AUTISM SPECTRUM DISORDERS: DEVELOPMENTAL TRAJECTORIES, NEUROBIOLOGICAL BASIS, TREATMENT UPDATE

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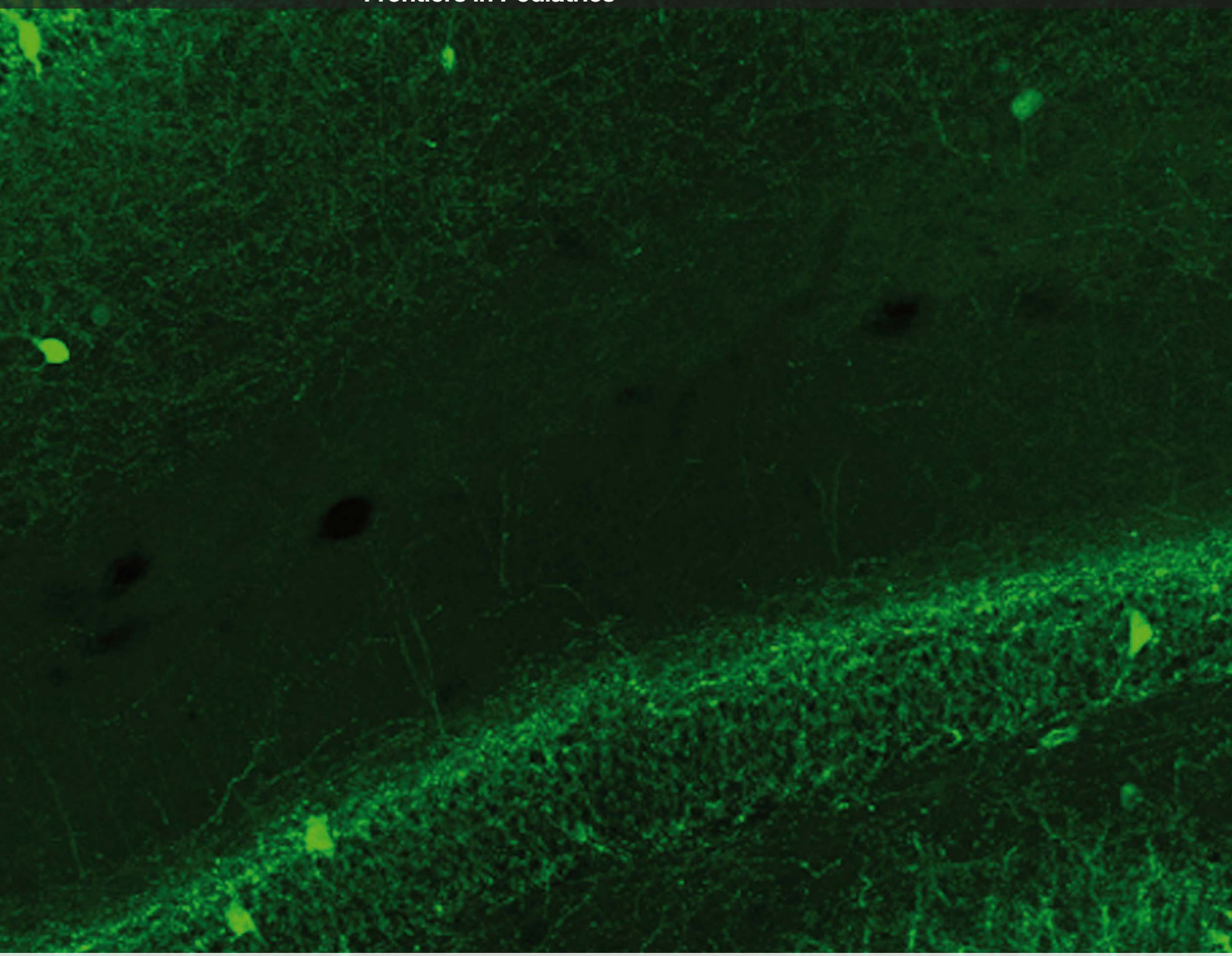

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AUTISM SPECTRUM DISORDERS: DEVELOPMENTAL TRAJECTORIES, NEUROBIOLOGICAL BASIS, TREATMENT UPDATE

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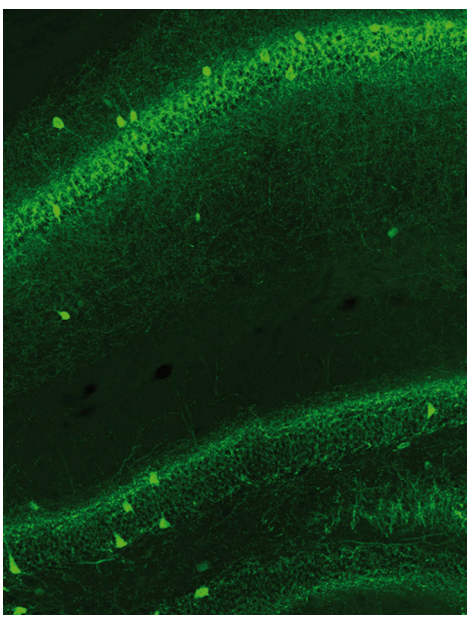

Cover image: Image shows parvalbumin-positive inhibitory neurons of the mouse hippocampus, labelled in green. Studies performed on human and mouse brain tissues that dysfunctions of parvalbumin neurons are associated to the pathogenesis of autism spectrum disorders.

This Research Topic has the aim to fill the gap of the many unresolved scientific issues on Autism Spectrum Disorders (ASD) that are still in need of investigation, Targeted treatments based on the understanding of the underlying pathogenic mechanisms of disease are still lacking. Further research is awaited and should be obtained through a significant effort on experimental treatment trials and neuroscience research.

This Topic is divided in two main sections, one covering clinical issues and another on basic neurosciences of Autism Spectrum Disorders. A more detailed description of the contents of the articles is provided in the editorial at the beginning of the issue.

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EDITORIAL
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Editorial: Autism Spectrum Disorders: Developmental Trajectories, Neurobiological Basis, Treatment Update

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Editorial on the Research Topic

Autism Spectrum Disorders: Developmental Trajectories, Neurobiological Basis, Treatment Update

This Research Topic stems from the urgent need to fill the gap of the many unresolved scientific issues on autism spectrum disorders (ASD) that are still in need of investigation, and follows another Research Topic we recently edited. As an example, targeted treatments based on the understanding of the underlying pathogenic mechanisms of disease are still lacking. Further research is awaited and should be obtained through a significant effort on experimental treatment trials based on consistent "proof-of-concept" preliminary results. In the first contribution listed in this editorial, a fundamental question is raised about the current limitations in preclinical models and the need for a thorough reappraisal of their application in clinical studies of ASD. This is one of the main reflections on current investigation that we like to draw attention upon. Though we are confident that we are providing valuable contributions on different themes, we are aware that many other important issues have not been dealt with. This Research Topic has therefore major limitations and does not include all the issues that deserve further research in the vast field of ASD research. The following is an overview of the articles collected in this Topic.

Loth et al. provided an overview of current issues in ASD diagnosis and treatment. The phenotypic and etiological variability between individuals with ASD, together with the lack of effective treatments, urgently point to a precision medicine approach. Such an approach aims to identify targeted treatments based on the understanding of the underlying pathogenic mechanisms of disease that might be tackled by specific interventions, both as pharmacotherapy or behavioral intervention. To this purpose, stratification biomarkers are useful tools to select or exclude patients for a particular treatment. Further, different factors that may impact on developmental outcome have been detailed. Common variants and genetic background are to be thoroughly considered. The environmental risk factors for ASD include maternal infections and prenatal exposure to teratogenic agents such as valproate acid, exposure to toxins, and dysfunction of the immune system. Finally, the timing of impact of genetic and environmental agents on neuronal development likely plays a crucial role in determining the outcome. An important remark on factors implicated in failed clinical trials in ASD points to the limited translatability from animal models to humans. As to the new perspectives of investigation, patient-derived induced pluripotent stem cells are an interesting new tool that overcomes inter-species differences and deserves further development.

Following this line of thought, other studies investigated key gene pathways involved in ASD pathogenesis. Among the potential candidate genes identified in ASD, those involved in Akt/mammalian target of rapamycin (mTOR) signaling and the downstream effects of this pathway are highly represented including *FMR1*, *PTEN*, *TSC1*, and *TSC2*. Aberrant Akt/mTOR signaling has the potential to impact cellular growth, proliferation, and cytokine production in the immune system, which can in turn affect behavior. The activity of the mTOR pathway in cells obtained from children with ASD and typically developing controls was investigated by Onore et al. Elevated higher activity of mTOR and lower activity of glycogen synthase kinase 3 α and tuberin (TSC2) in cells from children with ASD were observed. In addition, the study showed a phosphorylation pattern supporting higher activity in the Akt/mTOR pathway in children with ASD, and not limited to known ASD-associated Akt/mTOR genetic mutations mentioned above. This common pathological pathway needs further investigation as the abnormalities in Akt/mTOR signaling observed in this study are likely not limited to T cells but would be detected also in other immune cells and have relevance to overall immune abnormalities observed in ASD. Transcriptome analyses highlighted that gene networks involved in synapse development, neuronal activity, and immune function are deregulated in ASD and prompts to carry out further research to shed light on these issues. Mouse models provide unique tools to investigate the neurobiological basis of ASD. In their study, Provenzano et al. used two well-recognized ASD mouse models, BTBR and Engrailed-2 knockout, to identify conserved clusters of ASD-related genes. Each of these clusters (modules) showed a specific enrichment profile in neuronal and glial genes, as well as in genes associated to ASD comorbidities such as epilepsy and schizophrenia. Significant transcriptional similarities and differences between the BTBR and *En2*$^{-/-}$ hippocampus were detected and thoroughly detailed. This study also underscored that transcriptome analysis of ASD mouse models may contribute to identify novel molecular targets for pharmacological studies. Indeed, ASD and schizophrenia spectrum disorders (SSD) share clinical and genetic components and co-occur more frequently than would be predicted by their respective prevalence. As detailed in the contribution of Canitano et al., a complex, multifactor association is implicated in both conditions. As a current hypothesis, social and cognitive disturbances in ASD and SSD derive from abnormalities in the ratio of excitatory to inhibitory cortical activity (E/I imbalance). Altered functions of genes coding for glutamatergic and GABAergic brain receptors and/or synaptic proteins would be at the origin of systems derangement. Current understanding of shared and divergent patterns between ASD and SSD from molecular to clinical aspects is far from clear. The Research Domain Criteria approach is promising to guide future progress and accomplishments in this field because it represents a new framework for carrying on research in neurodevelopmental disorders that diverge significantly from current standards. The aim is to build a research literature that reflects advances in genetics and neurosciences including behavioral science to provide a consistent foundation for precision diagnosis and treatment of ASD.

An original conceptualization of the emergence of language disturbances in ASD has been proposed by Benitez-Burraco et al. It has been hypothesized that language appearance would be linked to changes in the human brain/skull associated to the process of self-domestication of the human species. Individuals with ASD would exhibit less marked domesticated traits at the morphological, physiological, and behavioral levels. In addition, many ASD candidate genes are represented among the genes known to be involved in the "domestication syndrome," e.g., the constellation of traits exhibited by domesticated mammals deriving from the hypofunction of the neural crest and also among the set of genes involved in language function. Some candidate genes for domestication and language development showed the same expression profile in people with ASD and chimps in brain areas involved in language processing. As a result, ASD may represent an abnormal ontogenetic trajectory for the human faculty of language resulting from mutations in genes important for the "domestication syndrome" and from the normal functioning of the neural crest.

Identification of molecular and structural biomarkers is crucial to identify novel, early diagnostic tools. A study on biomarkers in 94 children with ASD was carried out by Frye et al., providing important clues in relation to distinct profiles as to folate receptor autoantibodies receptors FRAA with implication for diagnosis and treatment. Folate receptor α (FRα) autoantibodies (FRAAs) are rather frequent in ASD and disrupt the transport of folate across the blood–brain barrier by binding FRα. Children positive for the binding FRAA were found to have higher serum B12 levels as compared to those negative for binding FRAAs. Conversely, children positive for the blocking FRAA were found to have relatively better redox metabolism and inflammation markers as compared to those negative for blocking FRAAs. In addition, ASD children positive for the blocking FRAA showed better communication on the Vineland Adaptive Behavior Scale, stereotyped behavior on the Aberrant Behavioral Checklist and mannerisms on the Social Responsiveness Scale. Altered zinc (Zn) homeostasis has been reported in fibroblasts from >60 years old Fragile X premutation carriers. Napoli et al. tested FMRP protein expression, brain bioenergetics, and expression of the Zn-dependent synaptic scaffolding protein SH3 and multiple ankyrin repeat domains 3 (Shank3) in a knockin premutation mouse model of Fragile X syndrome. Significant deficits in brain bioenergetics, Zn levels, and Shank3 protein expression were observed in these mice, and the authors provided evidence that in premutation carriers, altered Zn homeostasis, brain bioenergetics, and Shank3 levels could be compounded by Zn-deficient milk, increasing the risk of developing emotional and neurological/cognitive problems. Liska and Gozzi describe the progress in mouse brain connectivity mapping by means of resting-state functional magnetic resonance imaging, which opens a new avenue of investigation in ASD. This technique allows to test mechanistic hypotheses about the abnormal connections observed in ASD and examples are illustrated of how this approach can be used to establish causal links between ASD-related mutations, developmental processes, and brain connectivity architecture. The role of insulin-like growth factor 1 (IGF-1) in treating neurodevelopmental

disorders including ASD is thoroughly dealt with by Vahdatpour et al. The promising potential of this polypeptide is ranging from Rett syndrome to X-fragile and ASD, due to the effects that IGF-1 exerts in the development, growth and maturation of the CNS and its synapses. Putative mechanisms of action of IGF-1 are delineated in the different disorders. In a double blind, placebo controlled Phase 2 trial, the safety and preliminary efficacy of IGF-1 treatment were reported on nine patients with Phelan and McDermid syndrome aged 5–15. The exact role of the IGF-1 in overall ASD is still unclear, as results of previous studies are conflicting. At present, clinical trials of IGF-1 in ASD are ongoing and will possibly shed light on this issue. Finally, Billeci et al. reviewed recent research findings on the neurostructural and neurofunctional substrates in parents of individuals with ASD (pASD). The primary hypothesis was that, like for the behavioral profile, the pASD express an intermediate neurobiological pattern between ASD individuals and healthy controls. The 13 reviewed studies showed that pASD are generally different from healthy controls at a structural and functional level despite often not behaviorally impaired.

Another series of studies described the importance of detailed behavioral characterization of ASD patients and the development of novel strategies of intervention. Orienting and social interest was investigated in a group of children with ASD (age >3 years) in an interesting study by Franchini et al. By means of an eye-tracking task, visual preference for social stimuli was measured in children with ASD compared to typically developing (TD) children. Reduced interest for biological motion in children with ASD compared to TD children was detected and was associated with better adaptive functioning in preschoolers with ASD. Moreover, longitudinal results showed that a preference for biological motion clearly predicted decreased severity of ASD symptoms. Eye-tracking technique confirms to be a very useful tool for collecting valuable information of developmental profile of children with ASD at early age. A thorough analysis of stereotypies in children with various disorders other than ASD was presented by Cardona et al. The complexity of stereotypies presentation urges a comprehensive standardized evaluation including developmental and clinical components that eventually provide the correct definition. Further, this approach would be fruitful to improve our knowledge of such a heterogenous field. A novel model of intervention called "Developmental and Sequenced One-to-One Educational Intervention" (DS1-EI) in 5- to 9-year-old children with ASD and intellectual disabilities (ID) was set up and presented by Tanet et al. Aim of this study was to describe a school-based intervention that was adapted to the French health and education system with the basis to implement the method and adapt it to a low-functioning population of ASD children. The treatment protocol was adapted for school implementation using an educational agenda, and the intervention was based on various principles such as intensity, regular assessments, updating objectives, encouraging spontaneous communication, promoting skills through play with peers, etc. The model was applied in 11 French institutions in small classrooms and holds promise for further application at school in this subgroup of children with ASD and ID. Bonn et al. described an automated gaming platform enabling intensive intervention in nomadic settings. The games involved application of visual and audio stimuli with multiple difficulty levels and a wide variety of tasks and actions pertaining imitation and joint attention. Performance of the platform was assessed in a 3-month open trial with 10 children with ASD, and parents highlighted enhancement in the child's concentration, flexibility, and self-esteem in 78, 89, and 44% of the cases, respectively. This pilot study shows the feasibility of using the developed gaming platform for home-based intensive intervention.

