increased OXTR expression selectively in the anterior nuclei of the amygdala. Average regional expression of OXTR defined by [^{125}I]-OVTA binding in *Oprm1*$^{-/-}$ mice in comparison with WT mice. **(A)** No differences in OXTR expression were detected in *Oprm1*$^{-/-}$ mice in several brain regions reported to co-express medium-high levels of MOR and OXTR. **(B)** OXTR expression is not influenced by the lack of MOR in the posterior nuclei of the amygdala whereas **(C)** an increase of OXTR is detectable in the anterior nuclei of the amygdala CeA and MeA. Data expressed as mean and SEM of three to four animals. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to WT controls.

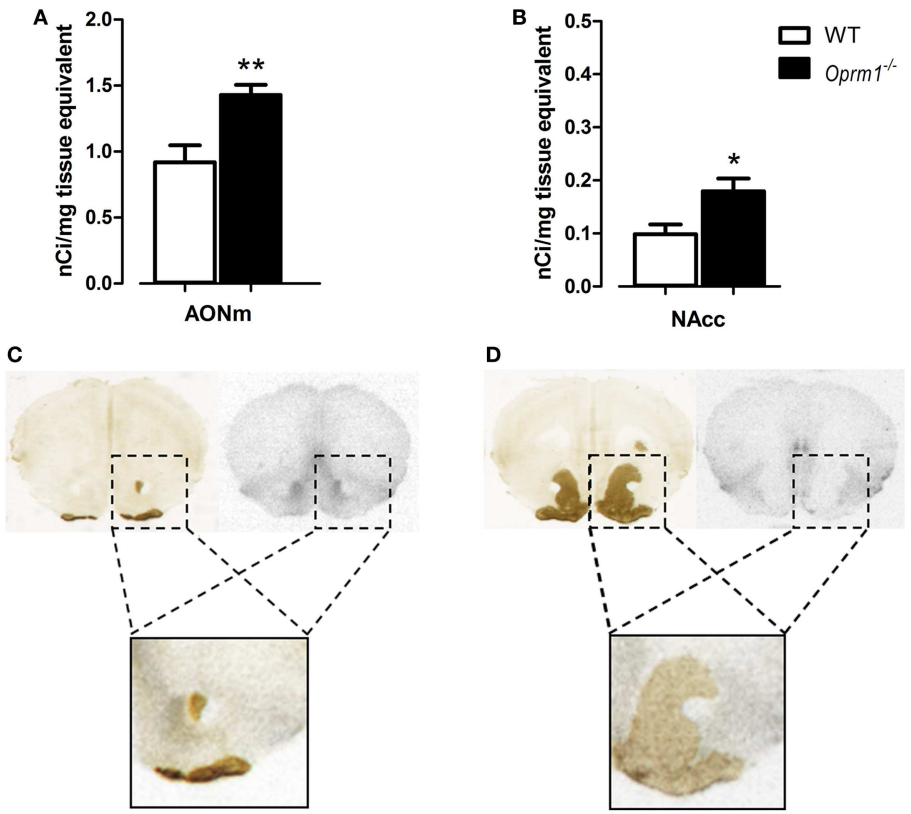

FIGURE 4 | *Oprm1*$^{-/-}$ mice display increased OXTR expression in the medial AON and the nucleus accumbens. Average regional expression of OXTR defined by [^{125}I]-OVTA binding in *Oprm1*$^{-/-}$ mice in comparison with WT mice. OXTR expression is increased in both **(A)** AONm and **(B)** NAcc of *Oprm1*$^{-/-}$ mice. At the zone of transition between AON and NAcc, distinction between the two structures was determined by AChE staining, which selectively labels the NAcc and the olfactory tubercle. **(C)** The spot of intense [^{125}I]-OVTA labeling evident in the autoradiographic picture and the AChE staining, apparently in the same position, do not overlap when the two images are merged (blow-up), indicating that the NAcc doesn't express high levels of OXTR in this area. **(D)** Superimposition of the autoradiogram and the AChE staining of a coronal section of the NAcc where the AONm is not present anymore (blow-up) confirms that this region does not express high levels of OXTR.

the difficulty, in this region, to discriminate between AONm and NAcc in absence of AChE staining, we cannot exclude that the area identified as NAcc in (34) may, instead, correspond to the AONm.

Finally, in the NAcc of *Oprm1*$^{-/-}$ mice, sampled at three different coronal planes, we found an OXTR level twice as high as that observed in WT animals {*t*-test; $[t_{(5)} = 2.767,\ p < 0.05]$} (**Figure 4B**), suggesting that the NAcc is an anatomical field of compensative interaction(s) between the oxytocinergic and the opioidergic systems.

BEHAVIORAL EFFECTS OF INTRANASAL ADMINISTRATION OF OXYTOCIN IN *Oprm1*$^{-/-}$ MICE

To evaluate the pro-social effects of OXT, we tested sexually naive males during courtship, before the initiation of sexual behavior, during the first minutes of interaction with a female. The behavioral profile of males interacting with females is shown in **Figure 5**. The MANOVA indicated no significant effect of main factors (genotype: $\lambda = 0.90$, $F_{(5/27)} = 0.58$, n.s.; treatment: $\lambda = 0.89$, $F_{(5/27)} = 0.61$, n.s.), but a significant genotype × treatment interaction ($\lambda = 0.62$, $F_{(5/27)} = 3.56$, $p < 0.05$), suggesting that OXT had different effect on mice behavioral profile according to the genotype. Univariate results confirmed significant genotype × treatment effect for locomotion $[F_{(1/31)} = 5.01,\ p < 0.05]$, social investigation $[F_{(1/31)} = 4.22,\ p < 0.05]$, and USVs $[F_{(1/31)} = 15.29,\ p < 0.001]$. *Post hoc* analysis showed a significant difference at basal conditions between *Oprm1*$^{-/-}$ and WT mice only in ultrasonic vocalizations (*Oprm1*$^{-/-}$ + Veh vs. WT + Veh: $p < 0.01$), and an increase in the number of USVs due to OXT treatment, selectively in the *Oprm1*$^{-/-}$ line (*Oprm1*$^{-/-}$ + Veh vs. *Oprm1*$^{-/-}$ + OXT: $p = 0.01$). By contrast, OXT did not to significantly modify WT males' vocalizations ($p = 0.13$, n.s.) (**Figure 5B**). The deficit shown by *Oprm1*$^{-/-}$ mice in USVs emission in response to social cues does not depend on a general impairment in their capacity to emit high levels of ultrasonic calls or in their sensitivity to olfactory stimuli, in infancy (16) as well as in adulthood (data presented here), but suggests lower motivation to interact with conspecifics.

The low impact of OXT on mutant males' general social behavior (**Figure 5A**) can be related to the role of their mutant (untreated) females that may prefer to avoid their partner. In this respect, the USVs test will result more susceptible to treatment, as

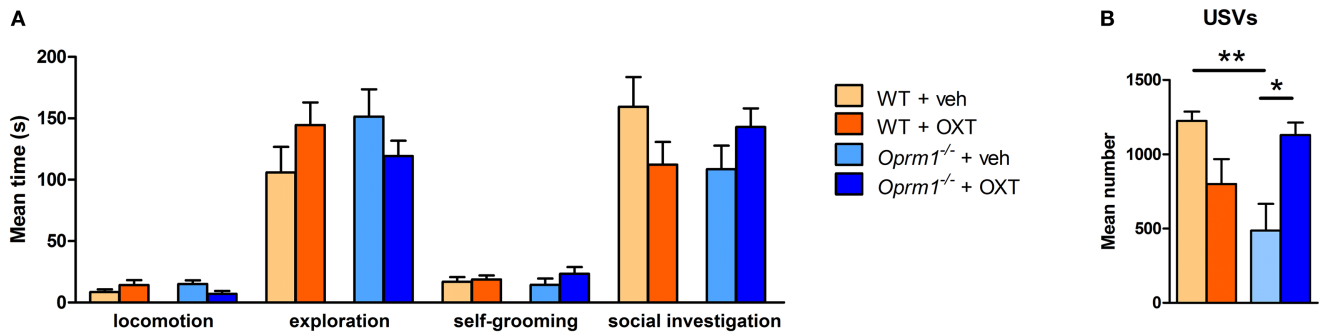

FIGURE 5 | Acute intranasal administration of OXT rescues the impairments in ultrasound emission in *Oprm1*$^{-/-}$ mice during male–female social interaction. Effects of acute intranasal OXT administration (600 ng) in *Oprm1*$^{-/-}$ and WT male mice during a 5 min male–female social interaction. **(A)** Genotype and OXT treatment do not affect the amount of time spent in locomotion, exploration, self-grooming, or social investigation during the test. **(B)** *Oprm1*$^{-/-}$ mice emit a reduced number of ultrasound vocalizations during the test in comparison with WT mice, and this deficit is completely rescued by OXT treatment. Data expressed as mean and SEM of eight to nine animals. $*p < 0.05$, $**p < 0.01$ compared to *Oprm1*$^{-/-}$ + veh group.