AUTHOR CONTRIBUTIONS

Equal contribution by RC and YB.

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PERSPECTIVE
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Defining Precision Medicine Approaches to Autism Spectrum Disorders: Concepts and Challenges

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The tremendous clinical and etiological variability between individuals with autism spectrum disorder (ASD) has made precision medicine the most promising treatment approach. It aims to combine new pathophysiologically based treatments with objective tests (stratification biomarkers) to predict which treatment may be beneficial for a particular person. Here we discuss significant advances and current challenges for this approach: rare monogenic forms of ASD have provided a major breakthrough for the identification of treatment targets by providing a means to trace causal links from a gene to specific molecular alterations and biological pathways. To estimate whether treatment targets thus identified may be useful for larger patient groups we need a better understanding of whether different etiologies (i.e., genetic and environmental risk factors acting at different critical time points) lead to convergent or divergent molecular mechanisms, and how they map onto differences in circuit-level brain and cognitive development, and behavioral symptom profiles. Several recently failed clinical trials with syndromic forms of ASD provide valuable insights into conceptual and methodological issues linked to limitations in the translatability from animal models to humans, placebo effects, and a need for mechanistically plausible, objective outcome measures. To identify stratification biomarkers that enrich participant selection in clinical trials, large-scale multi-modal longitudinal observational studies are underway. Addressing these different factors in the next generation of research studies requires a translatable developmental perspective and multidisciplinary, collaborative efforts, with a commitment to sharing protocols and data, to increase transparency and reproducibility.

Keywords: autism spectrum disorder, biomarkers, precision medicine

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INTRODUCTION

When parents first receive a diagnosis of autism spectrum disorder (ASD) of their child, some of their most pressing questions are: what is the prognosis of my child? What has caused his/her autism? And what are the treatment options?

Autism spectrum disorder is a clinically and etiologically heterogeneous condition, currently estimated to affect between 1 and 1.5% of children and adults worldwide (1, 2). Diagnostic ascertainment is based on the behavioral symptom profile alone; the co-occurrence of

social-communicative deficits, repetitive and restricted behaviors and interests, and sensory processing anomalies [DSM-5 (3)]. In addition, up to 70% of individuals have one or more psychiatric and/or medical comorbidities, such as intellectual disability, ADHD, irritability, aggression, anxiety, depression, epilepsy, and sleep anomalies (4).

The prognosis is very variable. A recent large-scale longitudinal study showed distinct developmental trajectories in children between the ages of 2 and 14 years (5). Children whose symptoms were least severe at first diagnosis showed the most symptom improvement. However, a subgroup of around 10% of children who presented with the most severe social deficits at age 3 years made significant gains in their social trajectory across childhood. Nevertheless, for the majority of people with ASD, outcome in adulthood has been estimated to be "poor" (46%) or even "very poor" (12%) (6). IQ and language level are widely considered the best predictors of outcome. Beyond this, it is currently largely unknown whether different developmental trajectories may reflect different biological subgroups, and why some individuals develop comorbidities but others do not.

To date, no effective medical treatments are available that significantly improve the core symptoms of ASD. Only two medications (the second-generation antipsychotics risperidone and aripiprazole) have been approved in ASD by the US Food and Drugs Administration (FDA) and one (risperidone) by the European Medicines Agency (EMA). Both medications are not specific for ASD and target associated symptoms, such as aggression or irritability. Instead, the management of ASD relies heavily on behavioral and educational interventions (7). Although several of these programs report significant improvements, difficulties in generalizing skills to "real-world" settings, and access to these treatments and their expense, remain common limitations (8).

THE CALL FOR A PRECISION MEDICINE APPROACH

Recognition of the phenotypic and etiological variability between individuals on the autism spectrum and the lack of effective treatments has called for a precision medicine approach. This approach aims to identify targeted treatments based on the understanding of the underlying pathophysiology and to then combine the drug (or intervention) with a companion diagnostic (stratification biomarker) to select or exclude patients for a particular treatment. Below we review current progress and discuss some of the challenges and requirements that still lie ahead.

Perhaps the biggest breakthrough for drug discovery in ASD came from the identification of syndromic and monogenic forms of ASD (where the disorder is thought to be caused by a highly penetrant single gene). Hundreds of ASD risk genes have been identified (9, 10), and more are expected to be found over the next years through whole-genome sequencing and studies with larger sample sizes. The significance of these discoveries lies in their potential ability to identify a causal link from a gene to cellular and molecular mechanisms underlying ASD symptoms (11). Moreover, although each of these monogenic forms is rare (i.e., found in less than 1% of individuals with ASD) different genes have been shown to converge on affecting a much smaller number of common pathways (12). This finding is crucial as it means that a particular biological pathway could be a treatment target rather than individual gene products. Treatments thus identified may therefore, potentially, be applicable for broader patient groups. Many of these risk genes modulate pathways involved in synapse formation and function, as well as other cellular functions, such as chromatin remodeling and transcription, protein synthesis and degradation, and receptor signaling (10). Any of these mutations may therefore alter essential developmental processes *in utero* or shortly after birth (13). For example, abnormalities in synapse development, function, and plasticity may broadly impact the balance between excitation (mainly modulated by glutamate) and inhibition (mainly modulated by gamma-aminobutyric acid, GABA) In particular, it has been suggested that (some forms of) ASD may be linked to disproportionally high levels of excitation and cortical network function (14).

Animal models of syndromic and monogenic forms showed significant pre-clinical promise such that several molecular aberrations and behavioral phenotypes could be reversed through pharamacological treatment or genetic rescue – sometimes even in adulthood (15, 16). Subsequent Phase I clinical trials using mGluR antagonists, or a $GABA_B$ agonist, also reported promising results.

However, well-powered (Phase IIb or Phase III) double-blind placebo-controlled clinical trials with individuals with Fragile X syndrome so far produced disappointing results (17). For example, two clinical trials, led by Roche and Novartis, respectively, reported a lack of efficacy of their mGluR-inhibiting drugs (RG7090 and mavoglurant) in Fragile X syndrome. Similarly, a trial with arbaclofen, led by Seaside Therapeutics, failed to find significant improvements in individuals with Fragile X and idiopathic autism relative to placebo. This highlights several factors.

First, even monogenic or syndromic forms also involve considerably heterogeneous symptom expression. For example, although a SHANK3 haploinsufficiency (thought to cause Phelan McDermid Syndrome) is one of most penetrant genetic risk factors for ASD [approximately 70% (18)], it can also lead to ADHD, schizophrenia, or bipolar disorder in a smaller number of individuals (19). About 30% of people with Fragile X syndrome have ASD (20). Others present with ADHD, anxiety and avoidance behavior, mood instability, or aggressive behavior (21). This variable expressivity appears to be not solely explained by the size or location of the deletion alone. What are the factors that may influence shared vs. distinct developmental outcomes; and at what level(s) can divergence or variability be observed?

FACTORS THAT IMPACT DEVELOPMENTAL OUTCOME

Figure 1 outlines the interplay between four factors that may impact on developmental outcome.

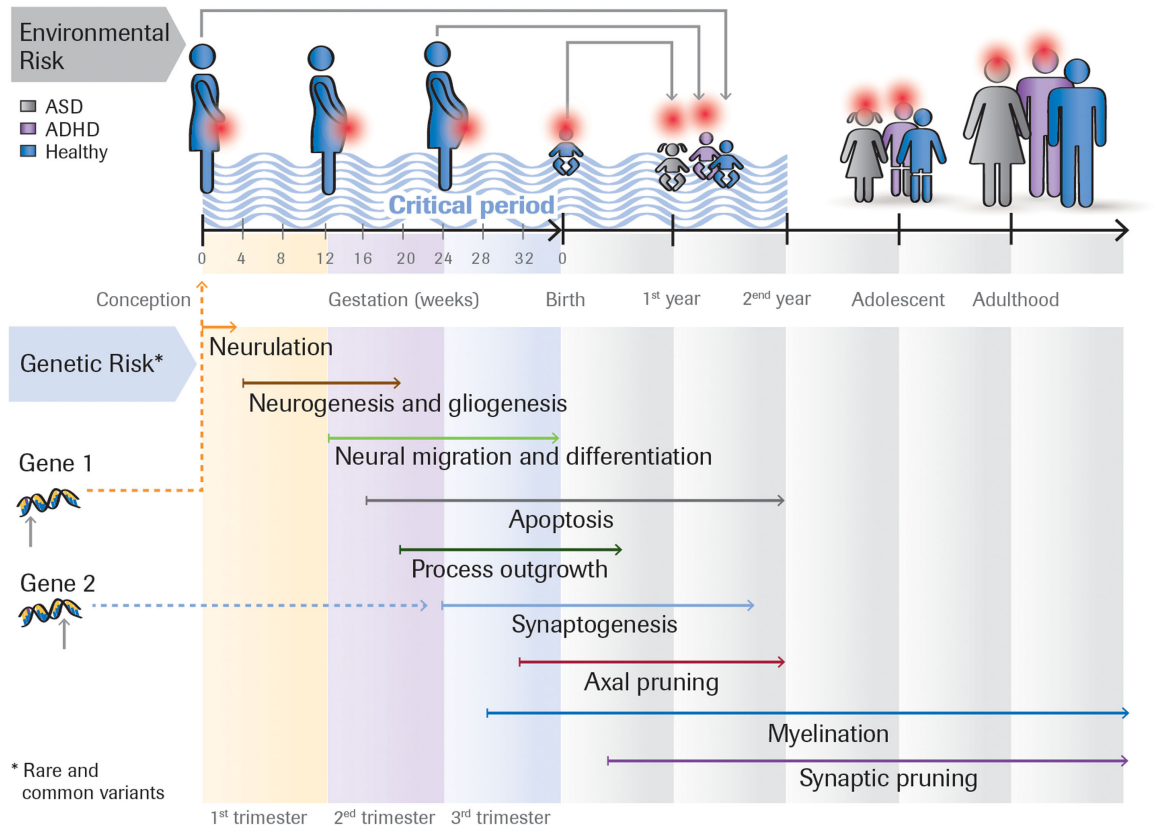

FIGURE 1 | Rare and common genetic variants, as well as environmental risk factors (e.g., toxins, maternal infection) acting at different time points impact on the developmental outcome. For example, the same rare genetic mutation can either lead to ASD, ASD + ADHD, or ADHD, depending on the person's genomic background (the sum of common variants). Or the effect of a genetic variant could be modulated by environmental insults that occur during the first, second or third trimester of the pregnancy. Many ASD risk genes are involved in essential developmental processes including proliferation, neuronal growth and differentiation, and synapse formation. These genes are expressed at different times in brain development.

Common Variants and Genomic Background

The impact of rare mutations on phenotypic outcome may depend on genomic background (the sum of common variants). It may act as a "buffer" that diminishes or increases the deleterious effect of rare variants (CNVs) – for example, by modulating synaptic homeostasis (10). For an individual with a genetic background that contains a high load of common ASD risk variants, a small burden of rare risk genes may suffice to cause ASD. By contrast, an individual whose genetic background only includes a small number of common risk variants may require a higher burden of deleterious mutations to cause ASD. The rare genetic mutation may still have some penetrance, but the effect may be "milder," including sub-threshold clinical symptoms (10).

Environmental Risk Factors

In addition to genetic factors, it is likely that environmental influences – notably those acting during the embryonic stage – modulate risk for ASD. This includes maternal infections and prenatal exposure to teratogenic agents such as valproate acid, exposure to toxins, and dysfunction of the immune system (22). For instance, human studies comparing monozygotic and dizygotic twins have consistently revealed significant environmental influences that account for approximately 5–6% (23) of the observed variance in ASD. Moreover, higher concordance rates of ASD and other neurodevelopmental disorders in dizygotic twins than (singleton) siblings suggest a specific role of the fetal environment because both the twins and singletons share 50% of their genes (24).

Critical Periods

The time when different factors impact on neuronal development likely plays a crucial factor in determining developmental outcome. Brain development is initially determined by distinct temporal and spatial stages of gene expression (25) and intrinsic neuronal activity (26) but then becomes actively refined by interactions with the environment. Timing could be influenced either by genetic factors as different genes are expressed at different times in brain development [e.g., in humans genes involved in cell proliferation are expressed earlier than those involved in synaptogenesis or myelination (25)] or the time when an environmental insult occurs.

By altering the developmental window during which genetic/environmental insults are applied, animal studies can trace these effects across cellular, molecular, brain systems, and behavioral levels. However, the vast majority of animal studies have only tested adult animals. Therefore, we only know the end state – and not how abnormalities developed or changed across development. To understand this is vital because it is possible that some treatment effects may be different in developing vs. adult brains. A treatment that is likely only effective in early development would raise important ethical implications for clinical trial designs that usually first test safety, efficacy and side-effects in adults.

TRANSLATABILITY

One potential factor in failed clinical trials could be limited translatability from animal models to humans. Patient-derived induced pluripotent stem cells (iPSCs) are an interesting new approach that overcomes inter-species differences, although the technology is still in need of further development. Recently, protocols have been developed to derive iPSCs from peripheral blood mononuclear cells (27) or hair roots (28). This makes sample collection significantly easier and more viable for vulnerable and larger patient groups than earlier protocols based on fibroplasts (derived from skin biopsies). In addition, the ability to freeze keratinocytes themselves now offers great flexibility in generating lines from patients with particular genetics and phenotypic characteristics. For example, by comparing lines from patients with a particular monogenic defect (e.g., with SHANK3), but different clinical symptoms we may be able to account for differences in genomic background (which is difficult in animal models) and identify which cellular alterations may be linked to the gene vs. particular phenotypic differences. Comparison of phenotypes between cells derived from patients with monogenic vs. "idiopathic" forms of ASD provides valuable information on how generalizable cellular or molecular alterations identified from specific genetic/environmental processes may be for wider patient groups. Currently, the iPSC methodology is very costly. However, by studying cell lines of patients that are also comprehensively characterized in terms of their systems level (MRI, EEG, and PET) and neurocognitive profile we will be able to advance our understanding of the relationship between cellular, morphological and molecular, and higher level phenotypes in the same person. New systems level features of brain anatomy, function, and connectivity are now developed that offer higher resolution and greater translatability to brain phenotypes studied in animal models (29).

WE NEED TO BRIDGE LEVELS OF ANALYSES – COGNITION AS A BLACK BOX

One of the current challenges for neurobiological hypotheses, such as the E/I imbalance hypothesis, lies in its broadness: both glutamate and GABA are ubiquitous in the brain. Is the E/I imbalance in ASD cell and/or circuit-specific? Or might phenotypic differences linked to E/I imbalances across disorders (e.g., ASD vs. schizophrenia, ID) depend on a critical period during which they occur? And how do E/I imbalances give rise to characteristic cognitive profiles of ASD that involves both weaknesses and strengths?

The immature brain undergoes progressive alterations in molecular composition and in synchronized currents that underpin the development of functional neuronal circuits. Synchronized patterns of neuronal activity engage many neurons of developing networks, possibly because of efficient feed-forward GABA-ergic inhibition. These immature signals stop at critical time points to enable behaviorally relevant brain activity to emerge, which requires sparse fired, time-locked oscillations. Whereas early perturbations during basic circuit refinement may lead to widespread abnormalities, later occurring ones may produce more specific and localized disruptions. Brain networks may also differ in their resilience to gene dosage such that the functional effects of abnormal gene dosage could be localized even if the genetic abnormalities are widespread (30). Brain networks involved in evolutionarily older biological processes are thought to have developed more compensatory mechanisms than those supporting more recent cognitive functions. For example, the timing of insults in synapse development differentially affects different cortical regions as the timeline for synaptogenesis is different across the cortex (31). Changes in synaptic function and timing might then particularly disrupt the connectivity of higher order association areas, including frontal–parietal, frontal–temporal, and frontal–striatal circuits (32). This coincides with brain systems supporting higher level social-cognitive function or language development, which spike in synaptogenesis and plasticity between 1 and 3 years of age (31); roughly the time when social and language-related symptoms often become first apparent in ASD. Transcriptomics studies of co-expression patterns showed enrichment of ASD genes in cortical projection neurons (33), including glutamatergic projection neurons in superficial cortical layers (34).

Recently, some efforts were also made in linking glutamate or GABA neurotransmission to sensory processing abnormalities. For example, deficits in binocular rivalry (35) as well as paradoxical motion perception (36) in ASD are taken as indirect proxies for reduced GABA-ergic signaling. However, this approach makes the strong assumption that anomalies in circuits underpinning sensory anomalies are "primary deficits" in autism (37, 38) with down-stream effects on networks supporting higher level (and later developing) cognitive and social-cognitive functions – a premise that remains to be further tested.

Finally, some brain circuits may be more affected than others because glutamate and GABA modulate and are modulated by other neurotransmitter systems in particular brain regions and that are crucial for cognitive functions. For example, at birth, GABA-ergic signaling shifts from excitation to inhibition due to a reduction in intracellular chloride concentration, which in turn is mediated by endogenous oxytocin release. This shift in GABA-ergic polarity is abolished in mouse models of fragile X syndrome and rodents treated with valproate *in utero*; and some evidence indicates that "immature" excitatory GABA-ergic activity may persist in people with ASD (39). This provides a

potential link of GABA to a cascade effect of social-motivational abnormalities that are thought to be modulated by oxytocin.

Well-Validated Translated Cognitive Tests for Large-scale Investigations

Cognitive measures that map onto specific circuits are needed to bridge our understanding between systems level and behavioral anomalies. For many commonly used tests, psychometric properties (e.g., test–retest reliability), age norms, are not available. We need to invest in well-validated cognitive batteries that are equally suitable and meaningful for both children and adults, or for which different comparable versions exist. As most syndromic and monogenic forms of ASD involve varying degrees of intellectual disability, these tests should also be sensitive across the ability ranges, including profound intellectual disability levels to understand whether mechanisms are shared or different between these forms of ASD.

WHAT ARE MECHANISTICALLY PLAUSIBLE CLINICAL ENDPOINTS?

International regulators, such as the FDA, require clinical trials to select only one assessment as the primary endpoint against which the success of a study is measured. Currently, the lack of a truly mechanistic understanding of the link between molecular, neuroanatomical/functional, and cognitive processes impedes informed selection of "primary endpoints." For example, in a pilot trial of insulin-like growth factor-1 (IGF-1) in Phelan McDermid Syndrome the Aberrant Behavior Checklist (ABC) social withdrawal subscale was chosen as the primary outcome measure because it is well validated in ASD and ID and accepted within pediatric psychopharmacology research (40). In the STX209 arbaclofen trial with volunteers with "idiopathic" ASD, the ABC-irritability subscale was used as the primary outcome measure; again because the measure is known to be sensitive to change in pharmacologic trials. However, in both examples there was no mechanistic reason why the growth-stimulating hormone should primarily affect social symptoms or a specific $GABA_B$ receptor agonist should "specifically" affect irritability (rather than core social deficits of ASD). Also, in some instances, the clinical outcome measure (irritability) did not directly translate to the most consistent behavioral changes identified in the mouse model (learning) (17).

PLACEBO EFFECTS

Placebo effects are a major difficulty for testing treatment efficacy in double-blind randomized control trials, which result from participants' expectation that they are receiving a treatment and may lead to conscious or unconscious behavioral changes. Even the support, attention, and interest from the research team compared to the person or family's everyday experience may have a "therapeutic" effect. The effect size of the placebo response in medication trials for ASD is estimated to be "moderate" (41). Recently, trial designs have been proposed that aim to address the interaction between those behavioral changes and treatment (42). In addition, demonstration of target engagement using objective measures may be one way forward to evaluate the efficacy of the treatment in clinical trials, even when – by virtue of the placebo effects – changes in overall outcome may not be significantly different between the treatment and control groups.

STRATIFICATION BIOMARKERS

The next important step is to select particular patients groups for clinical trials that are more likely to respond to the treatment under consideration. Despite an explosion of biomarker research over the past years, we currently do not have a single validated or clinically useful biomarker for ASD. Biomarkers have been defined as "a characteristic that is objectively measured and evaluated as an indication of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (43). In the past, using case–control designs, the majority of research studies focused on the identification of *diagnostic biomarkers* to provide a discrete and objective indication of diagnostic status (i.e., whether or not someone has an ASD). Although many studies reported significant *mean group* differences in, for example, performances on a range of cognitive tests, or in brain structure, function, or connectivity (using MRI methods), inconsistencies in findings and failure to replicate are common problems. Moreover, it is important to note that a mean group difference alone (especially with moderate effect sizes) by no means indicates that that measure has diagnostic biomarker utility. This further requires an almost complete non-overlap of the distributions of individuals in the case and control groups (and, according to traditional categorical classifications, individuals with other neurodevelopmental or neuropsychiatric conditions).

Stratification biomarkers divide a group of patients into subgroups with shared biological characteristics. These subgroups may differ in terms of their clinical symptom profile and/or etiology. Stratification markers may be primarily clinically relevant if they have either *prognostic* value, i.e., they assess the (untreated) progression and outcome of the disorder, or *predictive* value, i.e., they estimate the probability of response to a given treatment. Stratification biomarker research in ASD is still in its infancy. Difficulties for stratification research have been primarily studies with small sample sizes, such that the majority of cognitive or neuroimaging studies include 15–30 participants per group – which are associated with limited power, especially if a group were divided into two or more subgroups.

Large-scale multi-center multidisciplinary observational studies, such as the EU-AIMS Longitudinal European Autism Project (LEAP) are currently underway that have sufficient power to identify stratification markers (44). Multi-modal assessments of each individual allow us to identify genetic, molecular, circuit-based, and behavioral markers.

One approach to stratification is to split the sample based on *a priori* participant criteria (e.g., sex, developmental level). For example, there is some evidence that females with ASD differ from males with ASD in terms of their cognitive profile, neuroanatomy, or function (45) and that females may require a

higher burden of genetic risk factors to develop ASD [i.e., being more protected from developing ASD (46)]. There may also be differences between individuals with ASD with/without distinct co-occurring conditions, such as ADHD or anxiety. In addition, unsupervised, data-driven methods may be particularly useful to identify subtypes based on differences in, for example, brain anatomy, function, and/or cognitive profile. To do this, hierarchical clustering methods (47) or normative modeling approaches to neuroimaging data (48), have recently been used. Larger-scale neuroimaging studies of ASD [e.g., EU-AIMS LEAP (44); Province of Ontario Neurodevelopmental Disorders Network, POND (49)] or efforts to aggregate neuroimaging data from different laboratories [Autism Brain Image Data Exchange, ABIDE (50)] now begin to provide cohorts of sufficient sizes and to enable replication. In contrast to the relative high costs of MRI scans, electrophysiological methods are relatively less expensive, and easy to use in young children and even infants, and individuals with intellectual disabilities (51). Valid EEG stratification markers may therefore be in principle more feasible to implement in clinical practice. Many circuits underpinning fundamental bio-behavioral dimensions affected in ASD (e.g., social cognition, reward processing) cut across different neurodevelopmental and neuropsychiatric disorders. Therefore, a biomarker that estimates deficits in, for example, (neural activation underlying) emotional reactivity or reward sensitivity does not necessarily have to be specific to ASD but instead may predict dimensional symptom severity (52).

Multi-modal biomarkers (e.g., combining resting-state EEG and fMRI) likely have improved prognostic as well predictive value relative to markers based on one modality. For example, abnormalities in gamma band oscillations could indicate either increased excitatory (e.g., glutamatergic) or reduced inhibitory (e.g., GABA-ergic) signaling. Additional information on glutamate vs. GABA concentrations as derived from MRS or behavioral proxies of GABA signaling may help to interpret an individual's score on the EEG measure. For a stratification biomarker to be predictive of treatment response those differences are critical as it may indicate whether – broadly – a GABA agonist or glutamate (receptor) antagonist may be more likely to be effective for a particular individual. Also a fuller understanding of an individual's cognitive profile across domains, and the relationship between cognitive strengths and weaknesses is crucial, as individuals with ASD and their families may be more likely to accept future medical treatments if they could be reassured that those strengths, which form an important part of a person's identity, are not blunted by them.

Longitudinal designs with at least three time points are needed that concurrently track changes in the clinical, neurocognitive, functional, and anatomical trajectories to ascertain the prognostic value of stratification biomarkers. This way, we can begin to model whether a person whose social-communicative skills improve between, say childhood and adolescence has a different neurobiological profile to someone whose symptoms stay the same or worsen. Identification of convergent/divergent pathways into the disorder (risk biomarkers) would be particularly useful to estimate whether a child will develop an ASD before it is clinically manifest and to offer intervention or treatments during early development. Research on risk biomarkers typically employs the high-risk infant sibling design, which capitalizes on the finding that siblings of a child with ASD have a 20-fold increased risk to also developing ASD. So far, the majority of infant-at-risk studies have treated "high-risk" infants as a relatively homogeneous group and only typically stratified by whether or not they developed ASD at age 2–3 years. Identification of "prodromal" subgroups is difficult given that this design is so costly and the number of children who develop ASD in these cohorts is relatively small.

Validation of stratification biomarker requires determining their accuracy (i.e., sensitivity, specificity, positive and negative predictive values), plausibility (causal or mechanistically understandable), reliability in relating to a certain clinical endpoint, and reproducibility across clinically relevant settings (53). To increase reproducibility, large-scale consortium studies, such as the IMI-funded EU-AIMS (54) and the NIMH funded Autism Biomarker Consortium-Clinical Trials, are committed to sharing protocols and data.

CONCLUSION

The current assumption that ASD involves multiple etiologies and pathophysiological mechanisms makes precision medicine the most promising approach to effective treatments for individuals with the overall umbrella condition. Because in the majority of individuals with ASD (including most with IQ in the normal range) the etiology is currently not known, the approach hinges on a better understanding of whether cellular or molecular mechanisms are shared. The recently failed clinical trials with monogenic forms of ASD point to further obstacles: conceptual and methodological issues linked to translatability from animal models to humans, clinical trial design, placebo effects, and selection of mechanistically plausible, objective outcome measures. Addressing these different factors in the next generation of research studies requires a translatable developmental perspective and multidisciplinary, collaborative initiatives.

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EL has produced the first and final draft of the paper. DM and WS have commented on and revised several versions of this manuscript.

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ORIGINAL RESEARCH
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Blocking and Binding Folate Receptor Alpha Autoantibodies Identify Novel Autism Spectrum Disorder Subgroups

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Folate receptor α (FRα) autoantibodies (FRAAs) are prevalent in autism spectrum disorder (ASD). They disrupt the transportation of folate across the blood-brain barrier by binding to the FRα. Children with ASD and FRAAs have been reported to respond well to treatment with a form of folate known as folinic acid, suggesting that they may be an important ASD subgroup to identify and treat. There has been no investigation of whether they manifest unique behavioral and physiological characteristics. Thus, in this study we measured both blocking and binding FRAAs, physiological measurements including indices of redox and methylation metabolism and inflammation as well as serum folate and B12 concentrations and measurements of development and behavior in 94 children with ASD. Children positive for the binding FRAA were found to have higher serum B12 levels as compared to those negative for binding FRAAs while children positive for the blocking FRAA were found to have relatively better redox metabolism and inflammation markers as compared to those negative for blocking FRAAs. In addition, ASD children positive for the blocking FRAA demonstrated better communication on the Vineland Adaptive Behavior Scale, stereotyped behavior on the Aberrant Behavioral Checklist and mannerisms on the Social Responsiveness Scale. This study suggests that FRAAs are associated with specific physiological and behavioral characteristics in children with ASD and provides support for the notion that these biomarkers may be useful for subgrouping children with ASD, especially with respect to targeted treatments.

Keywords: folate receptor autoantibody, autism spectrum disorders, folinic acid, redox metabolism, glutathione

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INTRODUCTION

Autism spectrum disorder (ASD) is a devastating neurodevelopmental disorder with life-long consequences that affects young children during critical times in their development. The Center for Disease Control estimates that 1 in 68 individuals in the United States (~1–2%) are affected by an ASD (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators; Centers for Disease Control and Prevention (CDC), 2014). Despite this alarming prevalence, the etiology of ASD is still poorly understood with several studies suggesting that both environmental and genetic components may contribute equally to the etiology of ASD (Hallmayer et al., 2011; Sandin et al., 2014).

Recent research has suggested that several physiological abnormalities such as dysfunctional mitochondrial and redox metabolism and immune abnormalities, alone, or in combination, are related to the underlying aberrant biological processes that results in ASD (Rossignol and Frye, 2012b). Folate is related to many of the physiological systems found to be abnormal in ASD (Frye and James, 2014) and has been shown to be protective against the development of ASD when supplemented in adequate amounts during or before pregnancy (Schmidt et al., 2012; Suren et al., 2013). Folate is a water-soluble B vitamin that is essential for numerous metabolic reactions and normal neurodevelopment (Greenblatt et al., 1994; Black, 2008).

In some individuals with ASD, the primary mechanism that transports folate across the blood-brain barrier may be compromised. The folate receptor alpha (FRα) along with the energy dependent endocytosis transports folate, attached to the FRα, from the apical to the basolateral side of the choroid plexus endothelium against a concentration gradient. Active transport of folate is necessary because the folate concentration in the central nervous system is several times higher than the folate concentration in the blood (Frye et al., 2013b).

A new neurometabolic disorder called cerebral folate deficiency (CFD) was described about a decade ago. CFD is characterized by abnormally low folate concentrations in the cerebrospinal fluid despite normal folate concentration in the serum (Ramaekers et al., 2002). CFD is associated with autoantibodies to the FRα which impair its function (Ramaekers et al., 2005). Serum titers of FRα autoantibodies (FRAAs) have been correlated with cerebrospinal fluid (CSF) folate concentrations in independent studies (Ramaekers et al., 2005; Frye et al., 2013b). Mitochondrial disorders can cause CFD because of the lack of energy for the active transportation of folate across the blood-brain barrier (Ramaekers et al., 2007b; Garcia-Cazorla et al., 2008; Frye and Naviaux, 2011). Early case-series of children with CFD described many with ASD features (Ramaekers and Blau, 2004; Ramaekers et al., 2005).

Recently, Frye et al. (2013b) measured blocking and binding FRAAs in 93 children with ASD using the assay developed by Dr Quadros (Ramaekers et al., 2005; Molloy et al., 2009). Overall, 60 and 44% were positive for the blocking and binding FRAAs, respectively. The high prevalence of FRAAs in ASD children was verified in a study from Belgium which demonstrated that 47% of children with ASD were positive for the blocking FRAA as compared to 3.3% of developmentally delayed non-ASD controls (Ramaekers et al., 2013b). These prevalence rates for blocking FRAAs are clearly higher than prevalence rates in healthy populations which vary from 10 to 15% (Frye et al., 2013b).