it only relies on the male behavior, independently on the female receptivity. Adult male mice emit USVs in the presence of female conspecifics and these vocalizations, promoting proximity, facilitate close social interactions, and sexual behavior. The advantage of testing males in this context stands in the consistent amount of ultrasonic calls that characterizes their first approach to the partner, which is independent from female's receptivity and closely associated to the male's motivational state (42–44). As a matter of fact, males emit USVs also when exposed to female's cage bedding or urine (47). When in close proximity, mice can acquire information on the partner's individual characteristics (e.g. sex, rank, sexual experience, and estrous condition) modulating their social behavior, and facilitating or avoiding social interactions. Moreover, the lack of strong deficits during sexual encounters in this mutant line confirms the entirety of their reproductive capabilities. Finally, the absence of significant effects of the intranasal OXT treatment in control male mice is in line with the Huang et al. study, showing only little changes in social investigation toward a female partner in C57BL/6J mice (34).

DISCUSSION

The aim of this study was to investigate, in *Oprm1*$^{-/-}$ mice, the altered molecular mechanisms underlying the well documented deficits in social behavior presented by this animal model (16–19). In particular, we focused on alterations in the expression of brain OXTRs because they are the target of intranasally administered OXT, a promising new treatment for social deficits in autism (35, 48, 49). Our data show an OXTR up-regulation in specific brain regions of MOR-null mice, such as AONm, CeA, MeA, and NAcc. All these regions are involved in the regulation of social behavior, but, in our study, not all regions involved in social behavior that express OXTR were found to up-regulate the receptor. Furthermore, no correspondence between a high expression level of the OXTR and its up-regulation was observed. In particular: (1) regions that display very high levels of OXTR such as LS and BNST did not show any OXTR up-regulation and (2) of the regions where we observed OXTR up-regulation, three (AON, MeA, and CeA) have a high level of OXTR expression, and one (NAcc) has a medium-low level. Interestingly, a region-selective OXTR up-regulation was also observed in a recent paper in which OXTR expression levels were analyzed in the mouse brain after morphine treatment and withdrawal (50). However, the regions found in the aforementioned pharmacological manipulation study did not overlap with those described in the present study, an observation consistent with multiple patterns of receptor modulation linked to specific genetic, developmental, and pharmacological conditions. AONm, MeA, CeA, and NAcc are, nevertheless, all characterized by a medium-high MOR expression in WT mice (**Table 1**), suggesting a local interplay between the two receptor systems, which may involve neuroanatomical, cellular, or even subcellular interactions.

An open question regards, which is the neurobiological mechanism at the basis of the OXTR up-regulation. It is difficult to answer to this question as the OXT content and capability of OXT release in the hypothalamus of *Oprm1*$^{-/-}$ mice has not been evaluated so far. If reduced, an up-regulation of the OXTR in target sites of the neuropeptide's action may represent a compensatory mechanism to normalize the neuronal responses to reduced levels of secreted OXT. Becker et al. (19) actually found a reduced level of OXT transcript in *Oprm1*$^{-/-}$ mice, but this impairment was restricted to the NAcc, leaving the overall levels of OXT undetermined. A second possibility implies direct molecular interactions between OXTR and MOR. G-protein coupled receptors such as OXTR and MOR, can form homo- (with another molecule of the same receptor) or hetero- (with receptors from other families) dimers and it has been shown that this physical association can modulate receptor binding and function (51). Interestingly, in mice lacking the alpha2A-adrenergic receptor, morphine has a reduced analgesic effect, suggesting that alterations in the noradrenergic system induce alterations in the opioid system (52). Molecular dimerization of MOR and alpha2A-adrenergic receptor has been demonstrated in both cell lines and primary neuronal cultures (53) and it has been shown that, in MOR–alpha2A-adrenergic receptor dimers, the activation of MOR by morphine

inhibits the adjacent alpha2A-receptor by blocking its ability to activate the G-proteins even in the presence of noradrenaline (54). If dimeric interactions occur between MOR and OXTR, the lack of MOR may result in specific alterations of OXTR pharmacology in the brain regions in which the two receptors physically interact. Even if only speculative at present, this hypothesis will deserve further investigation.

A second question is: what are the neuroanatomical basis of the specific OXTR up-regulation in NAcc, CeA, MeA, and AONm of MOR-null mice?

As the NAcc is involved in the processing of social motivation and reward, it is not surprising that alterations in OXTR levels in NAcc have been found to be linked to altered social behaviors. First of all, natural variability in OXTR expression in the NAcc has been associated to social organization and mating behavior in voles, being the level of OXTR in the NAcc higher in monogamous voles displaying greater maternal care (55). Many other studies associated OXTR density in the NAcc to the degree of maternal and affiliative behavior and partner preference formation (56–60). Regardless of whether differences in OXTR expression are intrinsic (due to individual or species-related variability) (55, 57, 58), exogenously induced by viral-mediated over expression (35, 59, 60), or physiologically caused by labor and lactation (56), an higher OXTR level in these brain regions correlates with more pronounced social behavior. On the contrary, reduced levels of OXTR binding in the NAcc were observed in voles after paternal deprivation (61). At the neurophysiological level, presynaptic OXTRs have been recently shown to be required for social reward in the NAcc (33). In particular, OXTRs located on axonal terminals of serotoninergic projections coming from the dorsal raphe have been found to elicit Long-Term Depression (LTD) in medium spiny neurons by activation of serotoninergic 5HT1B receptors (33). NAcc neurons express high levels or MOR, but the neuronal populations that mediate all the distinct opiate effects remain elusive; recently, a subpopulation of striatal direct-pathway neurons was shown to support opiate reward-driven behaviors (62), and specific "hotspots" involved in the modulation of hedonic "liking" responses in which opioid receptors might function as hedonic enhancers have been identified (63). Furthermore, activation of MORs within the dorsal striatum appears to be critical to partner preference formation by generating socially motivated behavioral responses (9). OXTR may thus be postulated to act on specific MOR positive NAcc subpopulation(s) to regulate specific aspects or components of social reward, possibly via a presynaptic mechanism similar to that observed for the OXT induced LTP at 5HT1B receptors.

The different nuclei of the amygdala are responsible for the processing of emotional content of both positive and negative experiences. The CeA, as part of the brain's reward circuitry, is important in processing positive emotions and, particularly, in the learning process of stimulus–reward association (64, 65). Moreover, the CeA is strongly implicated in the expression of fear. In mice, a neural circuit within the CeA that gates fear responses activating active strategies of risk assessment or passive expression of fear (freezing) has been described and, in this circuit, the OXTR was found to be a key player (66). Interestingly, as it has been observed that excessively aggressive animals show highest OXTR binding in the CeA (67), it is tempting to speculate that the increased aggressive behavior observed in *Oprm1*$^{-/-}$ mice (19) may be linked to the selective up-regulation of OXTR in the CeA. The MeA is highly activated during social encounters and recognition, thus representing a critical neuroanatomical substrate of social cognition. The recognition of conspecifics is an initial and crucial condition for the establishment of social and sexual behavior. Odor, scent, and pheromones mediate sexual and competitive interactions, and are important in individual and kin recognition and mate selection. In rodents, odor signals are processed by two systems: the main olfactory pathways and the vomeronasal pathway, both directly and indirectly projecting to the MeA, a key region for the processing of chemosensory information (68). OXT-deficient mice are not able to recognize a previously encountered, familiar conspecific, and this social recognition behavior can be restored with a direct microinjection of OXT into the MeA of OXT KO mice, indicating that OXT in the MeA is both necessary and sufficient for social recognition (69, 70). Furthermore, male social interest correlated positively with OXTR in the MeA, while female social interest correlated negatively with OXTR expression in the same region (71). Finally, it has recently been shown that OXT-mediated LTD in the AOB–MeA pathway is directly involved in long-term social recognition memory formation (72). The up-regulation of OXTR in MeA may thus be involved in the pharmacological rescue of intranasal OXT on the social interaction test used in our study.

The up-regulation of OXTR in AONm of *Oprm1*$^{-/-}$ remains of difficult interpretation. The *pars medialis* of the AON can be distinguished from the rest of the AON by cytoarchitectural features and projection patterns (73, 74), suggesting a different role than a station along the main olfactory and the vomeronasal pathways, but the role of OXTR in this region is still undefined.