The importance of the FRα is related to the targeted treatment which can bypass the FRα when it is blocked and/or dysfunctional. Folinic acid can cross the blood-brain barrier using the reduced folate carrier (RFC) when the FRα is blocked by FRAAs or non-functional due to mitochondrial dysfunction and/or genetic mutations. The RFC has a lower affinity for folate than the FRα, so a high-dose of folinic acid is required for treatment. Case-reports (Moretti et al., 2005) and series (Ramaekers et al., 2005, 2007a) have described neurological, behavioral and cognitive improvements in children with CFD and ASD with high-dose folinic acid (0.5–2 mg/kg/day), including complete recovery of ASD symptom in some and substantial improvements in communication in many (Ramaekers et al., 2005, 2007a).

Frye et al. (2013b) treated 44 children positive for at least one FRAA with high-dose folinic acid in an open-label fashion. After 4 months of treatment, significant improvements in verbal communication, receptive and expressive language and stereotypical behavior were noted in the treated children as compared to a wait list control group who were also FRAA positive. About two-thirds (66%) of treated children showed some improvement in language, verbal communication and stereotyped behavior, with one-third (∼33%) demonstrating moderate or much improvement in these areas.

Although, this promising evidence suggests that FRAAs are biomarkers that can identify an important subset of children with ASD who may respond to a specific treatment, an investigation into whether the subgroup of ASD children with FRAA have particular characteristics that can distinguish them from others with ASD, has not been conducted. Thus, in this study we examine a sample of children with ASD to determine the correspondence between FRAA status and behavior and developmental characteristics. Since the folate pathway is closely connected with methylation and redox regulation pathways (Frye and James, 2014), markers of glutathione and methylation metabolism are also examined. Since antibody production can be associated with immune activation and increases in inflammation can be associated with redox and methylation abnormalities (Rose et al., 2012; Rossignol and Frye, 2014), a marker of inflammation is also examined.

Since there are two different FRAAs, blocking and blinding, our analysis determines whether the characteristics investigated are an effect of blocking or binding FRAAs independently of one another. Since some individuals will have both blocking and binding FRAAs, a general linear model is used to investigate these independent effects. This will be the first time a difference between these two FRAAs has been investigated. Such information can not only help better identify children with ASD who are positive for FRAAs but can help us understand the consequence and significance of each FRAA in ASD.

MATERIALS AND METHODS

The data from the 94 children with ASD were obtained from two research protocols approved by the Institutional Review Board at the University of Arkansas for Medical Science (Little Rock, AR). Written informed consent was obtained from parents of participants and assent was waived.

A diagnosis of ASD, required for study entry, was defined by one of the following: (i) a gold-standard diagnostic instrument such as the Autism Diagnostic Observation Schedule and/or Autism Diagnostic Interview-Revised (ADI-R); (ii) the state of Arkansas diagnostic standard, defined as agreement of a physician, psychologist and speech therapist; and/or (iii) Diagnostic and Statistical Manual for Mental Disorders

diagnosis by a physician along with standardized validated questionnaires and diagnosis confirmation by the Principal Investigator (REF). Reconfirmation of the diagnosis using the ADI-R by an independent research reliable rater was requested for a portion of participants to confirm that the criteria used for including the participants was equivalent to other high-quality studies. Excluded from the study were children on antipsychotic medications as well as children with well-defined genetic syndromes.

Folate Autoantibody Assay

About 1 ml of serum was analyzed at the State University of New York, Downstate (Brooklyn, NY) for blocking and binding FRAAs using the assay previously described (Ramaekers et al., 2005; Molloy et al., 2009). Blocking FRAAs are expressed as pmoles of folic acid blocked from binding to FRα per ml of serum, and binding FRAAs are expressed as pmoles of IgG antibody per ml of serum.

Redox, Methylation, Immune and Vitamin Biomarkers

Redox and methylation potential was measured by the total and free reduced-to-oxidized glutathione redox ratio (tGSH/GSSG and fGSH/GSSG) and the S-adenosylmethionine to S-adenosylhomocysteine ratio (SAM/SAH), respectively. 3-Chlorotyrosine (CT), a measure of myeloperoxidase activity, was used as a marker of immune system activation. Fasting blood (4 ml) was collected into an EDTA-Vacutainer tube, chilled on ice and centrifuged at 1500 × g for 15 min at 4°C. Plasma was stored at −80°C and analyzed by HPLC with electrochemical detection as previously described (Melnyk et al., 1999) within 2 weeks of collection. Total plasma folate and vitamin B12 were measured using MP Diagnostics SimulTRAC-SNB Radioassay Kit (Cat# 06B264806).

Cognitive and Behavioral Assessments

The Preschool Language Scale-4 (PLS) and two versions of the Clinical Evaluations of Language Fundamentals (CELF) were used to assess language ability. The CELF is one of the only standardized, well-validated language assessment instruments that spans the age range of most participants (using both CELF-preschool-2 and CELF-4; Semel et al., 2003; Wiig et al., 2004). It assesses a wide range of language skills that are only partially measured by other language tests, including high-level language skills that are abnormal in individuals with ASD, such as language pragmatics (Condouris et al., 2003) and has been used in several studies focusing on core language deficits in ASD (Verly et al., 2014; Edgar et al., 2015). The PLS is also a standardized, well-validated language assessment instrument that can measure subtle changes in language in children with poor language abilities (Zimmerman et al., 2002; Volden et al., 2011). Both instruments provide a standardized core language score which was used as the index of language ability. For each participant the most ability appropriate instrument was used in order to prevent floor and ceiling effects.

Adaptive behavior was assessed using the Vineland Adaptive Behavior Scales, 2nd Edition, Interview Edition, Survey Form (VABS), an instrument that has demonstrated good reliability and validity (Sparrow et al., 2005). Standardized scores for summary scales were examined: communication, daily living skills, social skills, motor skills and adaptive behavior composite.

The Aberrant Behavior Checklist (ABC) was designed to measure disruptive behaviors in individuals with developmental disabilities (Aman et al., 1985). The ABC has been shown to have convergent and divergent validity in ASD (Kaat et al., 2014) and has been used in multiple autism clinical trials (Frye et al., 2015).

The Social Responsiveness Scale (SRS) measures the severity of social skill deficits (Constantino, 2002). It has been validated and shown to be reliable and to have good correspondence to the gold-standard ADI-R, while being more time efficient and cost effective (Murray et al., 2011).

Statistical Analysis

The "glm" procedure of SAS 9.1 (SAS Institute Inc., Cary, NC) was used with a two-tailed alpha of 0.05. FRAA status was dichotomized into positive or negative for both blocking and/or binding FRAAs. These variables were entered into a general linear model to determine if differences in the dependent variable were related to blocking and/or binding FRAA status. In this sense, the analysis examined the independent effects of blocking and bindings FRAAs on the dependent variable while controlling for the effect of each FRAA. Because of the limited sample size, the interaction of the two FRAAs was not examined. Planned orthogonal contrasts were used to determine whether the blocking and binding FRAAs demonstrated significant differences in the dependent variable. Since ASD symptoms are often associated with developmental level, the VABS Adaptive Behavior Composite was used as a covariate in the analysis that examined behavior and developmental indices.

RESULTS

Participants

Basic participant characteristics are outlined in **Table 1** stratified across FRAA status. The average age and gender did not differ across the FRAA groups. Race and ethnicity was not different across FRAA groups. Overall, 84% were Caucasian, 7% African American, 5% Asian and 3% mixed race and 95% were non-Hispanic.

Participants were recruited from our research registry (35%), autism clinic (27%), word-of-mouth (15%), physician referrals (13%), and community advertisement and social media (11%). All participants evaluated by an independent research reliable rater exceeded the threshold for the autism diagnosis.

Overall, immune and neurological abnormalities did not differ across FRAA status. Of the children with immune disorders, 79% had recurrent infections, 35% were diagnosed with immune disorder not otherwise specified, 32% had eczema, 21% had Pediatric Acute-onset Neuropsychiatric Syndrome, 11% had hypogammaglobulinemia and 5% had complement deficiency. Of the children with neurological abnormalities, 42% had epilepsy, 27% had migraines, 23% had abnormal electroencephalograms without seizures, 20% had visual problems, 15% had macrocephaly, 8% had Chiari malformation,

TABLE 1 | Demographic and clinical characteristics by folate receptor alpha autoantibody groups.

Variable	FRAA Negative ($n = 40$)	FRAA Blocking Positive ($n = 16$)	FRAA Binding Positive ($n = 48$)
Age, years months	7 years 0 months (3 years 4 months)	6 years 5 months (3 years 0 months)	7 years 4 months (3 years 2 months)
Males, N (%)	34 (85%)	15 (94%)	39 (81%)
Vineland adaptive behavior composite,	64.9 (11.7)	66.7 (10.8)	63.2 (10.4)
Only blocking autoantibody positive, N (%)		6 (38%)	
Only binding autoantibody positive, N (%)			38 (79%)
Both folate autoantibodies positive, N (%)		10 (63%)	10 (21%)
Blocking titer (pmol/ml),		0.52 (0.36)	0.54 (0.44)
Binding titer (pmol/ml),		1.03 (0.81)	0.85 (0.66)
Glutathione redox ratio, total	30.72 (1.09)	29.67 (1.07)	34.92 (2.06)
Glutathione redox ratio, free	9.06 (1.83)	9.82 (1.82)	8.75 (1.62)
Methylation SAM/SAH Ratio	2.65 (0.48)	2.67 (0.42)	2.60 (0.46)
3-Chlorotyrosine	37.7 (7.6)	33.4 (6.6)	37.1 (7.7)
Folate (ng/ml) [Normal 5-21],	17.7 (4.3)	18.9 (5.3)	18.0 (4.1)
B12 (pg/ml) [Normal 200-900],	828.3 (451.3)	1426.0 (1589.8)	1383.0 (1495.4)
LANGUAGE TESTING, N (%)			
Preschool language scales	12 (30%)	4 (25%)	17 (35%)
Clinical evaluation of language fundamentals 2	14 (35%)	6 (38%)	10 (21%)
Clinical evaluation of language fundamentals 4	13 (33%)	6 (38%)	21 (44%)
DIAGNOSTIC DOCUMENTATION, N(%)			
Autism diagnostic observation schedule	20 (50%)	8 (50%)	22 (46%)
3 Practitioner agreement	30 (75%)	10 (63%)	31 (65%)
Single practitioner with standardized questionnaires	5 (13%)	5 (31%)	12 (25%)
AUTISM DIAGNOSTIC INTERVIEW-REVISED			
Participated in confirmation testing, N(%)	27 (68%)	11 (69%)	37 (77%)
Social interaction score,	21.41 (5.44)	22.18 (4.40)	22.76 (4.96)
Communication score: verbal,	16.64 (4.40)	18.88 (1.46)	19.43 (3.89)
Communication score: non-verbal,	12.69 (2.36)	12.33 (2.08)	13.07 (1.59)
Restricted and repetitive play score,	5.85 (1.83)	5.82 (2.71)	5.84 (2.39)
Summary score,	4.41 (0.93)	4.64 (0.50)	4.41 (0.93)
MEDICATIONS (CONCURRENT TREATMENTS), N (%)			
Melatonin	17 (43%)	1 (6%)	10 (21%)
Allergy/Asthma medications	13 (33%)	4 (25%)	10 (21%)
Gastrointestinal medications	15 (38%)	2 (13%)	9 (19%)
Alpha-adrenergic agonists	7 (18%)	1 (6%)	10 (21%)
Stimulant	6 (15%)	0 (0%)	8 (17%)
Antiepileptic medication	4 (10%)	0 (0%)	6 (13%)
Antimicrobial medications	5 (13%)	1 (6%)	4 (8%)
Selective serotonin reuptake inhibitors	0 (0%)	1 (6%)	6 (13%)
Other psychotropic medications	4 (10%)	0 (0%)	2 (4%)
Immunomodulatory medications	2 (5%)	1 (6%)	2 (4%)
Antipsychotic	2 (5%)	0 (0%)	0 (0%)
Beta blocker	1 (3%)	0 (0%)	0 (0%)
SUPPLEMENTS (CONCURRENT TREATMENTS), N (%)			
Multivitamin	13 (33%)	6 (38%)	12 (25%)
Minerals	8 (20%)	3 (19%)	11 (23%)
Fatty acids	7 (18%)	4 (25%)	10 (21%)
Folate	8 (20%)	0 (0%)	7 (15%)
Vitamin B-12	2 (5%)	2 (13%)	12 (25%)
Carnitine	6 (15%)	3 (19%)	7 (15%)
Other antioxidants	4 (10%)	1 (6%)	8 (17%)
Other vitamins	7 (18%)	1 (6%)	5 (10%)

(Continued)

TABLE 1 | Continued

Variable	FRAA Negative ($n = 40$)	FRAA Blocking Positive ($n = 16$)	FRAA Binding Positive ($n = 48$)
Other B vitamins	3 (8%)	3 (19%)	8 (17%)
CoEnzyme Q10	4 (10%)	1 (6%)	5 (10%)
Other supplements	1 (3%)	0 (0%)	3 (6%)
Amino acids	1 (3%)	1 (6%)	2 (4%)
Thyroid supplements	1 (3%)	0 (0%)	1 (2%)
COMORBID MEDICAL CONDITIONS, N (%)			
Allergic disorders	18 (45%)	6 (38%)	19 (40%)
Gastrointestinal disorders	18 (45%)	6 (38%)	17 (35%)
Neurological disorders	9 (23%)	5 (31%)	15 (31%)
Copy number variants	7 (18%)	4 (25%)	14 (29%)
Psychiatric disorders	5 (13%)	3 (19%)	14 (29%)
Immune abnormality	8 (20%)	1 (6%)	10 (21%)

Mean values with standard deviation in parenthesis.

8% had non-specific magnetic resonance imaging abnormalities of the brain, and 4% had microcephaly.

Folate Receptor Alpha Autoantibodies

Fifty seven percent of the participants were positive for either the blocking or binding FRAAs, with 17% positive for blocking FRAA and 51% positive for the binding FRAA; 11% were positive for both FRAAs. There was no significant difference in age across participants who were FRAA blocking positive vs. negative or FRAA binding positive vs. negative.

Folate and B12

B_{12} [$F_{(1, 92)} = 4.35$, $p = 0.03$], but not folate, was significantly higher in the binding FRAA positive participants as compared to binding FRAA negative participants (See **Figure 1**). Participants positive for the blocking FRAA were not, as a group, significantly different than those negative for the blocking FRAA with respect to B_{12} or folate. Neither, B_{12} nor folate was significantly different in those positive for blocking FRAAs vs. those positive for the binding FRAAs.

Redox, Methylation and Inflammation Biomarkers

As depicted in **Figure 2**, total and free GSH/GSSG was significantly higher in participants positive for the blocking FRAA as compared to those participants negative for the blocking FRAA [$F_{(1, 92)} = 9.52$, $p = 0.003$ and $F_{(1, 92)} = 5.35$, $p = 0.02$, respectively]. Total and free GSH/GSSG were significantly higher in participants positive for blocking FRAAs as compared to those positive for binding FRAAs [$F_{(1, 92)} = 9.06$, $p = 0.003$ and $F_{(1, 92)} = 6.76$, $p = 0.01$, respectively].

CT was significantly lower in participants positive for the blocking FRAA as compared to those negative for the blocking FRAA [$F_{(1, 92)} = 4.83$, $p = 0.03$] but CT was not significantly different between those positive for blocking FRAA vs those positive for binding FRAAs.

Since supplemental B_{12} can improve the GSH/GSSG ratio in clinical studies, we reanalyzed the difference in GSH/GSSG ratio across groups with B_{12} as a covariate but the results did not changed, indicating that the difference in GSH/GSSG ratio across groups was not due to differences in serum B_{12} concentrations.

The SAM/SAH methylation ratio was not significantly different between blocking FRAA positive and negative groups. Additionally, participants positive for the binding FRAA were not, as a group, significantly different than those negative for the binding FRAA with respect to GSH/GSSG or SAH/SAH ratio or CT.

Behavior and Cognition

The VABS Adaptive Behavior Composite was not significantly different in those positive for the blocking FRAA vs. those negative for the blocking FRAA or those positive for the binding FRAA vs. those negative for the binding FRAA, suggesting that any behavioral differences found across FRAA status were not due to developmental level.

As depicted in **Figure 3**, VABS Communication [$F_{(1, 86)} = 5.95$, $p = 0.02$], ABC Stereotyped Behavior [$F_{(1, 84)} = 5.30$, $p = 0.02$] and SRS Mannerisms [$F_{(1, 82)} = 5.19$, $p = 0.03$] were significantly better in participants positive for blocking FRAA as compared to those negative for blocking FRAA. Total SRS Score was significantly better in those positive for the blocking FRAA as compared to those positive for the binding FRAA [$F_{(1, 82)} = 4.04$, $p = 0.05$]. None of the behavioral or developmental measures were different across those positive vs. negative for the binding FRAA.

DISCUSSION

FRAAs (both blocking and binding) are believed to have pathologic consequences in ASD because of their role in blocking the transport of folate into the brain by interfering with FRα function and reducing folate availability in the brain (Frye et al., 2013b). Several studies have documented that individuals with FRAAs, including those with CFD, have ASD characteristics (Ramaekers and Blau, 2004; Ramaekers et al., 2005) and the prevalence of FRAAs appears to be high in individuals with ASD (Frye et al., 2013b; Ramaekers et al., 2013b). However, until

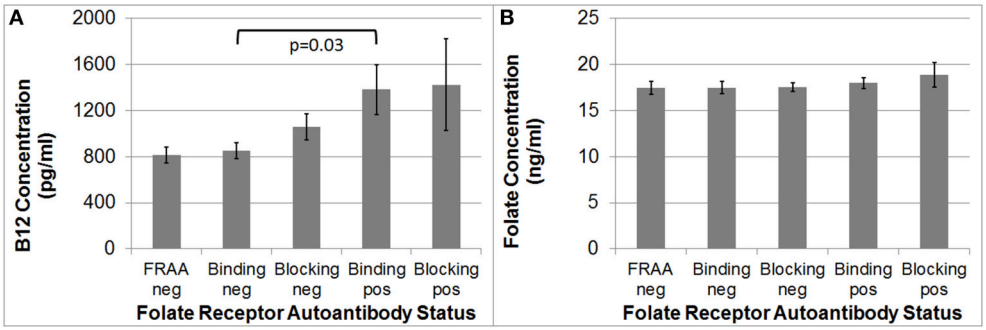

FIGURE 1 | (A) Children with Autism Spectrum Disorder who are positive for the binding Folate Receptor Alpha Autoantibody have higher serum B12 concentrations than children negative for the binding Folate Receptor Alpha Autoantibody; **(B)** Serum Folate concentration does not differ across Folate Receptor Alpha Autoantibody status in children with Autism Spectrum Disorder.

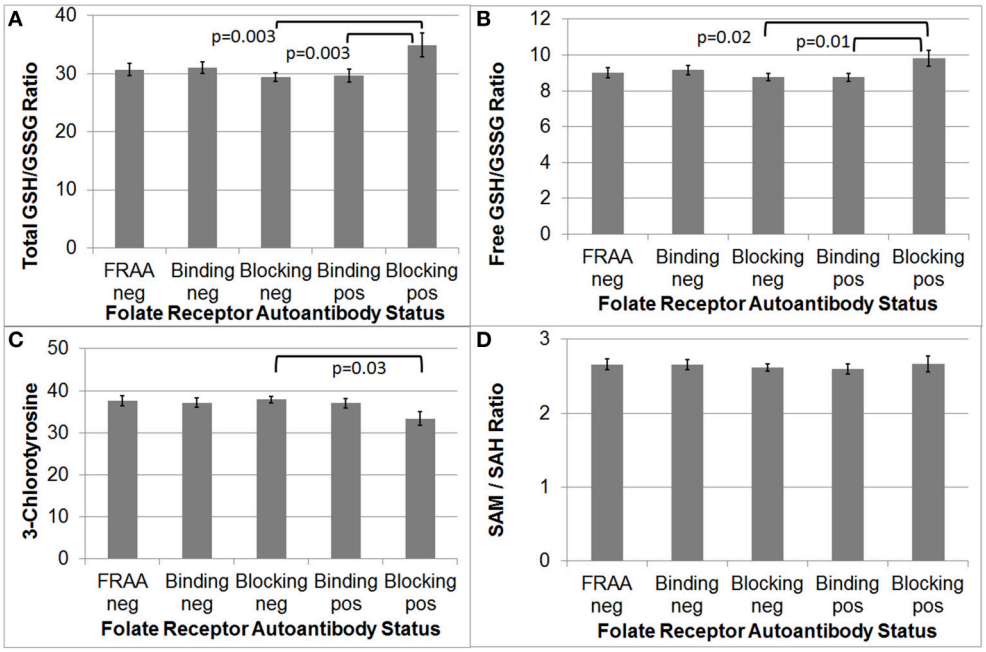

FIGURE 2 | Children with Autism Spectrum Disorder who are positive for the blocking Folate Receptor Alpha Autoantibody have more favorable total and free glutathione redox ratios (A,B) and marker of inflammation (C) as compared to those negative for the blocking FRAA. (D) Methylation metabolism was not different across Folate Receptor Alpha Autoantibody status.

this study no one has examined whether individuals with ASD who are FRAA positive have behavioral, developmental and/or physiological characteristics that are distinct from individuals with ASD who are negative for FRAAs.

In this study we found that individuals with ASD who were positive for the blocking FRAA demonstrated behavioral and physiological differences as compared to those negative for the blocking FRAA and as compared to those positive for the binding FRAA. Interestingly this subset of children appears to have better physiological and behavioral profiles, although their behavioral scores are well within the range for children with ASD. There is evidence that children positive for the FRAAs respond to folinic acid therapy, suggesting that these children are a subset of children with ASD that may be particularly responsive to therapy that addresses physiological abnormalities. The fact that children with FRAAs, at least blocking FRAAs, may have less severe ASD symptoms, suggests that these children may be a group to target early as they may be very likely to substantially improve their ASD symptoms and attain optimal outcomes with treatment. This may explain several reports of children with CFD recovering from ASD symptoms with high-dose folinic acid therapy—since they may have started out with milder ASD symptoms it may have been easier for them to recover.

Interestingly, ASD children with the blocking FRAA appear to have a more favorable redox and inflammation profile with relatively better glutathione and CT indices than FRAA blocking

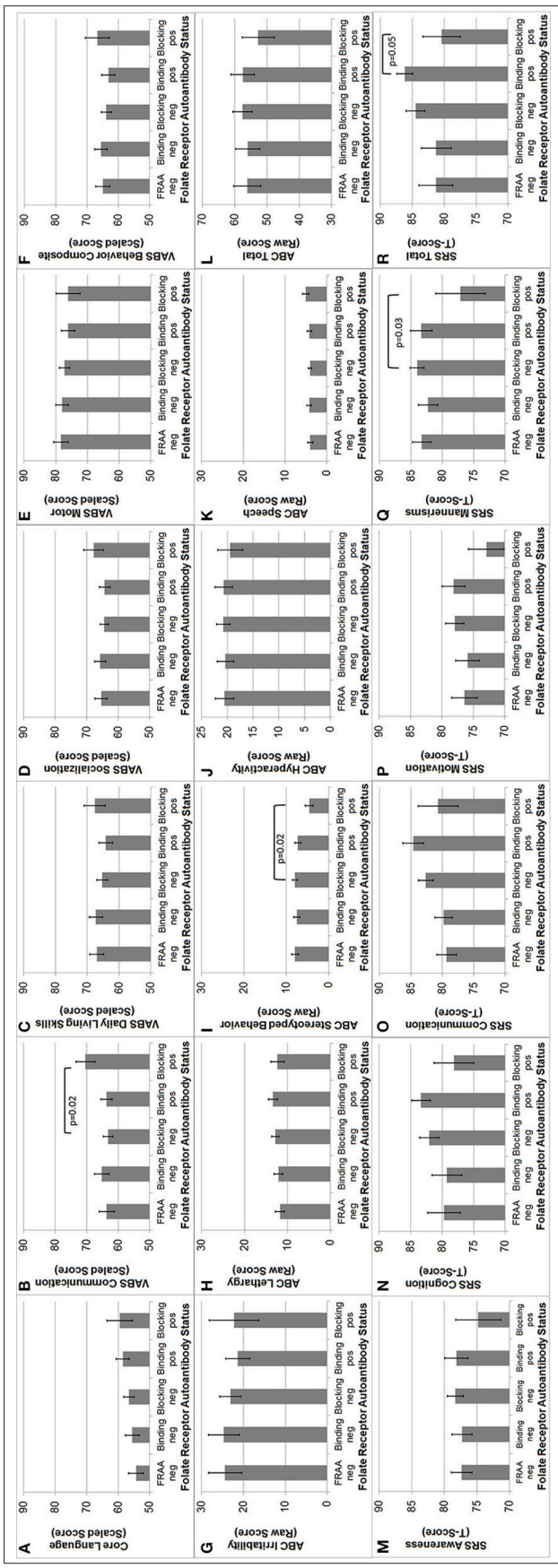

FIGURE 3 | Children with Autism Spectrum Disorder who are positive for the blocking Folate Receptor Alpha Autoantibody have more favorable (B) communication on the Vineland Adaptive Behavior Scale, (I) Stereotyped behavior on the Aberrant Behavior Checklist (ABC) and (Q) Mannerisms on the Social Responsiveness Scale (SRS) as compared to children negative for the blocking Folate Receptor Alpha Autoantibody. (R) In addition, children positive for the blocking FRAA have better total Social Responsiveness Scale score than those positive for the binding Folate Receptor Alpha Autoantibody. This figure depicts behavioral and Cognitive differences across Folate Receptor Alpha Autoantibody (FRAA) groups including difference in **(A)** Language, **(B–F)** Vineland Adaptive Behavioral Scale, **(G–L)** ABC, and **(M–R)** SRS.

negative children. A group of children with ASD and relatively unfavorable redox profiles have been described as having a distinct metabolic endophenotype in previous studies (James et al., 2006). It would appear that the group of ASD children with blocking FRAAs may be a complimentary metabolic endophenotype to the group with unfavorable redox metabolism that may have abnormalities specific to folate metabolism rather than redox metabolism. Importantly, this study demonstrates how multiple biomarkers can be used to differentiate subgroups of children with ASD who could potentially respond to different and/or synergistic treatments.

Children with ASD who are positive for the binding FRAA did not appear to have significant differences in behavioral, developmental or physiological measures when compared to children with ASD who were negative for the binding FRAA but the group did demonstrate significantly higher serum B12 concentrations. This may suggest that the binding FRAAs may interfere with cellular B12 uptake, resulting in a higher concentration of B12 in the blood. Indeed, polymorphisms in the B12 binding protein have been linked to an increased risk of ASD (James et al., 2006). Thus, the binding FRAAs may yet be another mechanism for interfering with B12 metabolism, resulting in methylation and glutathione abnormalities. Abnormalities in B12 metabolism, especially interference with B12 uptake into the cell, could result in the glutathione abnormalities seen in participants with the binding FRAAs. As B12 supplementation can correct glutathione metabolism abnormalities ASD (James et al., 2009) and improve adaptive behavior (Frye et al., 2013a) in children with ASD, it is very possible that a deficit in the cellular uptake of B12, either through a polymorphism in the B12 binding protein or binding FRAA interfering with B12 transport, could result in abnormal glutathione metabolism. Although, we used B12 as a covariate in the analysis of glutathione differences across groups, the covariate would only have been significant if it represented higher levels of intracellular B12. If indeed the higher B12 levels represent a failure of B12 entering the cell, then the covariate would have no relation to glutathione levels (or may have had the opposite predicted relationship). Thus, this is another indication that the elevated B12 levels associated with the binding FRAA do not represent intracellular levels and may be a reflection of a lack of cellular B12 uptake. If this is the case, the binding FRAA may interfere with both folate and B12 transportation, resulting in a more severe phenotype. Indeed, individuals positive for the binding FRAA appear to have worse social skills (as measured by the SRS) than children positive for the blocking FRAA.

Because of the limited sample size we might not have been able to detect small differences in physiology and behavior between the groups. Larger sample sizes could be helpful in detecting more subtle physiological and behavioral differences between groups. Perhaps an interesting study would be to measure the variation in FRAA titers within individuals. Titers tend to vary over short intervals of time (Ramaekers et al., 2013a), but this study only characterized the participants as FRAA positive or negative. It may be very useful to monitor changes in both FRAA titers and behavior within an individual over time as a more sensitive measure of the relationship between FRAAs titers and behavior, as examining intra-subject variation can control for the variation in physiology between individuals. Indeed, one study has demonstrated that the close relationship between aggression and blocking FRAA titers over a 6 week period in a 8 year old girl with ASD (Ramaekers et al., 2007a). Although, this study did not find any relationship between aggressive behavior and FRAA status, this may have been due to the large number of factors that can result in aggressive behavior within an individual with ASD (Matson and Jang, 2014). Thus, intra-subject studies may be most sensitive, especially if considering correlating FRAA titers.

It is important to appreciate that FRAAs could also work in concert with the defects in folate metabolism that have been associated with ASD, including polymorphisms in dihydrofolate reductase (Adams et al., 2007), the reduced folate carrier (James et al., 2006) and methylenetetrahydrofolate reductase (Frustaci et al., 2012), and mitochondrial dysfunction which appears to be rather common in children with ASD (Frye and Rossignol, 2011; Rossignol and Frye, 2012a). In addition, emerging evidence suggests that the enteric microbiome also has an important role for regulating the bioavailability of vitamins such as folate and B12 (Frye et al., 2015). Thus, a study which comprehensively assesses multiple folate pathway abnormalities in relationship to ASD behavior could be very insightful.

This study has helped define the importance of FRAAs in ASD and the effect of the FRAAs on physiology and behavior. It appears that blocking and binding FRAAs are associated with slightly different physiological and behavioral effects in children with ASD. Further, research will help better define their physiological roles and significance. Studying clinical subgroups in other disorders associated with FRAAs such as schizophrenia (Ramaekers et al., 2014) and subfertility (Berrocal-Zaragoza et al., 2009) may also yield important information. Ultimately, this information may be important to determining optimal treatments for certain children with ASD.

AUTHOR CONTRIBUTIONS

RF designed the study, analyzed the data and wrote the manuscript; LD collected and analyzed the data and edited the manuscript; JS collected and analyzed the data and designed the study and edited the manuscript; MT collected the data and edited the manuscript; RW collected and analyzed the data; SR collected and analyzed the data and edited the manuscript; SK edited the manuscript; SB analyzed the data; SM collected and analyzed the data and designed the study and edited the manuscript; JS collected and analyzed the data; EQ designed the study and wrote the manuscript.

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Autism Spectrum Disorders and Schizophrenia Spectrum Disorders: Excitation/Inhibition Imbalance and Developmental Trajectories

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Autism spectrum disorders (ASD) and schizophrenia spectrum disorders (SSD) share clinical and genetic components that have long been recognized. The two disorders co-occur more frequently than would be predicted by their respective prevalence, suggesting that a complex, multifactor association is involved. However, DSM-5 maintains the distinction between ASD, with core social and communication impairments, and SSD, including schizophrenia (SCZ), with hallucinations, delusions, and thought disorder as essential features. ASD and SSD have common biological underpinnings that may emerge early in development and unfold over time. One of the hypotheses supporting the similarities in the social and cognitive disturbances of ASD and SSD relates to abnormalities in the ratio of excitatory to inhibitory cortical activity (E/I imbalance). E/I imbalance in neurodevelopmental disorders could be the consequence of abnormalities in genes coding for glutamatergic and GABAergic receptors or synaptic proteins followed by system derangements. SSD and ASD have been characterized as polygenic disorders in which to the onset and progression of disease is triggered by interactions among multiple genes. Mammalian target of rapamycin signaling is under intense investigation as a convergent altered pathway in the two spectrum disorders. Current understanding of shared and divergent patterns between ASD and SSD from molecular to clinical aspects is still incomplete and may be implemented by the research domain criteria approach.