Finally, what is the pathophysiological meaning of the OXTR up-regulation in *Oprm1*$^{-/-}$ mice? As *Oprm1*$^{-/-}$ mice do have severe defects in social interaction and social memory (19), the up-regulation of OXTR binding sites observed in *Oprm1*$^{-/-}$ mice is apparently unable to normalize social cognition in these animals. Nevertheless, the up-regulation of OXTR may contribute to the rescue effects observed with intranasal OXT in MOR-null animals, as these KO animals completely recovered from communication deficits during the first minutes of social interaction, not differing from wild type animals anymore. Indeed, the present study confirms the efficacy of OXT treatment in reverting social deficits in a mouse model in which the oxytocinergic system underwent a compensatory modulation. These findings are of high relevance in the autism field as they further validate the use of OXT in ASD regardless of the primary etiopathogenetic cause(s) but solely on the basis of the social endophenotype. To fully understand the role of OXTR compensatory roles, it will be very interesting to investigate in detail the time-course of OXTR up-regulation in crucial periods of prenatal and post-natal life, including birth, weaning, and sexual maturity. Similarly, we only investigated male animals, but, due to the sexual dimorphism of OXTR expression in different species and brain areas (75, 76), similar analysis in female animals is warranted, as this may have a profound impact on the design of therapeutic schemes and doses.

In conclusion, the OXT system may act as a downstream effector toward social behaviors, compensating and/or bypassing genetic alterations in upstream circuits that produce social deficits. Such a hypothesis would justify the use of this neuropeptide in a wide variety of human conditions characterized by defects in social cognition and/or reward, such as ASDs, upon demonstration of OXT/OXTR modification(s) in the brain of affected individuals. Moreover, the results presented here further corroborate the hypothesis that alterations in the OXTergic system are involved in the pathophysiology of autism and that administration of OXT to such altered system might restore a balance in the neural circuitries involved in social behavior.

AUTHOR CONTRIBUTORS

Valentina Gigliucci: performed experiments (autoradiography, Nissl staining, and AChE) and data analysis; wrote the manuscript. Marianna Leonzino: performed data analysis and wrote the manuscript. Marta Busnelli: performed experiments (autoradiography and AChE staining) and data analysis. Alessandra Luchetti: performed experiments (pharmacological treatment and behavioral tests). Viola Stella Palladino: performed experiments (animal genotyping and behavioral tests). Francesca R. D'Amato: conceived and supervised behavior experiments and wrote the manuscript. Bice Chini: conceived and supervised the study and wrote the manuscript.

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Mapping pathological phenotypes in reelin mutant mice

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Autism Spectrum Disorders (ASD) are neurodevelopmental disorders with multifactorial origin characterized by social communication deficits and the presence of repetitive behaviors/interests. Several studies showed an association between the *reelin* gene mutation and increased risk of ASD and a reduced reelin expression in some brain regions of ASD subjects, suggesting a role for *reelin* deficiency in ASD etiology. Reelin is a large extracellular matrix glycoprotein playing important roles during development of the central nervous system. To deeply investigate the role of *reelin* dysfunction as vulnerability factor in ASD, we assessed the behavioral, neurochemical, and brain morphological features of reeler male mice. We recently reported a genotype-dependent deviation in the ultrasonic vocal repertoire and a general delay in motor development of reeler pups. We now report that adult male heterozygous (Het) reeler mice did not show social behavior and communication deficits during male–female social interactions. Wildtype and Het mice showed a typical light/dark locomotor activity profile, with a peak during the central interval of the dark phase. However, when faced with a mild stressful stimulus (a saline injection) only Het mice showed an over response to stress. In addition to the behavioral studies, we conducted high performance liquid chromatography and magnetic resonance imaging and spectroscopy to investigate whether *reelin* mutation influences brain monoamine and metabolites levels in regions involved in ASD. Low levels of dopamine in cortex and high levels of glutamate and taurine in hippocampus were detected in Het mice, in line with clinical data collected on ASD children. Altogether, our data detected subtle but relevant neurochemical abnormalities in reeler mice supporting this mutant line, particularly male subjects, as a valid experimental model to estimate the contribution played by *reelin* deficiency in the global ASD neurobehavioral phenotype.

Keywords: autism spectrum disorders, reeler mice, ultrasonic vocalizations, social interaction, stress response, dopamine, glutamate, circadian cycle

INTRODUCTION

Autism Spectrum Disorders (ASD) are neurodevelopmental disorders with multifactorial origin characterized by persistent deficits in social communication and interaction and restricted and repetitive patterns of behavior, interests, or activities (1). Several studies showed that abnormal reelin expression in the brain is involved in a number of neuropsychiatric disorders including lissencephaly, schizophrenia, and autism (2–8).

Clinical studies have shown reduced levels of reelin protein in blood serum and in post-mortem brain of ASD patients (9–12). Genetic variants in RELN have been investigated as risk factors of ASD in numerous epidemiologic studies but with inconclusive results (13–19). However, recent data collected on much larger samples and with more advanced genetic approaches indicated a relationship between *reelin* gene mutation and increase risk of autism, suggesting that *reelin* deficiency may be a vulnerability factor in the etiology of this neurodevelopmental disorder (20–27).

Animal models in which reelin expression is reduced or absent, provide important information about the role of *reelin* deficiency in the onset of neurodevelopmental disorders such as ASD. Homozygous reeler mice show decreased brain volume, increased ventricles volume, (28–30), a non-foliated cerebellum (30), reduced number of Purkinje cells (31), deficits in lamination of the hippocampus (Hip), and disorganization of the amygdala (30). Some of these abnormalities are comparable with the ones found in post-mortem studies on autistic brain such as: increased ventricle volume, altered cortical lamination, heterotopias, dysplastic changes, and reduced number of Purkinje cells (32–39). These morphological changes in homozygous reeler mice are also associated with serious physical impairments and for this reason these mice are not considered as a reliable animal model for basic behavioral research but their use has been so far limited to the study of neuronal migration and of etiology of human lissencephaly (4, 5).

Heterozygous reeler mice, which exhibit the 50% reduction in reelin expression, do not display a reeler phenotype but express a number of abnormal traits including loss of Purkinje cells of the cerebellum (40, 41) and decrease in the number of dendritic spines in cortical and hippocampal neurons (42). Reduced levels of reelin are also associated with an increased anxiety profile (43, 44), cognitive deficits in the operant conditioning (44, 45), executive functions (46), fear conditioning learning (47, 48), olfactory conditioning learning (49), latent inhibition (50), and attentional set-shifting (51).

Surprisingly, only limited studies have investigated the contribution of *reelin* deficiency to the establishment of the social/communicative deficits, first ASD core symptom as indicated in the DSM 5 (1). Adult social responses in heterozygous (Het) reeler mice have been tested so far in two studies assessing either direct male–male and female–female social interactions (52) or performance in a modified version of the three-chamber sociability test (51). In both studies, only social behavioral performances have been assessed but a detailed evaluation of the ultrasonic vocalizations (USVs) emitted during the interaction was missing. To this aim, we deeply investigated the social and vocal repertoire of wildtype (Wt) and Het reeler mice during courtship (53), to evaluate the presence of qualitative alterations in social interaction and communication in this mutant line. In addition, we evaluated the baseline circadian locomotor activity in the home-cage as well as the response to a mild stressful stimulus represented by a saline injection (54–57) to check for abnormalities in the spontaneous locomotor activity that could affect the behavioral performances. To investigate whether *reelin* mutation influences brain metabolism, brain morphology, and levels of monoamines and their metabolites into selected brain regions involved in ASD and social behavior, we performed *in vivo* quantitative magnetic resonance imaging (MRI), spectroscopy, and high performance liquid chromatography (HPLC) analyses.

MATERIALS AND METHODS

ANIMALS AND HOUSING

Breeding pairs were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in our laboratory at ISS. About 2 weeks after pairing for breeding (15 Het × Het crosses), the females were individually housed and subsequently inspected daily for pregnancy and delivery. After weaning on postnatal day (pnd) 25, mice were housed by sex in mixed genotype groups (B6C3Fe Wt and Het) of two to three per cage. All mice were housed in a colony room with temperature maintained at 21 ± 1°C and humidity at 60 ± 10% with food (Enriched standard diet purchased from Mucedola, Settimo Milanese, Italy) and water available *ad libitum*. The colony room was maintained on a 12:12 light/dark cycle with lights on at 18.30 h. Mice genotype was determined at pnd 21 by polymerase chain reaction (PCR) analysis on tale samples and the animals were marked by an ear punching for identification. Consistent with the higher prevalence of autism in human males, only male mice were tested. Homozygous reeler mice were not tested due to their serious physical impairments after weaning. The same cohort of adult male mice was tested for male–female reciprocal social interaction with concomitant USVs (3 months of age), locomotor activity in the home-cage (6 months), and HPLC (7 months). A separate cohort of mice was subjected to *in vivo* quantitative MRI and spectroscopy at 4 months of age. All procedures were conducted in strict compliance with the European Communities guidelines (EC Council Directive 86/609), Italian legislation on animal experimentation (Decreto L.Vo 116/92).