Keywords: autism spectrum disorders, schizophrenia spectrum disorders, psychosis, children and adolescents, excitation/inhibition imbalance

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AUTISM SPECTRUM DISORDER (ASD) AND SCHIZOPHRENIA SPECTRUM DISORDER (SSD): CURRENT UNDERSTANDING

The clinical interplay and overlap between SSD and ASD have long been recognized as the two classes of disorder that share phenotypic and clinical features and a number of individuals diagnosed with ASD subsequently develop SSD symptoms (1). Currently, the relationship is further emphasized after controversies on the shared patterns and differences of the two disorders.

It has been demonstrated that the two types of disorder co-occur more frequently than would be that predicted by their respective prevalence. DSM-5 maintains a nosological distinction between ASD and schizophrenia (SCZ) in spite of overlapping in clinical characteristics. Examining the specific definition of core symptoms, there are two major criteria for ASD: "(1) persistent deficits in social communication, social interactions, social-emotional reciprocity and communicative behaviors and

(2) restricted, repetitive patterns of behavior, interests or activities, including stereotyped or repetitive movements, behavioral rigidity, odd or intense interests," and as an important additional criterion, abnormally high or low reactivity to sensory stimuli. On the other hand, the DSM-5 diagnostic criteria for SCZ specify that at least two of the following symptoms must be present: "hallucinations, delusions, disorganized speech, grossly disorganized or catatonic behavior and negative symptoms." Furthermore, in DSM-5, the criteria have been reorganized to emphasize the variability in the severity of the psychopathology and the severity dimensions have been updated (2).

Childhood-onset schizophrenia (COS) is a subtype of SCZ defined by onset of psychotic symptoms before 13 years and the absence of any other neuropsychiatric diagnosis. Re-examination of the overlap between COS and ASD has highlighted the clinical and genetic commonalities. Remarkably, in almost half the cases of COS identified in the largest longitudinal study to date, a pervasive developmental disorder was present before the onset of psychosis (3). In contrast, and somewhat unexpectedly, prospective longitudinal studies following children with ASD into young adulthood rarely report the appearance of psychotic symptoms. SSD includes SCZ, schizofreniform disorder, schizoaffective disorder, and schizotypal personality disorder. In ASD, it is not rare to detect unusual preoccupations, unusual perceptual experiences, odd thinking, and speech. Both the shared clinical features and frequent co-occurrence point to a close relationship between SSD and ASD. To further strength this relationship, it has been reported that about 30% of children and adolescents with COS had co-morbid ASD (4). In addition, the well-known difficulty to recognize social cues from the actions of others is tightly related to deficit in theory of mind that is a characteristic feature common to both SSD and ASD (5).

In this context, it is critically important to underscore that the negative symptoms of SSD are often more disabling and more resistant to treatment than the so-called positive ones, e.g., hallucinations and delusions (6). These negative symptoms include social avoidance and emotional flatness and might be regarded as closely linked to impairments in social communication and motivation. These so-called negative symptoms of SSD might be considered to fall within the same domain of social impairment as the social difficulties characteristic of ASD. Furthermore, the disorganized or abnormal behaviors characteristic of SSD include behaviors which would meet ASD Criterion B, e.g., repeated and stereotyped movements and verbal expressions, as to DSM-5. Other pathognomonic features common to both conditions include impairments in facial recognition and emotion processing (7, 8). Patients with both ASD and SSD have been shown to have significant difficulties in interpreting social cues associated with eye gaze and deficits on theory of mind tasks—one of the hallmarks of ASD.

Autism spectrum disorders and SSD share biological underpinnings that may emerge in early neural development and unfold during subsequent childhood development (9). Abnormal neural development has been ascertained in cortical projection neurons from different brain areas including prefrontal and somatosensory regions in ASD and dorsolateral/ventrolateral prefrontal regions in SSD. It has to be mentioned that neurodevelopmental disorders are associated with known genetic abnormalities both in ASD and SSD phenotypes, as detailed in **Table 1**. Furthermore, epigenetic effects and alterations in copy number variants (CNVs) have been reported to contribute to abnormalities of neural circuits associated with SSD and ASD (**Table 2**). The risk of both disorders is increased by advanced paternal age and maternal infection/immune activation during pregnancy (10, 11). These shared patterns suggest that the two spectra are likely to represent

TABLE 1 | Candidate genes validated in autism spectrum disorders (ASD) and schizophrenia spectrum disorders (SSD).

Gene	Function	Other phenotypes
RELN	Neuronal migration, polarization	Lissencephaly, Alzheimer's disease
DISC1	Neural development, synaptic plasticity, mammalian target of rapamycin (mTOR) regulation	Depression, bipolar disorder
FOXP2	Regulates DISC1, CTNAP2, language, and neural development	Developmental verbal dyspraxia
BDNF	Neurotrophic factor, regulates mTOR/AKT	Alzheimer's disease, Huntington disease
MECP2	Epigenetic regulator	Rett syndrome
UBE3A	Epigenetic regulator	Angelmann syndrome
NLGN3	Postsynaptic component coupled with NRXN	Undefined
NLG4	Postsynaptic component coupled with NXRN	ID
NRXN1	Presynaptic component coupled with NXRN	Pitt–Hopkins phenotype
SHANK3	Postsynaptic protein in glutamatergic neuron	Phelan–McDermid syndrome
CNTAP2	Cell adhesion and differentiation	ID, epilepsy, language impairment
CNTAP4		
GRIN2B	NMDA receptor subunit	ID, epilepsy
NTGN1	Axon guidance	Bipolar disorder
GABRB3	GABA receptor subunits	Bipolar disorder
GABRA5		
GAD	Conversion of glutamate to GABA	Epilepsy
CACNA1C	Voltage-dependent calcium channel subunit	Bipolar disorder, Brugada, and Timothy syndromes
SLC25A12	Mitochondrial membrane, solute channel protein	Mitochondrial disorders
OXTR/OXT	Oxytocin receptor/oxytocin gene	Undefined
ZNF804A	Transcription regulator of PRSS16, COMT	Bipolar disorders

Modified by de Lacy and King (9).
ID, intellectual disability.

TABLE 2 | Copy number variants (CNVs) implicated in ASD and SSD.

Region and type	Candidate genes	Phenotypes
1q21.1 Del	Unidentified	SCZ, ASD, ID, ADHD, deficit IGE
1q21.1 Dup	Unidentified	ASD, ID, ADHD
2p16.3 Del	NRXN1	SCZ, ASD, ID
3q29 Del	PAK2	ACZ, ID, ADHD
3q29 Dup	Unidentified	ID
15q11.2 Del	CYF1P1	ID, DD, SCZ, ASD, IGE, OCD, MDD
15q11-13 Dup	GABRA5, GABRB3, GABG3, and others	SCZ, ASD, ID, Ataxia
15q13.3 Del	CHRNA7	SCZ, ASD, ID
16p11.2 Del	DOC2A, ERK1	SCZ, ASD, ID, learning disorder
16p11.2 Dup	DOC2A, ERK1	SCZ, ASD, ID, DD
16p13.11 Del	NDE1	SCZ, ASD, ID
16p13.11 Dup	NDE1	SCZ, ASD, ID, ADHD, IGE
17q12	Undefined	SCZ, ASD, ID
22q11.2	PRODH, COMT, DGCR6, TRX1	SCZ, ASD, ID, epilepsy
22q11.21	PRODH, COMT, DGCR6, TRX1	ID, DD
22q13.3	SHANK3	ID, DD, ASD, SCZ

Modified by de Lacy and King (9).
ID, intellectual disability; ADHD, attention-deficit hyperactivity disorder, DD, developmental delay; OCD, obsessive–compulsive disorder; MMDD, major depressive disorder; IGE, immunoglobulin E deficit.

outcomes of common pathophysiological mechanisms. The next sections describe the E/I imbalance as a candidate mechanism possibly involved.

E/I IMBALANCE IN ASD AND SSD

An emerging hypothesis for the similarities in the social and cognitive disturbances associated with ASD and SSD is based on alterations in the ratio of excitatory to inhibitory cortical activity (E/I imbalance). Glutamate and GABA are, respectively, the two main neurotransmitters involved in excitatory and inhibitory signaling in the brain. Increased glutamatergic signaling alongside decreased GABAergic signaling would represent an E/I imbalance. Such imbalances may arise from disturbances in neural circuit formation or, abnormalities in the genes which code for proteins involved in these processes and linkage and association studies have been implicated in ASD and SSD (12). Postmortem studies have reported structural changes in both excitatory glutamatergic and inhibitory GABAergic circuits in individuals with ASD and SCZ (13–15).

In neurodevelopmental disorders, an E/I imbalance could arise directly through alterations in genes coding for glutamatergic receptors or synaptic proteins (16–18). The synapse organizers neurexins and their binding neuroligins are implicated in the formation and maintenance of excitatory and inhibitory synapses. Heterozygous deletions eliminating exons of the neurexin-1α gene in patients with ASD and SCZ have been detected and the functional significance of this recurrent deletion is still unclear. However, the availability of mice with deletion of the promoter and first exon of neurexin-1α provided evidence of the effects of neurexin-1α disruption on phenotypes relevant to ASD and SCZ and supported the role of neuroxins in neurodevelopmental disorders (19, 20).

In addition to the synaptic dysfunction, there is increasing evidence that E/I balance is also modulated by glial mechanisms that regulate glutamate activity (21, 22). Abnormalities in astrocyte gene expression in both ASD and SCZ have been detected (23), and reduced numbers of oligodendrocytes, impaired cell maturation, and altered gene expression of myelin/oligodendrocyte-related genes have been ascertained in SCZ (24). In turn, an increased number of activated microglia cells in adults with ASD have been found (25). As a result, glia deserves specific attention in the evaluation of E/I imbalance in these conditions.

ASD AND E/I IMBALANCE

The net effect of changes in glutamatergic and GABAergic systems in ASD may be an overall increase in the ratio of excitation to inhibition (E/I). Such an increase is likely to be implicated in seizures, macrocephaly, and core ASD symptoms (26). The E–I ratio in neocortical structures is determined by pyramidal glutamatergic neurons and inhibitory GABAergic parvalbumin (PV)-positive interneurons that are modulated and fine-tuned by minicolumns (groups of functionally autonomous neurons whose afferent and efferent connections influence the functioning of microcircuits) which have been found to be abnormal in ASD (27, 28). There are a number of candidate mechanisms for glutamatergic hyperactivity-driven hyperexcitability. Neuroligins (NL1–4) and neurexins (Nrxns 1–3) have been linked with ASD via point mutations and truncations, and chromosomal rearrangements have been identified in the region of interest (29–31). SHANK1, SHANK2, and SHANK3 are scaffolding proteins which influence the postsynaptic density of glutamatergic synapses and are of primary importance in ASD. SHANK3 is reported to be involved in Phelan–McDermid syndrome a form of ASD associated with moderate to severe intellectual disability (ID) and poor language skills (32). Regarding SHANK2 and SHANK1, they were found altered in ASD associated with mild ID as well as in high functioning individuals (33).

As to the mechanisms of GABAergic inhibitory dysfunction, the link with core ASD symptoms in humans is still under

investigation. Deficit in binocular rivalry, a visual function that is thought to rely on the balance of excitation/inhibition in visual cortex has been observed in ASD individuals. The link between GABA and binocular rivalry dynamics was found specifically absent in ASD pointing to an insufficient GABA inhibitory function (34). Postmortem studies have provided evidence of alterations in GABAergic circuits in ASD individuals; there have been reports of significantly reduced GAD65/GAD67 levels in the parietal cortex and cerebellum (35).

Induced pluripotent stem cells (iPSCs) have been used to investigate putative abnormalities in neural substrate of individuals with ASD. Even if no known underlying genomic mutation could be identified in a new study herein presented, interestingly, transcriptome and gene network analyses revealed upregulation of genes involved in cell proliferation, neuronal differentiation, and synaptic formation. The main finding was that overexpression of the transcription factor FOXG1 was responsible for the overproduction of GABAergic neurons, shifting the E/I balance toward inhibition (36).

SSD AND E/I IMBALANCE

Several postmortem studies detected lower levels of PV mRNA and GAD67, the principal synthesizing enzyme for GABA, in dorso-lateral-prefrontal cortex (DLPFC) PV neurons of patients with SCZ. Markers of GABA neurotransmission between chandelier neurons and their synaptic targets are altered in the DLPFC of subjects with SCZ (37, 38).

NMDA receptors are ionotropic glutamate receptors involved in synaptic regulation of E/I balance and there are multiple subtypes of NMDA receptor with different functions and distributions (39). Dysfunction of NMDARs has been documented in SCZ both in experimental models and human studies. In the NMDA-hypofunction model of the disease, changes in E/I balance and the resulting changes in behaviors have been hypothesized (40). Disrupted NMDAR function is implicated in altered neurodevelopment and may play a role in the progression of symptoms for SCZ especially for cognitive deficits (41–43). NMDA receptor hypofunction has been proposed in ASD as well and the NR2A, NR2B, and NR2C genes abnormalities have been associated with ASD (44).

Remarkably, two *de novo* mutations in the *GRIN2A*-coded subunit of NMDA receptors have been detected in patients with SCZ and one *de novo* mutation in *GRIN2B*-coded subunit in a patient with ASD. Truncating mutations in *GRIN2C*, *GRIN3A*, and *GRIN3B* were identified in both patients and controls, but no truncating mutations were found in the *GRIN1*, *GRIN2A*, *GRIN2B,* and *GRIN2D* genes (45).

NRG1and ErbB4 genes deserve attention, are expressed at excitatory synapses, and regulate spine structure and function. ErbB4 deletion is associated with neurodevelopmental abnormalities that are consistent with SSD (46, 47). The disrupted in SCZ 1 gene (*DISC1*) is another important candidate gene implicated at different levels of neurodevelopment through a scaffolding protein and different mutations have been detected in SCZ emphasizing its role (48, 49).

E/I imbalance has been proposed as a mechanism for hallucinations, one of the main positive symptoms of SSD. Hallucinations have been linked to inhibitory deficits such as impaired GABA transmission unfolding in a series of abnormalities such as impaired NDMA receptor plasticity, reductions in gamma frequency oscillations, sensory cortical hyperactivity, and cognitive inhibition deficits. However, the mechanisms by which E/I dysfunctions at the cellular level might be linked to clinical symptoms and cognitive deficits remain unclear (50).

The 22q11 microdeletion syndrome is the most common CNV associated with SCZ as it is present in 1–2% of cases, further there is a very high association of the syndrome with SCZ, up to 30–40%. This elevated risk is not associated with any other neurogenetic syndrome. Social cognition is impaired in 22q11.2 deletion syndrome and remarkably this feature is correlated with psychotic symptoms. The role of this microdeletion as a potential contributor to E/I imbalance is undefined (51).

CONVERGENT PATHWAYS VS. DIVERGENT PHENOTYPE IN ASD AND SSD

Schizophrenia spectrum disorders and ASD have been described as polygenic disorders in which the onset and progression of disease are triggered by interactions among multiple susceptibility genes.

Overlaps of risk genes among ASD and SSD have been documented. Two lines of mutant mice with *Shank3* mutations linked to ASD and SSD have been documented with shared and distinct synaptic and behavioral phenotypes. Mice with the ASD-linked InsG3680 mutation manifest striatal synaptic transmission defects before weaning age and impaired juvenile social interaction, coinciding with the early onset of ASD symptoms. On the other hand, adult mice carrying the SCZ-linked R1117X mutation demonstrated synaptic defects in prefrontal cortex and social dominance behavior. This is a paradigmatic example of different alleles of the same gene that have distinct phenotypes at molecular, synaptic, and circuit levels which may inform exploration of these divergences in human patients (52).