ADULT MALE–FEMALE SOCIAL INTERACTIONS

Three-month-old male mice ($N = 9$ Wt, $N = 21$ Het) were evaluated in the male–female social interaction test as in Ref. (53). Each male subject was isolated 1 h before testing and the vaginal estrous condition of each stimulus female was assessed as in Ref. (58). Only females in estrous were selected for the test. The unfamiliar stimulus C57BL/6J female mouse was placed into the home-cage of the isolated male mouse and behaviors and USVs were recorded for a 3-min test session. Stimulus mice (C57BL/6J females) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in our colony room in social groups of three per home-cage. Each female was used only twice and were matched to the subject mice by age and body weight.

Social interaction test was conducted between 09.00 and 13.00 h, during the dark phase, under red light. In addition to the isolated mouse, the cage contained litter (1.5-cm deep) and the lid was removed during the test. For video recordings, the videocamera (Panasonic monochrome charge-coupled device camera) was mounted facing the side of the cage and the subsequent scoring of social investigation parameters was conducted with Noldus Observer 10XT software (Noldus Information Technology, Leesburg, VA, USA).

Social interactions were scored from the videotapes for the frequencies and durations of the following behavioral responses performed by the subject mouse: *anogenital sniffing* (direct contact with the anogenital area), *body sniffing* (sniffing or snout contact with the flank area), *head sniffing* (sniffing or snout contact with the head/neck/mouth area), *locomotor activity*, *rearing* up against the wall of the home-cage, *digging* in the bedding, and *grooming* (self-cleaning, licking any part of its own body). No observations of *mounting*, *fighting*, *tail rattling*, and *wrestling* behaviors were observed. Scoring was conducted by two investigators uninformed of the genotype. Inter-rater reliability was 98%.

For audio recordings, the ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) was mounted 20 cm above the cage and the USVs recorded using Avisoft RECORDER software version 3.2. Settings included sampling rate at 250 kHz; format 16 bit. The ultrasonic microphone was sensitive to frequencies between 10 and 180 kHz. For acoustical analysis, recordings were transferred to Avisoft SASLabPro (version 4.40) and a fast Fourier transformation (FFT) was conducted as previously described (59). Start times for the video and audio files were synchronized. Parameters analyzed included number and mean duration of calls, qualitative and quantitative analyses of sound frequencies measured in terms of frequency, and amplitude at the maximum of the spectrum. Waveform patterns of calls [a total of 17195 (Wt) and 8454 (Het) calls] were examined in depth in the sonograms collected from every mouse tested. Each call was identified as one of eight distinct categories, based on internal

pitch changes, lengths, and shapes, as in our previously published studies (53, 59, 60).

Inter-rater reliability in scoring the call categories was 98%. Scoring was conducted by two investigators blind to the mouse genotype. Call category data were subjected to two different analyses: (1) Genotype-dependent effects on the probability of producing calls (proportion of calls in each category for each subject) from each of the eight categories of USV, as described below under statistical analysis; (2) a descriptive analysis that included genotype-dependent effects on the percentage of calls emitted by each subject in each of the eight categories of USV.

LOCOMOTOR ACTIVITY IN THE HOME-CAGE

At 6 months of age, male mice ($N = 9$ Wt, $N = 10$ Het) were individually housed in standard cages (33 cm × 13 cm × 14 cm) and assigned to a continuous monitoring of spontaneous locomotor activity. The assessment of daily spontaneous activity in the home-cage was carried out by means of an automatic device using small passive infrared sensors positioned on the top of each cage (Activiscope system, see the website: www.newbehavior.com) (61–63). The system operated continuously for 13 days and after 2 days of acclimation the experimental procedure began. The sensors (20 Hz) detected any movement of mice. Data were recorded by an IBM computer with dedicated software. No movements were detected by the sensors when mice were sleeping, inactive, or performed moderate self-grooming. Scores were obtained during 30-min intervals and expressed as counts per minute (cpm). The 24-h profile of activity was obtained by averaging 7 days of continuous registration. The position of Wt and Het mouse cages in the rack was equally distributed in rows and columns. Animals were provided with tap water and food pellets *ad libitum*. After the first 7 days of spontaneous activity, all animals were subjected to an injection of saline (a mild stressful stimulus), at 11 h (dark phase), and locomotor activity monitored up to 3 days later. The analysis of the locomotor profile over a period of 7 h (11–18) after saline injection was performed to evaluate the immediate stress response.

MONOAMINES AND THEIR METABOLITES: HPLC DETERMINATION

Subsequently to behavioral studies, male mice ($N = 9$ Wt, $N = 10$ Het) were sacrificed, their brains removed and rapidly dissected on ice to obtain the olfactory bulb, frontal cortex, striatum, Hip, and cerebellum for HPLC analysis. All samples were immediately flash frozen on dry ice, and then stored at −80°C until further processing. HPLC was performed according to Ref. (64). In particular, each brain region was weighed, ultrasonicated in 0.1 M perchloric acid, centrifuged for 20 min at 15,000 g (4°C) and the supernatant was used for monoamine neurotransmitters and their metabolites detection. The endogenous levels of 5-HT and 5-HT metabolite (5-hydroxyindolacetic acid; 5-HIAA), dopamine (DA) and final DA metabolite (homovanillic acid; HVA), and norepinephrine (NA) and NA metabolite (4-hydroxy-3-methoxyphenyl-glycol, MOPEG) were assayed by HPLC using a SphereClone 150 mm × 2 mm column (3-μm packing). Detection was accomplished with a Unijet cell (BAS) with a 6-mm-diameter glassy carbon electrode at +650 mV versus an Ag/AgCl reference electrode, connected to an electrochemical amperometric detector (INTRO, Antec Leyden, The Netherlands). For each analysis, a set of standards containing various concentrations of each compound (monoamines and their metabolites) was prepared in the perchloric acid solution, and calibration curves were calculated by a linear regression. The retention time of calibration standards was used to identify peaks, and areas under each peak were used to quantify monoamine levels. Results were normalized to the weight of wet tissue.

MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY

At 4 months of age, a separate cohort of male mice ($N = 7$ Wt, $N = 7$ Het), was subjected to *in vivo* MRI and magnetic resonance spectroscopy (MRS). During the MR analyses, animals were anesthetized with 2.5–2.0% isoflurane in oxygen 1 l/min (Isoflo, Abbott SpA, Latina, Italy). An integrated heating system allowed maintaining the animal body temperature at 37.0 ± 0.1°C. All MRI and MRS experiments were conducted on a 4.7 T Varian/Agilent Inova animal system (Agilent Inc., Palo Alto, CA, USA), equipped with actively shielded gradient system (max 200 mT/m, 12 cm bore size). A 6-cm diameter volume coil was used for transmission in combination with an electronically decoupled receive-only surface coil (Rapid Biomedical, Rimpar, Germany). Spin-echo sagittal anatomical images (TR/TE = 3000/60 ms, 13 consecutive slices of 0.8 mm thickness, FOV = 20 mm × 20 mm, matrix of 128 × 128, 2 averages) were acquired for accurate positioning the voxel for the MRS study. Single voxel localized ^{1}H MR spectra (PRESS, TR/TE = 4000/23 ms, ns = 256 or 512) were collected from relevant brain areas: Hip (11.7 μl), striatum (STR, 10.4 μl), thalamus (Th, 12.96 μl), and cerebellum (Cb, 7.45 μl), as shown in **Figure 1A** and defined in the mouse brain atlas (65). Quantitative MRS protocol, including water T2 measurements, was applied (66) and T2 measurements were performed on water signal in order to identify any change in the mutant mice. Unsuppressed water signal was used for metabolite quantification (assuming 79.9% for gray matter water content). Spectra were analyzed using LCModel (67). Only those metabolites that were estimated to have Cramer–Rao lower bounds (CRLB) <20%, which corresponded to an estimated concentration error <0.2 μmol/g, were included into the quantitative analysis. In some cases, metabolites that have resonance overlapped or very close are also given as their sum. An example of spectra and its LCModel analysis is shown in **Figure 1B**.