MAMMALIAN TARGET OF RAPAMYCIN (mTOR) SIGNALING IN ASD AND SSD

The mTOR pathway is directly involved in the physiological maintenance of the synaptic E/I ratio and is implicated in ASD by virtue of its role in upstream signaling and downstream regulatory mechanisms (12). Dysregulation of mTOR increases excitability and decreases inhibition thus contributing to E/I imbalance. mTOR activation is found in tuberous sclerosis complex mutations (TSC1/TSC2) occurring in tuberous sclerosis, which is frequently associated with ASD. Dysregulation of the mTOR pathway in these conditions provides clues to the molecular pathophysiology of ASD as the synaptic and cellular alterations involved may converge to produce the core social impairment of these disorders (53). In addition, mTOR

inhibitor compounds have the potential to reverse many of the behavioral and neurophysiological abnormalities associated with ASD (54).

Recent investigations have linked SSD to the mTOR signaling cascade (55). Dysfunction of diverse upstream activators and environmental stressors, that have been previously implicated in SCZ, can lead to either over-activation or inhibition of the signaling pathway. Alterations in GABA signaling may be involved in the dysfunction of inhibitory circuits in SSD through the DISC1–Akt–mTOR pathway. As well, a putative depression of mTOR signaling with possible variation between and within brain regions affecting neuronal functioning in variable fashion has been proposed. Consistently, a preponderant decrease in glutamatergic activity with respect to GABAergic activity has been reported (56). In this functional and still undefined background, abnormal synaptic function may be related to positive and negative symptoms of SSD (57). Lastly, mTOR signaling undergoes variations as neurodevelopment unfold and environment plays a significant role especially through early life experiences that needs to be thoroughly considered (58).

FINAL REMARKS AND FUTURE DIRECTIONS

There is epidemiological, clinical, neurobiological, and genetic evidence for a close relationship between ASD and SSD, and significant overlap in symptoms is frequently observed; however, there are also differences in clinical presentation, behavioral phenotype, and developmental trajectory.

The complex pathways that control E/I balance provide a framework for understanding how different genetic alterations implicated in these two distinct disorders can interact to disrupt excitatory and inhibitory neuronal function, neuronal circuit organization thus eventually influence complex social and cognitive behaviors. Nonetheless, it has to be clearly stated that current knowledge of the mechanistic relationships between E/I imbalance and the two spectrum disorders is still exploratory and need further evidence. The ways in which these shared mechanisms contribute to specific phenotypes such as ASD and SSD are still largely unknown. There are a number of open questions that need to be addressed such as whether there is a critical period for an E/I imbalance that mediates ASD- and SSD-associated behavior, or whether the E/I imbalance is circuit specific. Furthermore, an E/I imbalance may arise not only from synaptic dysfunction but also from altered cell fate that can lead to abnormal proportions of inhibitory and excitatory cells.

Shedding light on the shared functions of candidate genes for involvement in ASD and SSD is the key to translating genetic findings into descriptions of developmental and clinical subtypes. As to neuronal dysfunction hypothesized, abnormalities might be specific affecting only a subset of synapses in a selective group of neurons responsible of distinct symptoms but all that is still an hypothesis as evidence on the brain circuits potentially involved is lacking.

Research domain criteria (RDoC) project seems particularly indicated to this scope as it is directed to implement all the above level of understanding. One of the main purpose is to investigate mental illness through the dimensional approach to the fundamental components of behavior, through individual symptoms or symptom clusters, that cut across diagnoses, in this case specifically in ASD and SSD domains (59). Aim and legacy of RDoC novel approach is to build a research perspective that reflects advances in genetics, neuroscience, and behavioral science to provide a foundation for precision diagnosis and treatment of complex mental disorders such as those herein examined. The details obtained by the use of RDoC matrix likely will help to shed light on ASD and SSD relationships as well as on the longitudinal monitoring of emerging convergent and divergent symptoms of the two spectra (60).

It should also be noted that there is a subset of individuals with complex neurodevelopmental disorders whose symptoms span multiple functional domains including cognition and social communication. These individuals do not fit under any of the current diagnostic labels listed under ASD and SSD and further research through an RDoC approach holds promise to describe the specific biobehavioral profiles and thus eventually establish the diagnostic category in which they should be included. Consistent developmental designs are awaited to capture changes in the underlying neural circuitry, molecular pathways including E/I balance, and other biological components in ASD and SSD, relating them to changes in their corresponding cognitive and affective determinants as they emerge over time and alter behavior under the influencing role of environment.

AUTHOR CONTRIBUTIONS

RC and Mauro Pallagrosi equally participated in the substantial contribution to the conception or design of the work; drafted the work and revised it critically for important intellectual content; approved the final version to be published; and are 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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Brief Report: A Preference for Biological Motion Predicts a Reduction in Symptom Severity 1 Year Later in Preschoolers with Autism Spectrum Disorders

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Recent research has consistently demonstrated reduced orienting to social stimuli in samples of young children with autism spectrum disorders (ASD). However, social orienting greatly varies between individual children on the spectrum. Better understanding this heterogeneity in social orienting may contribute to our comprehension of the mechanisms underlying autistic symptoms thereby improving our ability to intervene. Indeed, children on the autism spectrum who show higher levels of interest in social stimuli demonstrate reduced clinical symptoms and increased adaptive functioning. However, longitudinal studies examining the influence of social orienting on subsequent outcome are critically lacking. Here, we aim to explore the relationship between social interest at the age of 3 and changes in severity of autistic symptoms over the subsequent year, in 20 children with ASD and 20 age-matched typically developing (TD) children. A visual preference for social stimuli was measured using an eye-tracking task at baseline, consisting of a previously studied visual preference paradigm presenting biological and geometric motion side-by-side. The task was altered for the current study by alternating presentation side for each type of stimuli to keep visual perseveration from influencing participants' first fixation location. Clinical data were collected both at baseline and 1 year later at follow-up. As a group, we observed reduced interest for biological motion (BIO-M) in children with ASD compared to TD children, corroborating previous findings. We also confirmed that a preference for BIO-M is associated with better adaptive functioning in preschoolers with ASD. Most importantly, our longitudinal results showed that a preference for BIO-M strongly predicted decreased severity of diagnostic symptoms. Participants who preferred social stimuli at the age of 3 showed drastic reductions in their severity level of autistic symptoms 1 year later, whereas participants who preferred geometric stimuli showed autistic symptoms that were unchanged

Abbreviations: ASD, autism spectrum disorders; BIO-M, biological motion; BVMP, biological motion visual preference task; GEO-M, geometric motion; GR, geometric responders; SR, social responders; TD, typically developing.

or more severe after 1 year. As a whole, our results suggest that a preference for BIO-M may be key to understanding the behavioral phenotype of young children with ASD, and may represent a promising candidate behavior for predicting early developmental trajectories and outcome.

Keywords: autism spectrum disorders, early development, social orienting, eye-tracking, symptom severity, adaptive functioning

INTRODUCTION

Autism spectrum disorders (ASD) comprise behavioral symptomatology with significant heterogeneity (1–3). This heterogeneity is evident from the first year of life, when behavioral signs of autism are first detectable (4). Also, developmental trajectories of young individuals with ASD have been shown to be varied and related on the behavioral features noted when the diagnosis of autism was established (5–7). For example, the rare, but consistent, longitudinal studies of outcome predictors, which are based on symptoms in children with ASD during early development, suggest that higher levels of early adaptive, cognitive and socio-communicative functioning predict better clinical outcomes [e.g., Ref. (5, 7)].

Several studies aimed at disentangling the heterogeneity of ASD symptoms have searched for biomarkers for autism with inconsistent results [for a review, see Ref. (8)]. Eye-tracking represents a promising way to measure early and specific visual exploration patterns in ASD that are different from those of children with typical development or developmental delay [for a review, see Ref. (9)]. Most eye-tracking studies on young children with autism have used stimuli with a high social content, such as faces or biological motion (BIO-M) (human movement) [e.g., Ref. (10–12)]. These investigations using social stimuli have consistently reported reduced attention to social stimuli in individuals with ASD [for a meta-analysis see Ref. (13)], though this reduction is inconsistent in preschoolers with ASD and appears to be related to adaptive or cognitive functioning [e.g., Ref. (14–17)]. These initial findings provide evidence for a relationship between reduced social interest in young children on the spectrum and their development. However, it is important to keep in mind that paradigm design also can greatly influence attention to social stimuli. An elegant meta-analysis by Chita-Tegmark (13) recently described different eye-tracking paradigms used to assess visual attention to social stimuli in ASD. This implies, for instance, social scenes used to measure preference for socially salient stimuli (e.g., faces) or on non-socially salient stimuli (e.g., objects). Also, existing research used visual preference paradigms opposing social stimuli to non-social stimuli (e.g., BIO-M vs. non-BIO-M). Measures of preference are both considered as visual exploration (e.g., time spent on stimuli) or latency to fixate social stimuli (e.g., reaction time). The authors point out that non-central aspects of these paradigms, including motion (e.g., static vs. dynamic stimuli), social content and communicative intention (e.g., number of people in a social scene and their social engagement), ecological validity, and multimodal presentation (e.g., conjunction with audio inputs) can significantly affect the allocation of social attention.

The *social motivation hypothesis of autism* [for a review, see Ref. (18)] considers a lack of social interest as the trigger for a behavioral cascade leading to the emergence of ASD symptomatology (19, 20). Accordingly, studies that further explore the relationship between reduced social interest and autistic symptoms deepen our understanding of early development of ASD in individuals. However, to the best of our knowledge, researchers have yet to explore the relationship between social interest and the development of severity of diagnostic symptoms in preschoolers with ASD over time.

Here, we explore how reduced social interest impacts ASD symptomatology in a group of 20 preschoolers with ASD and in 20 typically developing (TD) children of the same age. Pierce and colleagues (10, 16) previously demonstrated the validity of using visual preference patterns for BIO-M to assess social interest in preschoolers with ASD. Their task inspired our eye-tracking paradigm of visual preference for BIO-M (Franchini et al., submitted)[1], redesigned for the current study. In addition to the eye-tracking paradigm, we also evaluated participants on severity of diagnostic symptoms during a diagnostic assessment and adaptive functioning using parent reports to investigate whether visual preference, clinical, and/or adaptive features predict severity of diagnostic symptoms 1 year later.

First, we would expect that preschoolers with ASD would spend a lower percentage of their time looking at BIO-M than TD preschoolers; second, that the time ASD and TD participants spend on the BIO-M would correlate with their clinical and adaptive functioning. Third, we hypothesize that a preference for BIO-M will be positively related to a reduction in severity of diagnostic symptoms after 1 year.

MATERIALS AND METHODS

A total of 20 participants with ASD (all boys) were included in the study. A first visit (Time 1) was planned as soon as possible after a clinical diagnosis was made and a follow-up visit (Time 2) was organized 12 months later. An age-matched group (all boys) of TD children was compared to our group of children with ASD at Time 1 only. Participants with ASD were recruited through French-speaking parent associations and specialized clinical centers. TD participants were recruited through announcements in the Geneva community. At Time 1, the children's ages ranged between 22 and 51 months. The groups did not differ by age ($t = 0.38$, $p = 0.71$, see **Table 1**). At Time 2, ASD participants

[1]Franchini M, Wood de Wilde H, Glaser B, Gentaz E, Eliez S, Schaer M. Social orienting and joint attention in preschoolers with Autism Spectrum Disorders. Submitted.

TABLE 1 | Comparison between ASD and TD children on their clinical, adaptive, and social orienting features at Time 1.

	ASD – mean (SD) $n = 20$, males	TD – mean (SD) $n = 20$, males	*t*-Value	*p*-Value
Age (in months)	35.00 (9.47)	33.84 (9.52)	0.38	0.71
Severity Score	6.80 (0.39)	1.05 (0.05)	14.31	<0.0001***
Adaptive Behavior Composite	73.15 (1.87)	102.6 (2.78)	8.87	<0.0001***
Communication	73.00 (2.08)	104.8 (2.28)	9.86	<0.0001***
Daily Living Skills	75.90 (2.45)	102.4 (2.43)	7.68	<0.0001***
Socialization	74.45 (1.59)	99.84 (3.16)	7.58	<0.0001***
Motor Skills	83.37 (2.64)	98.95 (2.57)	4.23	0.0002***
Preference for BIO-M (%)	44.83 (4.98)	68.30 (2.29)	4.21	0.0002***

****p < 0.001.*

were between 33 and 63 months of age (mean age = 47.13, SD = 9.54). As expected, the children in the ASD group and the TD groups differed on measures of severity of symptoms and adaptive behavior (further details in **Table 1**). The Institutional Review Board of the University of Geneva approved the study protocol for all participants, and participants' parents gave their informed consent prior to inclusion in the study.

Measures

To measure adaptive behavior, we used the Vineland Adaptive Behavior Scales, 2nd edition (21). The VABS-II is a standardized parent report interview that provides a standardized Adaptive Behavior Composite and four domain scores: Communication, Daily Living Skills, Socialization, and Motor Skills.

To assess the severity of autistic symptoms, we used the Autism Diagnostic Observation Schedule – Generic (ADOS-G) (22), which gives a diagnostic score that is derived from the sum of each symptom score according to diagnosis of ASD in the DSM-5 (23). The ADOS-G diagnostic score was then transformed according to Gotham et al.'s algorithm (24) to obtain a Severity Score ranging from 1 to 10. Severity Scores allow further comparison of the severity of autism spectrum symptoms between the ADOS-G evaluations of each participant. All participants performed the Module 1 of the ADOS-G (module adapted to children with "Few to No Words" or "Some Words") or Module 2 (adapted to children with "Phrase Speech").

Finally, to quantify participants' preference for BIO-M, we designed a Biological Motion Visual Preference eye-tracking task (BVMP, see text footnote 1), inspired from the paradigm proposed by Pierce et al. (10). This passive 1-min task consists of the simultaneous presentation of dynamic geometric motion (GEO-M) on one side of the eye-tracking screen, and dynamic BIO-M on the other side. For the GEO-M, we used moving geometric shapes, similar to the classic abstract screen savers taken from MacOS screensavers or available under General Public License.[2] For the BIO-M, we recorded standardized sequences of one child moving and dancing in front of a white wall (to minimize opportunities for distraction). In contrast to the stationary sequence of 60 s used by Pierce et al. (10), the stimuli in our study alternated between the left and right sides. Between each segment, a turning wheel brought the child's gaze to the middle of the screen. Of the six segments of children moving and dancing, three sequences were of a boy and three were of a girl.

The task was administered using Tobii Studio software[3] with the TX300 Tobii eye-tracker. The sampling rate of the machine was 300 Hz and the video resolution was 1920 × 1080 pixels. As recommended by Tobii, participants sat on a parent's lap or alone on a chair at approximately 60 cm from the screen to minimize the impact of head movement on the gaze data. Before administering the task, all participants completed a five-point calibration procedure adapted to toddlers to detect eye motion and eye gaze.

Data analysis was done with Tobii Studio, version 3.1.6. The software automatically counts a fixation point every time a participant spends at least 100 ms within a 30-pixel circle. Areas of interests (AOIs) were drawn on the paradigm videos to delineate BIO-M and GEO-M. For each kind of stimuli (BIO-M and GEO-M), we calculated the total sum of fixation duration (the total looking time). The percentage of time spent on each stimulus type (BIO-M and GEO-M) was calculated by dividing the total fixations on in each AOI (or per stimulus type) by the total fixations on the entire screen. Visual preference for BIO-M was defined by whether a child looked at least 50% of the time at the video of a child moving and dancing.