Multislice fast spin-echo axial images (TR/TEeff = 3200/60 ms, ns = 4, slice thickness 0.6 mm, 24 slices, matrix 256 × 256, FOV = 25 mm × 25 mm, which correspond to voxel resolution of 98 × 98 × 600 μm^3) were also acquired for volumetric analyses.

Motor cortex thickness was measured at +1.32 from bregma as shown in **Figure 1C**. Volumetric analyses of the whole brain have been performed from olfactory bulb to cerebellum excluded. Ventricles and cerebellum volumes were also measured. Brains were manually segmented for forebrain, ventricles, and cerebellum using Varian/Agilent Imaging Browser, which perform a 3D-volume calculation by summing the pixels areas on the center of each slices and interpolating the cross sectional areas between the center of the other slices (Agilent Inc., Palo Alto, USA) on MR images. Manual segmentation of the ventricles was facilitated by

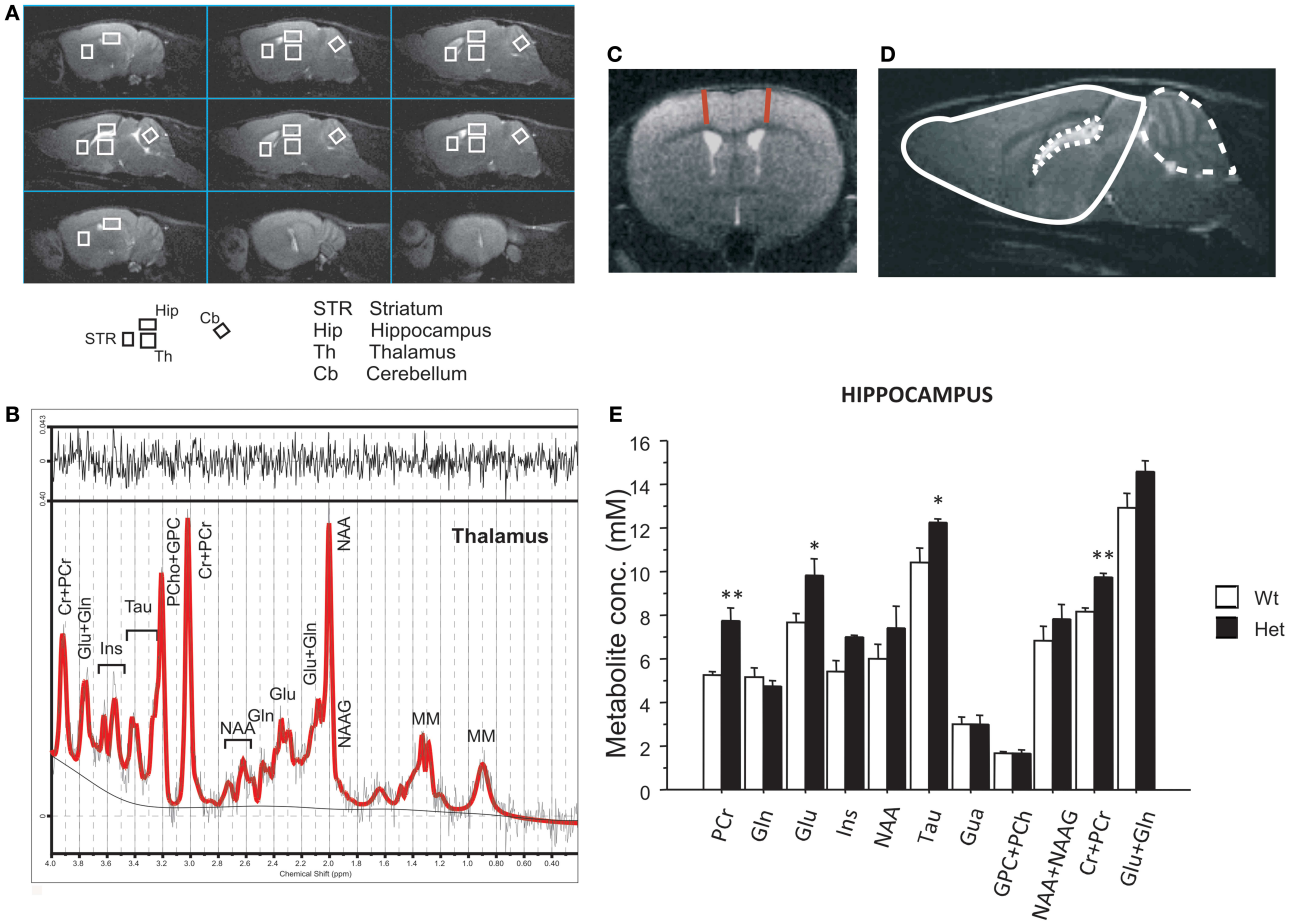

FIGURE 1 | Magnetic resonance imaging and spectroscopy performed in 4-month-old reeler mutant mice. **(A)** MRI panel – example of *in vivo* sagittal T2-weighted spin-echo images (TR/TE = 3000/70 ms, slice thickness 0.8 mm, NS = 2, FOV = 20 mm × 20 mm, matrix 128 × 128). Voxels localized on STR, Hip, Th, and Cb are indicated by the white rectangles. **(B)** MRS panel – examples of *in vivo* 1H spectra (as a black trace), acquired from the thalamus (PRESS, TR/TE = 4000/23 ms, NS = 256). The result of LCModel fit is shown as a red trace superimposed on the spectrum. Metabolite assignments: Ins, inositol; Cr, creatine; PCr, phospho-creatine; Glu, glutamate; Gln, glutamine; Tau, taurine; PCho, phospho-choline; GPC, glicero-phospho-choline; NAA, *N*-acetyl-aspartate; NAAG, *N*-acetyl-aspartyl-glutamate; MM, macromolecules. **(C)** Examples of axial fast T2-weighted MR images from reeler heterozygous (Het) mice, respectively (TR/TEeff = 3200/60 ms, ns = 4, slice thickness 0.6 mm, 24 slices, matrix 256 × 256, FOV = 25 mm × 25 mm, which correspond to voxel resolution of 98 × 98 × 600 μm^3). The red lines show the motor cortex thickness measure. **(D)** Example of brain segmentation for volumetric analyses of brain (plain line), cerebellum (dashed line), and ventricles (dotted line). **(E)** The histogram shows metabolite concentrations in hippocampus (Hip) for the two groups. Data are expressed as mean + SEM, $*P < 0.05$, $**P < 0.005$ between wildtype and heterozygous reeler mice. $N = 7$ Wt and $N = 7$ Het.

the high contrast that cerebrospinal fluid has in the MR images. **Figure 1D** shows an example of segmentation (slice central to the brain in sagittal images).

STATISTICAL ANALYSIS

A mixed-model ANOVA with repeated measures was used to analyze: (1) sniffing of different body areas (anogenital, body, or head), (2) spontaneous locomotor activity in the home-cage, (3) number of USVs for each minute of interaction, and (4) probability of vocalizations in eight call categories with genotype as between-subject factor. Probability of vocalizations within each genotype was calculated as number of calls in each category for each subject/total number of calls analyzed in each subject and standardized by angular transformation.

Data relative to MRI and MRS were analyzed by a one way ANOVA with genotype as the independent factor and MRI/MRS parameters (values of water T2, metabolite levels in each brain region and volume of each brain region) as dependent factor. Differences between genotypes in each brain region with respect to serotoninergic, dopaminergic, and noradrenergic systems (5-HT, 5-HIAA, and 5-HT turnover for serotoninergic system; DA, HVA, DOPAC, and DA turnover for dopaminergic system; and NA, MOPEG, and NA turnover for noradrenergic system) were determined by a multivariate analysis of variance (MANOVA), due to the potentially high correlation between these dependent variables within each system. Pillai's statistic was used. Univariate ANOVAs were conducted for each variable (Statview II, Abacus Concepts, CA, USA).

For all comparisons, data are expressed as mean ± SEM and significance was set at $P < 0.05$. *Post hoc* comparisons were performed using Tukey's test only when a significant F-value was determined.

RESULTS

MALE–FEMALE SOCIAL INTERACTION TEST

To assess the presence or absence of a social communication deficit in Het reeler mice, we evaluated the behaviors and the USVs emitted by a male mouse during the interaction with an estrus C57BL/6J female. Analysis of the social sniffing response on different body areas (head, body, and anogenital) did not reveal significant effects of genotype [frequency, $F(2,56) = 0.15$, $P = 0.858$, (data not shown); duration, $F(2,56) = 1.74$; $P = 0.183$, **Figure 2A**]. No genotype effect was detected on explorative behaviors such as *rearing* [frequency, $F(1,28) = 0.27$, $P = 0.610$ and duration, $F(1,28) = 0.30$, $P = 0.589$] and *digging* [frequency, $F(1,28) = 0.75$, $P = 0.392$ and duration, $F(1,28) = 1{,}15$, $P = 0.292$] (data not shown).

Analysis of the USVs emitted by male mice during the social interaction test did not detect significant differences between Het reeler and Wt mice: number of USVs [number of calls × genotype, $F(2,56) = 0.89$, $P = 0.41$, **Figure 2B**], mean duration [$F(2,54) = 0.79$, $P = 0.457$ (data not shown)], peak frequency [$F(2,54) = 0.43$, $P = 0.650$ (data not shown)], and peak amplitude [$F(2,54) = 0.14$, $P = 0.863$ (data not shown)]. As a whole, the pattern of sonographic structures did not differ between Het reeler and Wt mice indicating a comparable vocal repertoire in both genotypes (see pie graphs in **Figure 2C**).

LOCOMOTOR ACTIVITY IN THE HOME-CAGE

Sleep problems and irregular sleep–wake cycles have been identified in several ASD children (68–71). Alterations in circadian rhythm lead to anxiety-like, impulsive, and depressive behaviors both in humans and mice (72–74). In the present study, we evaluated baseline circadian locomotor activity in the home-cage as well as response to a mild stressful stimulus represented by a saline injection to check for abnormalities in the spontaneous locomotor activity that could affect the behavioral performances.

Analysis of spontaneous locomotor activity measured in the home-cage for 7 days revealed, as expected, an increased activity in mice of both genotypes during the dark phase of the light/dark cycle [phase effect, $F(1,17) = 239.05$, $P < 0.001$] (see **Figure 3**). No genotype differences were found [light phase: genotype, $F(1,17) = 2.99$, $P = 0.102$; dark phase: genotype, $F(1,17) = 1.34$, $P = 0.263$].

The analysis of the locomotor profile over a period of 7 h after saline injection (11–18, dark phase) was performed to evaluate the immediate stress response. Het reeler mice increased significantly their locomotor activity as compared to Wt mice [genotype, $F(1,17) = 5.63$, $P = 0.029$] thus revealing a genotype-dependent increased sensitivity to mild stress challenge (see graph on the top right of **Figure 3**). After this 7-h-period, locomotor activity goes back to the normal activity profile [genotype effect, $F(1,17) = 0.226$, $P = 0{,}64$; genotype × hours, $F(1,23) = 0.38$, $P = 0.99$; data not shown].

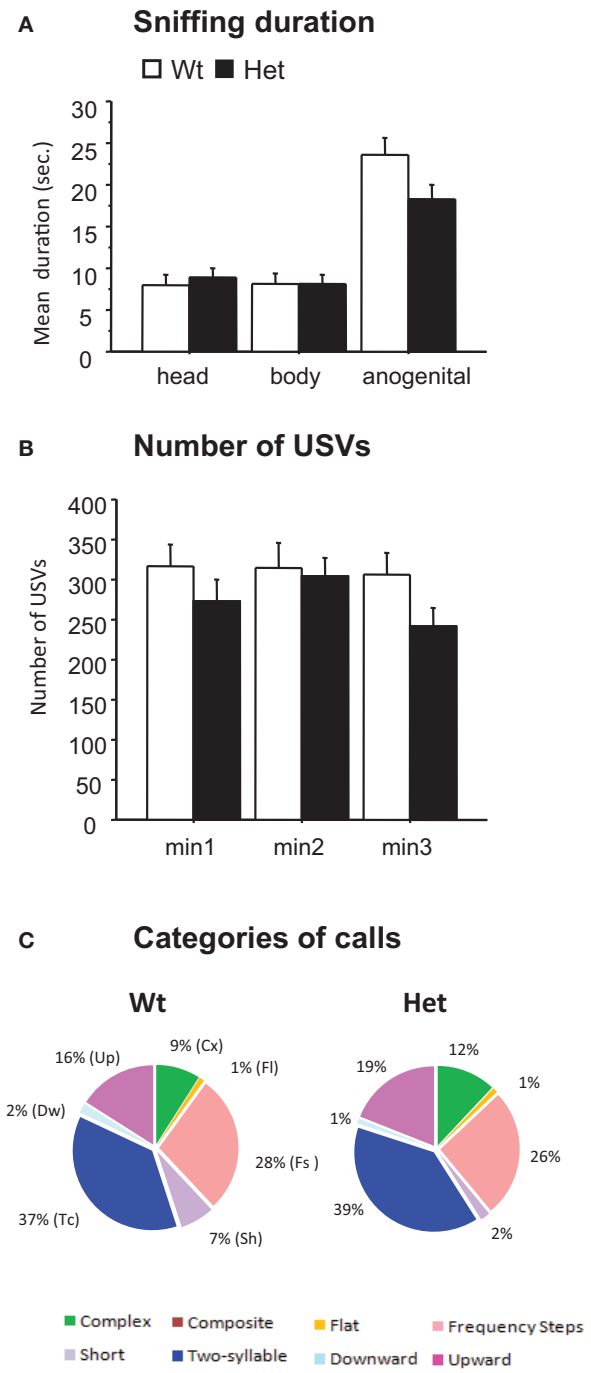

FIGURE 2 | Male–female social interaction test (3-min-session) performed in 3-month-old reeler mutant mice. Parameters measured during a direct interaction between a male with a sexually receptive C57BL/6J female. **(A)** Sniffing duration. **(B)** Number of ultrasonic vocalizations. Data are expressed as mean + SEM. **(C)** Pie graphs show the percentages of the different call categories emitted by Wt and Het reeler mice. Percentages were calculated in each genotype as number of calls in each category for each subject/total number of calls analyzed for each subject. Number of calls analyzed: 17195 in Wt and 8454 in Het. $N = 9$ Wt and $N = 21$ Het.

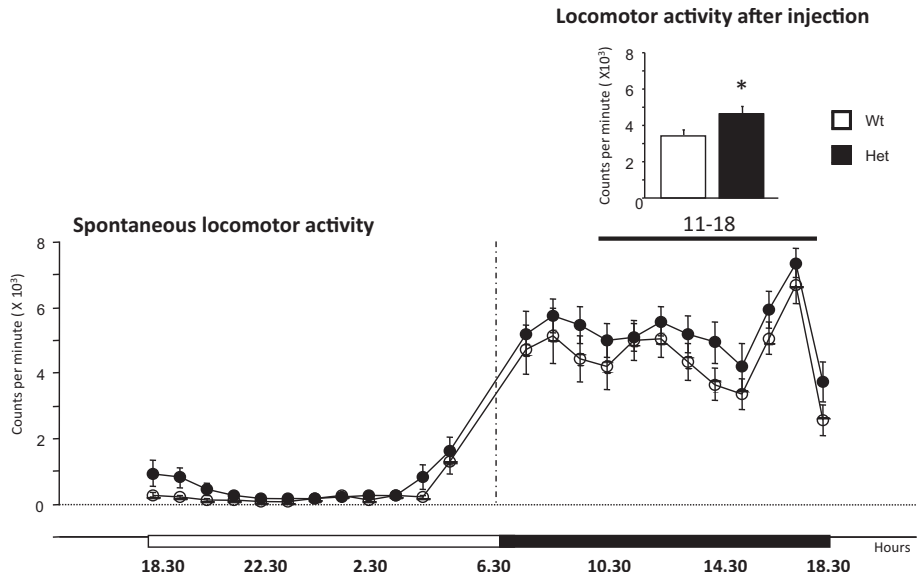

FIGURE 3 | Locomotor activity expressed spontaneously in the home-cage by 6-month-old wildtype and heterozygous reeler male mice. The main graph indicates spontaneous locomotor activity monitored for 1 week. The graph on the top right shows the spontaneous locomotor activity monitored for 7 h after a mild stressful stimuli represented by a saline injection at 11 h (dark phase). Data are expressed as mean ± SEM, $*P < 0.05$, between wildtype and heterozygous reeler mice. $N = 9$ Wt and $N = 10$ Het.

MONOAMINES AND THEIR METABOLITES: HPLC DETERMINATION

High performance liquid chromatography determination has been applied to investigate whether *reelin* mutation influences the different monoamine systems. MANOVA revealed a significant genotype effect on several components of the dopaminergic system in hypothalamus [Pillai's Trace: $F(4,13) = 9.73$, $P < 0.001$] and in Hip [Pillai's Trace: $F(4,13) = 4.97$, $P = 0.005$]. ANOVA showed a strong reduction of DA levels [genotype effect, $F(1,16) = 5.29$, $P = 0.035$] and a consequent increase in the DA turnover [genotype effect, $F(1,16) = 7.43$, $P = 0.015$] in the cortex of Het reeler as compared to Wt mice (see **Table 1**). Moreover, Het reeler mice showed an increase of DOPAC and HVA levels [genotype effect: DOPAC, $F(1,16) = 18.88$, $P < 0.001$; HVA, $F(1,16) = 11.47$, $P = 0.003$] in hypothalamus. ANOVA evidenced also a decrease of HVA levels [$F(1,16) = 6.43$, $P = 0.022$] and an increase of DA turnover [genotype effect, $F(1,16) = 6.14$, $P = 0.025$] in Hip of Het mice.

Heterozygous mice showed an higher DA levels [genotype effect, $F(1,16) = 4.38$, $P = 0.052$] and a lower DA turnover in olfactory bulb [genotype effect, $F(1,16) = 6.87$, $P = 0.018$] than Wt mice. No genotype related differences were found on noradrenergic and serotoninergic systems in each brain areas analyzed.

MAGNETIC RESONANCE IMAGING

To acquire deeper information into the functional state of brain areas involved in ASD, we assessed a ^{1}H magnetic resonance in adult reeler male mice. Enlarged ventricles and reduced cerebellum are typical features of reeler mice (75). Volumetric analyses confirmed a cerebellum reduction [genotype effect, $F(1,10) = 15.50$, $P = 0.002$] and an enlargement of ventricles volume [genotype effect, $F(1,11) = 8.01$, $P = 0.016$] in Het reeler when compared to Wt mice. No genotype differences were detected in volume [genotype effect, $F(1,11) = 0.86$, $P = 0.374$] and medial motor cortex (MC) thickness [genotype effect, $F(1,11) = 3.62$, $P = 0.083$] (see **Table 2**).

MAGNETIC RESONANCE SPECTROSCOPY

To investigate the possible alterations in brain metabolism of adult reeler male mice, we performed MRS. The high quality spectra allowed reliable quantification (%SD <20%) not only for the commonly observed *N*-acetyl-aspartate (NAA), total creatine (Cr + PCr) and total choline resonances (NAA + NAAG), but also for glutamine (Gln), glutamate (Glu), taurine (Tau), and inositol (Ins) in all the investigated brain regions.

Water T2 analyses confirmed that no changes between the genotypes occurred in the T2s in Hip [$F(1,10) = 2.52$, $P = 0.143$], Striatum [$F(1,10) = 0.02$, $P = 0.883$], Thalamus [$F(1,10) = 1.15$E-5, $P = 0.977$], and Cerebellum [$F(1,10) = 0.32$, $P = 0.584$] (data not shown).

Metabolic changes were detected in Hip while no differences have been found for any metabolite in thalamus, striatum, and cerebellum. Het reeler mice showed increased levels of Glu [genotype effect, $F(1,11) = 4.61$, $P = 0.044$], Tau [genotype effect, $F(1,11) = 4.82$, $P = 0.050$], PCr [genotype effect, $F(1,7) = 18.08$, $P = 0.003$], and total amount of PCr + Cr [genotype effect, $F(1,11) = 24.68$, $P < 0.001$] in Hip as compared to Wt mice (see **Figure 1E**).

DISCUSSION

Reelin is a glycoprotein playing a crucial role during development: it regulates neuronal migration and brain lamination (6, 8, 29, 30, 76, 77) and its reduced or complete lack of signaling impairs

Table 1 | Levels of monoamines and their metabolites detected *ex vivo* in cortex, bulbs, hypothalamus, striatum, hippocampus, and cerebellum (mean ± SEM: picogram per milligram of wet tissue).

Brain region	Genotype	Neurotransmitter, metabolite concentration picogram per milligram wet brain weight)						
		Noradrenergic system		Dopaminergic system			Serotoninergic system	
		NA	MOPEG	DA	DOPAC	HVA	5-HT	5-HIAA
Cortex	Wt	469.60 ± 22.99	102.74 ± 7.53	874.14 ± 268.95	134.50 ± 19.85	252.97 ± 35.58	523.10 ± 29.72	341.42 ± 17.59
	Het	440.84 ± 28.76	102.47 ± 6.20	296.37 ± 69.32*	108.65 ± 18.65	184.52 ± 40.76	470.09 ± 31.15	285.37 ± 14.32
Bulbs	Wt	291.82 ± 24.28	46.35 ± 5.94	312.11 ± 33.66	100.26 ± 8.63	152.87 ± 15.49	247.65 ± 25.63	191.88 ± 16.88
	Het	346.10 ± 14.98	66.26 ± 6.28	396.15 ± 23.84*	102.06 ± 6.83	154.78 ± 8.95	131.53 ± 20.97	192.13 ± 9.76
Hypothalamus	Wt	1789.74 ± 80.36	205.08 ± 23.06	394.87 ± 16.83	116.63 ± 6.12	362.10 ± 13.19	518.33 ± 22.43	934.82 ± 42.78
	Het	1742.52 ± 81.41	183.74 ± 7.50	540.86 ± 163.65	144.44 ± 5.48**	444.55 ± 13.27**	487.36 ± 29.79	864.22 ± 19.73
Striatum	Wt	129.71 ± 32.78	339.05 ± 49.89	12521.13 ± 1661.54	2718.95 ± 326.72	4310.08 ± 383.59	453.07 ± 30.88	654.30 ± 23.09
	Het	128.12 ± 19.16	261.70 ± 25.90	12275.97 ± 1376.40	2847.63 ± 196.75	4583.34 ± 398.19	109.34 ± 26.48	679.84 ± 24.65
Hippocampus	Wt	508.02 ± 33.64	115.37 ± 6.77	82.16 ± 7.61	18.21 ± 1.33	69.47 ± 8.48	505.52 ± 61.78	540.99 ± 37.90
	Het	423.67 ± 39.12	108.17 ± 8.75	84.80 ± 13.48	25.16 ± 4.81	42.01 ± 6.93*	580.54 ± 33.18	503.31 ± 53.05
Cerebellum	Wt	402.46 ± 28.48	48.85 ± 3.12	5.09 ± 1.06	12.52 ± 2.38	80.66 ± 5.09	115.60 ± 17.51	129.05 ± 6.03
	Het	433.56 ± 17.08	46.17 ± 3.42	6.75 ± 2.68	18.77 ± 7.55	79.52 ± 3.59	118.94 ± 21.13	126.85 ± 6.04

Brain region	Genotype	Neurotransmitter turnover		
		NA	DA	5-HT
Cortex	Wt	0.22 ± 0.01	0.24 ± 0.05	0.66 ± 0.03
	Het	0.24 ± 0.01	0.45 ± 0.05*	0.62 ± 0.03
Bulbs	Wt	0.16 ± 0.02	0.33 ± 0.02	0.80 ± 0.05
	Het	0.19 ± 0.01	0.26 ± 0.02*	0.86 ± 0.05
Hypothalamus	Wt	0.11 ± 0.01	0.30 ± 0.02	1.81 ± 0.09
	Het	0.11 ± 0.01	0.35 ± 0.04	1.83 ± 0.11
Striatum	Wt	4.09 ± 1.01	0.23 ± 0.03	1.47 ± 0.06
	Het	2.86 ± 0.7E	0.26 ± 0.03	1.71 ± 0.10
Hippocampus	Wt	0.23 ± 0.01	0.23 ± 0.02	0.95 ± 0.11
	Het	0.26 ± 0.02	0.29 ± 0.02*	0.87 ± 0.07
Cerebellum	Wt	0.12 ± 0.01	2.83 ± 0.51	1.25 ± 0.13
	Het	0.11 ± 0.01	6.76 ± 2.45	1.36 ± 0.23

*$P < 0.05$, **$P < 0.005$ between wildtype and heterozygous reeler mice.*

Table 2 | Analysis for forebrain, cerebellum, and ventricles volume.

	Forebrain volume (μ1)	Ventricles volume (μ1)	Cerebellum volume (μ1)	Medial cortex thickness (mm)
Wt	366.17 ± 2.6	3.29 ± 0.5	59.18 ± 1.2	1.16 ± 0.1
Het	369.85 ± 3.0	5.25 ± 0.4*	53.03 ± 1.1*	1.22 ± 0.1

*Data are expressed as mean ± SEM. *P < 0.05, between wildtype and heterozygous reeler mice.*

neuronal connectivity and synaptic plasticity (43, 78). Moreover, recent data suggest that a defect in reelin signaling pathway confers greater susceptibility to autism (20–25, 27).

For these reasons, we consider Het reeler mice, haploinsufficient for *reelin* and sharing some neurochemical and behavioral features with autistic patients, a suitable animal model for studying the effects of *reelin* deficiency in determining social communication deficits and in changing brain monoamine and brain metabolites levels. Unfortunately, no comparison can be drawn with homozygous mutant mice, since adult knockout reeler mice did not survive longer than weaning (79–82).

NO DEFICITS IN SOCIAL AND VOCAL REPERTOIRES DURING COURTSHIP

To our knowledge, this is the first time that a detailed analysis of the adult male vocal repertoire has been performed in this mutant line. Only behavioral data on same-sex interactions or approaching/recognizing a conspecific have been collected (44, 51, 52).

Recently, we characterized vocal and motor repertoires on homozygous and Het reeler pups (60) evidencing a general delay in vocal and motor development during the first 2 weeks of postnatal life, in line with the alterations in the same two systems observed in children with ASD. In addition, a preferential use of a specific call category (two-components) at pnd 2 and 6 was detected in both mutants (Het and homozygous), whereas an increased number of vocalizations characterized only Het pup's emission.

Contrary to what we found in pups, adult Het male mice did not show deficits on USVs emitted during courtship of a female in estrous. Social behaviors, generally associated to this vocal emission, were not affected either. These results are in contrast with the reduction in anogenital sniffing and/or the number of USVs found in other ASD animal models such as BTBR, En2, NMDA-Nr1, NLG3, NLg4, Dlg4, and FmR1 mice (53, 83–87), but in line with data collected on Shank3 mice, carrying a mutation strongly implicated in autism and Phelan-McDermid 22q13 deletion syndrome, where male knockout mice did not present alterations in social communication and interaction (88).

These data thus confirm that adult Het reeler mice present deficits on cognitive performances but not on social domains (44–47, 49–52). It is worth of notice that intellectual disabilities are present in about 50% of autistic individuals. Due to the cerebellar alteration leading to death shortly after weaning, no data could be collected on mice with the complete deletion in the reelin gene, thus we cannot exclude an impairment of the social domain only related to the complete deletion of *reelin*.

OVER RESPONSE TO A MILD STRESS STIMULI

Previous studies indicated that Het reeler mice have several abnormalities in their brain architecture (40–42), but, at a first sight, their phenotype is absolutely "normal" (7, 44, 89). Some behavioral deficits become evident only after a "second hit" (7, 63, 90, 91) supporting the "double-hit" theory postulating a gene–environment interaction in the pathogenesis of several neurodevelopmental disorders such ASD (89). Depending on the features of environmental factors and the time-window of insult interacting with reelin expression, an individual could thus develop one neurodevelopmental disorder rather than another one (i.e., schizophrenia versus ASD).

Our previous evidence shows that either an environmental pollutant or, for example, an activated stress reactivity caused by repeated separation from the dams, elicits different responses as a function of the mouse genotype (91). In line with these data, in the present study, no significant genotype differences were found in basal activity levels of mice monitored in their home-cages for 1 week. By contrast, after a saline injection (a mild stressful stimulus), the Het male displayed a higher locomotor activity profile as compared to Wt male mice. Already in a previous study, our group showed a hyperactive profile in Het adolescent reeler mice following handling plus saline injections (63). Altogether, these data indicate that Het reeler mice show a different response to environmental stimuli, confirming the suitability of such mutant line for the study of gene–environment interactions (7, 92).

Moreover, a deficit in behavioral inhibition has been reported as a core alteration of Het reeler mice, associated with dysfunctions of mesolimbic DA transmission (93) and reduced GABAergic transmission in central nervous system (40, 94–96).

IMPAIRMENT IN THE DOPAMINERGIC PATHWAY

To correlate observed behavioral abnormalities to the neural systems reportedly affected by *reelin* mutation, we conducted HPLC analyses in different brain areas involved in autism, detecting impairments in the dopaminergic system. Specifically, Het reeler mice had decreased DA levels in cortex and increased levels in the olfactory bulb, whereas DA turnover was altered in cortex, bulb, and Hip.

A disruption of DA maturation in *reelin* haploinsufficient mice had been already suggested: a reduced locomotor activation by D-amphetamine in reeler mice was associated with an exaggerated drug-induced stereotyped behavioral syndrome (90). Moreover, Ballmaier et al. (93) reported alterations in the mesolimbic DA pathway of Het reeler mice. In particular, they found that Het mice exhibit a reduction in DA transporter immunoreactivity and D2 receptor mRNA in the limbic striatum and the ventral tegmental area (93). In agreement with our study, they did not find any significant alteration in the dopaminergic markers examined in the nigrostriatal pathway of Het reeler mice.

Alterations in DA levels and its turnover have been found in brain areas primarily associated to reward. Individuals with ASD show reduced responsiveness to reward stimuli, a feature that appears to be especially prominent with social reinforces such as facial expressions, spoken language, and gestures (97, 98). No effects have been found in the striatum where DA contributes to motor performances.

In addition, the neurotransmitter DA plays a pivotal modulatory role on executive functions and learning (99, 100), thus a dysfunctional DA system could underlying the cognitive deficits detected in Het mice.

GLUTAMATE AND TAURINE INCREASED LEVELS IN HIPPOCAMPUS

To gain deeper insights into the functional state of brain areas involved in ASD, we carried out a ^{1}H MRI guided spectroscopy examination in adult reeler mice. MRS is a powerful, non-invasive tool for monitoring neurological diseases (101) and it is also used in clinical studies on autistic individuals (102). Abnormalities in neurotransmitter pathways have been associated to ASD, with evidence for a possible implication of glutamatergic, GABAergic, and serotonergic imbalances (102).

In the Hip, as compared to Wt in Het mice, MRS showed increased levels of glutamate, taurine, phospho-creatine, and of the total amount of phospho-creatine plus creatine. Glutamate is the main brain excitatory neurotransmitter involved in cognitive functions, although in excessive quantities can cause neuronal damages typical of neurodegenerative diseases (for example, Alzheimer's and Huntington's diseases) (103–105). The higher levels of glutamate in the Hip of Het reeler mice are in agreement with previous findings reporting an increase of glutamate in Hip (106) and cortex (107) of ASD patients; altogether these evidences support the hypothesis of an imbalance between excitatory and inhibitory (GABA) systems as one of the possible causes of autism (107).

Recently, clinical trials with glutamate antagonists have been initiated, since they have been proved to be effective in rescuing social deficits and repetitive behaviors in selected animal models of autism (108). Also the presence of high levels of taurine in the Hip could be correlated to high levels of glutamate. In fact, taurine appears to have a protective action against glutamate excitotoxicity (109) and it is widely considered a general index of neuronal functionality.

The largest meta analysis performed on ASD patients showed evidence that ASD is characterized by age-dependent fluctuations in metabolite levels across the whole brain. In particular, significant reduction in the level of a cerebral metabolites, NAA, a specific neuronal marker, in whole gray matter of ASD children as well as significant increase in the total pool of creatine (phospho-creatine plus creatine) in adult subjects were observed (110). The observed differences in creatine as a function of age and brain region, suggest caution in the use of Cr-based ratio measures of metabolites. For this reason, we adopt a quantitative approach for brain metabolites level determination, which has been validated on phantom (111) as well as on other animal models of psychiatric and neurodevelopmental diseases (51, 112–114).

CONCLUSION

In the literature, Het reeler mice are widely considered a reliable animal model of either autism or schizophrenia. Genetic and molecular evidences showed that reelin messenger-RNA and its protein are downregulated in cortical, hippocampal, and cerebellar neurons of patients suffering of schizophrenia and autism (3, 8, 10, 20, 115, 116). In particular, these mutant mice are characterized by decreased contextual fear conditioning (48), prepulse inhibition (43, 117), impaired executive functions (45), and associative learning (48), all typical traits of schizophrenia. In addition, Het reeler mice yielded autistic-like deficits in social behavior and communication in the first two postnatal weeks of age (60) and perseverative (51) and hyperactive behaviors (44) at adulthood. Discordant evidences exist on this model, possibly associated with differences in the genetic background, age of mice, training and testing protocols, and rearing conditions (52, 89).

Overall, our results, together with data previously collected by our (Laviola and collaborators) and other groups suggest that Het reeler mice have common behavioral traits to both these neurodevelopmental disorders. Moreover, these studies indicate the suitability of this mutant line to investigate the role of *reelin* as vulnerability factor on the etiology of both disorders. In addition, Het reeler mutant mice may represent a useful animal model to develop novel treatment strategies for these devastating human disorders. For example, our HPLC and MRS results favor further studies to evaluate the effects of DA agonist or glutamate antagonist treatments on behavioral and neurochemical responses.

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