Analysis Strategy

We first performed descriptive analyses of the groups' behavior and scores at Time 1. Children with ASD and TD children were compared on time spent on BIO-M at Time 1 by means of a *t*-test. To explore the relationship between variables in the ASD and the TD groups at Time 1, we correlated preference for BIO-M with standard VABS-II domain scores. In the ASD group, we additionally correlated Severity Score from the ADOS-G with VABS-II domain scores and with a preference for BIO-M (this was not performed in the TD group because all but one TD participant received the lowest possible Severity Score of 1). Subsequently, according to previous work [Pierce and colleagues (10, 16) and see text footnote 1], we split our group of children with ASD into two sub-groups: Social Responders (SR-ASD, who spent more than 50% of their total time on BIO-M) and Geometric Responders (GR-ASD, who spent more than 50% of their total time on GEO-M). Using *t*-tests, we compared GR-ASD and SR-ASD groups on

[2]http://www.reallyslick.com/screensavers

[3]www.tobii.com

the VABS-II domain scores and on the Severity Score from the ADOS-G.

Second, in the ASD group only, we correlated VABS-II scores and percent time spent on BIO-M at Time 1 with percent of change in Severity Score between Time 1 and Time 2. Correlation analyses were performed using Pearson correlation coefficients. Using a *t*-test, we then compared GR-ASD and SR-ASD children on their percent of change in Severity Score between Time 1 and Time 2. We then observed GR-ASD and SR-ASD improvements on each ADOS-G item contributing to the Severity Score. We, therefore, separated GR-ASD and SR-ASD into three groups for chi-square analysis of each item in the Severity Score: participants who showed a reduction on the item, participants who demonstrated no change on the item and participants with increased severity on the item. Three items were not included in our analyses because they contributed to the Severity Score in either Module 1-Few to No Words or Module 1-Some Words and, thus, did not allow for sufficient data. The specific items were "Pointing" ($n = 7$), "Responding to Joint Attention" ($n = 4$), and "Intonation of Vocalizations or Verbalizations" ($n = 4$). Results were considered significant at a *p*-value of 0.05. For the correlations between the VABS-II domains and related variables (preference for BIO-M, Severity Score, and percent change in Severity Score), we applied a Benjamini–Hochberg correction for multiple comparisons.

RESULTS

Cross-Sectional Observations: A Reduced Preference for Biological Motion in ASD Preschoolers Compared to TD Preschoolers

The ASD and TD groups differed on time spent on BIO-M ($t = 4.21$, $p < 0.001$) during the BVMP task (see **Table 1**). All TD children showed a preference for BIO-M (they spent more than 50% of the total time on BIO-M). By contrast, in the group of children with ASD, 12 preferred GEO-M (thus comprising the GR-ASD sub-group) and 8 preferred BIO-M (comprising the SR-ASD sub-group).

Time spent on BIO-M correlated with the VABS-II Adaptive Behavior Composite scores in the group of children with ASD ($r = 0.75$, $p < 0.001$, $df = 18$) but not in the TD group ($r = 0.45$, 0.05, $df = 18$) in which we observed a trend toward significance. We then examined the relationship between a preference for BIO-M and VABS-II domain scores in both groups.

After applying a Benjamini–Hochberg correction ($p < 0.0125$), we observed a positive relationship in the ASD group between time spent on BIO-M and scores on the Daily Living Skills ($r = 0.55$, $p = 0.0117$, $df = 18$) and the Socialization ($r = 0.84$, $p < 0.001$, $df = 18$) domains. We observed a non-significant trend between time spent on BIO-M and scores on the Motor Skills ($r = 0.51$, $p = 0.02$, $df = 18$) and Communication ($r = 0.68$, $p = 0.047$, $df = 18$) domains. In the TD group, we did not observe any significant relationship between preference for BIO-M and scores on the Communication ($r = 0.29$, $p = 0.22$, $df = 18$), Daily Living Skills ($r = 0.03$, $p = 0.89$, $df = 18$), Socialization ($r = 0.16$, $p = 0.51$, $df = 18$) or Motor Skills ($r = 0.51$, $p = 0.03$, $df = 18$) domains, though we observed a trend toward significance in the latter.

When we correlated severity of symptoms and VABS-II scores in the ASD group, we observed a trend toward a negative correlation between Severity Score and the Adaptive Behavior Composite ($r = -0.40$, $p = 0.08$, $df = 18$). Correcting the *p*-values for multiple comparisons at the threshold of $p < 0.0125$, the correlation between the Severity Score and the VABS-II socialization score did not survive ($r = -0.44$; $p = 0.05$, $df = 18$); none of the other correlations survived either between the Severity Score and the VABS-II Communication ($r = -0.29$, $p = 0.22$, $df = 18$), Motor Skills ($r = -0.38$, $p = 0.11$, $df = 18$) or Daily Living Skills ($r = -0.41$, $p = 0.07$, $df = 18$) scores. Time spent on BIO-M in the ASD group was not correlated with Symptom Severity ($r = -0.22$, $p = 0.36$, $df = 18$).

We subsequently compared SR-ASD and GR-ASD children. Despite the small sample sizes, we observed a significant difference between the groups. The SR-ASD children showed higher adaptive functioning compared to GR-ASD for the Adaptive Behavior Composite, Communication, and Socialization domains ($t = 2.13$, $p = 0.047$; $t = 2.14$, $p = 0.047$; $t = 3.39$, $p < 0.01$), but not for the Daily Living Skills and Motor Skills domains ($t = 1.17$, $p = 0.26$; $t = 1.01$, $p = 0.33$). We did not, however, observe a significant difference between SR-ASD and GR-ASD children ($t = 0.67$, $p = 0.51$) on Severity Score.

Longitudinal Analyses: A Preference for Biological Motion Predicts Reduced Symptom Severity 1 Year Later

We first correlated time spent on BIO-M at T1 and VABS-II adaptive behavior scores at T1 with percent change (between T1 and T2) in Severity Score on the ADOS-G (results are shown in **Table 2**). The results suggest that time spent on BIO-M at T1 predicts reduction in Severity Score 1 year later in the ASD group ($r = -0.67$, $p = 0.001$, $df = 18$, see **Figure 1**). In other words, children who spent the majority of their time on BIO-M during the eye-tracking BMVP task at baseline were the individuals who showed the most clinical improvement, as measured by the Symptom Severity score 1 year later.

A correlation between VABS-II Communication score and percent change in Severity Score survived the uncorrected

TABLE 2 | Predictors of changes in symptom severity 1 year later.

Adaptive behavior or preference for BIO-M at Time 1	Reduction in Severity Score from Time 1 to Time 2	
	r	*p*-value
Adaptive Behavior Composite	−0.31	0.18
Communication	−0.47	0.037
Daily Living Skills	−0.07	0.78
Socialization	−0.31	0.19
Motor Skills	−0.10	0.66
Preference for BIO-M (%)	−0.67	0.001**

$^{**}p < 0.01$.

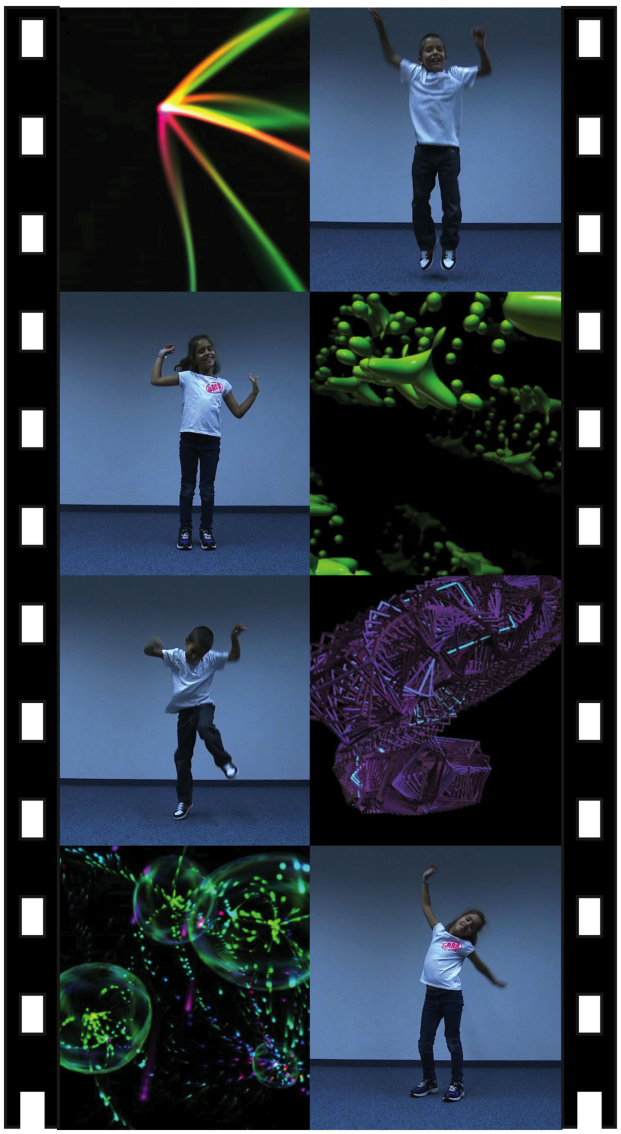

FIGURE 1 | Screenshots representing examples from the BMVP task.

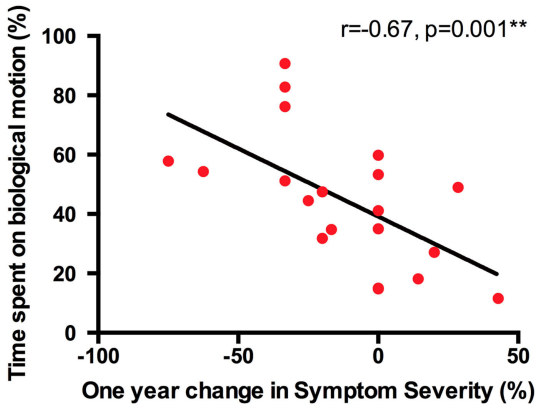

FIGURE 2 | Time spent on biological motion strongly predicts 1-year reduction of Symptom Severity in the group of preschoolers with ASD.

significance threshold, but did not survive our correction for multiple comparisons ($r = -0.47$, $p = 0.037$, $df = 18$).

We then looked at differences between the SR-ASD and GR-ASD sub-groups on the change in Symptom Severity between Time 1 and Time 2, and found a group difference. SR-ASD children showed greater improvement in Symptom Severity than GR-ASD children ($t = 3.38$, $p = 0.003$). After that, we looked at inter-group differences between GR-ASD and SR-ASD children for each item that contributed to the Symptom Severity score. Two participants were administered a Module 2 ADOS-G at one or both of the two visits and were, thus, excluded from analysis of items "Frequency of Spontaneous Vocalization Directed to Others" and "Integration of Gaze and Other Behaviors during Social Overtures," items that count toward the Severity Score in Module 1 only. A significant difference, with SR-ASD children showing more improvement than GR-ASD children, was detected on the following items: "Shared Enjoyment in Interaction" ($X^2 = 7.78$, $p = 0.02$), "Showing" ($X^2 = 13.13$, $p = 0.001$), and "Spontaneous Initiation of Joint Attention" ($X^2 = 7.82$, $p = 0.02$). For details, see **Table 3**.

DISCUSSION

Our results showing reduced interest in BIO-M in preschoolers with ASD compared to TD children, corroborate previous results by Pierce and colleagues (10, 16) and by our research group (see text footnote 1). Our ASD group also demonstrated a wide variance in their preferences for BIO-M, with percentage of visual preference for BIO-M ranging from 11.6 to 90.17% between participants. As previously observed in children with autism [(10, 16), see text footnote 1], this heterogeneity was related to their adaptive functioning. Based on these cross-sectional observations, we subsequently explored whether measures of adaptive functioning and social interest could predict symptom development in preschoolers on the spectrum. Longitudinal analyses revealed that a child's preference for BIO-M predicted a reduction in the severity of symptomatology after 1 year. Moreover, children with ASD with a stronger preference for BIO-M (SR-ASD) showed improvement on "Shared Enjoyment in Interaction," "Showing," and "Spontaneous Initiation of Joint Attention."

The implications of reduced social interest overall during the early development of children with ASD and the relationship between social interest (a preference for BIO-M) and reductions in certain ASD symptoms will be discussed in the following sections.

Reduced Preference for Biological Motion in ASD Children Compared to Their TD Children

In the present study, we confirmed previous findings (10, 16) that preschoolers with ASD orient less often toward BIO-M than TD children. As discussed in Franchini et al. (see text footnote 1),

TABLE 3 | Change in ASD Symptom Severity after 1 year according to preference for BIO-M at Time 1.

		Change in Symptom Severity				
ADOS-G Symptoms	**Preference for BIO-M**	**Reduction (*n*)**	**No change (*n*)**	**Increasing (*n*)**	**X^2**	***p*-Value**
SOCIAL AFFECT						
Communication						
Frequency of Spontaneous Vocalization Directed to Others	SR-ASD (*n* = 6)	5	1	0	4.06	0.13
	GR-ASD (*n* = 12)	4	7	1		
Gestures	SR-ASD (*n* = 8)	5	2	1	1.25	0.54
	GR-ASD (*n* = 12)	5	6	1		
Reciprocal social interaction						
Unusual Eye Contact	SR-ASD (*n* = 8)	1	7	0	0.06	0.79
	GR-ASD (*n* = 12)	2	10	0		
Facial Expression Directed to Others	SR-ASD (*n* = 8)	2	6	0	0.06	0.79
	GR-ASD (*n* = 12)	3	6	3		
Integration of Gaze and Other Behaviors during Social Overtures	SR-ASD (*n* = 6)	4	2	0	2.00	0.38
	GR-ASD (*n* = 12)	5	4	3		
Shared Enjoyment in Interaction	SR-ASD (*n* = 8)	5	3	0	7.78	0.02*
	GR-ASD (*n* = 12)	1	7	4		
Showing	SR-ASD (*n* = 8)	6	1	1	13.13	0.001**
	GR-ASD (*n* = 12)	0	9	3		
Spontaneous Initiation of Joint Attention	SR-ASD (*n* = 8)	5	1	2	7.82	0.02*
	GR-ASD (*n* = 12)	1	8	3		
Quality of Social Overtures	SR-ASD (*n* = 8)	3	5	0	2.55	0.28
	GR-ASD (*n* = 12)	1	11	0		
RESTRICTED AND REPETITIVE BEHAVIOR						
Unusual Sensory Interest in Play Material/Person	SR-ASD (*n* = 8)	2	6	0	3.34	0.16
	GR-ASD (*n* = 12)	3	5	4		
Hand and Fingers and Other Complex Mannerisms	SR-ASD (*n* = 8)	2	6	0	4.47	0.11
	GR-ASD (*n* = 12)	2	5	5		
Unusually Repetitive Interests or Stereotyped Behavior	SR-ASD (*n* = 8)	4	2	2	3.61	0.16
	GR-ASD (*n* = 12)	2	8	2		

*$p < 0.05$, **$p < 0.01$.

these results demonstrate that visual preference paradigms for BIO-M can serve as consistent and valid biomarkers for visual exploration patterns in young children with ASD. Moreover, presentation side of BIO-M and GEO-M stimuli changed often during the BMVP task to avoid visual exploration patterns associated with "sticky attention," which are characterized by a difficulty disengaging from GEO-M, a behavior that may be characteristic of individuals on the autism spectrum (25). Pierce and collaborators (10, 16) did not alternate the stimuli presentation during their task, which could potentially result in the young participants' attention to get "stuck." Accordingly, social orienting may, thus, be a better indicator of an initial preference for social or geometric stimuli, rather than a participant's main interest during the task. As we hypothesized, a preference for BIO-M was related to adaptive functioning in participants on the autism spectrum, lending support to the idea that interest in social stimuli is related to the behavioral phenotype of young children with ASD.

It is important to note that there are studies on social attention in ASD that did not report a difference between the visual preferences of ASD and TD children for social stimuli [for reviews, see Ref. (13, 26)]. Specific social content of stimuli used in tasks, as well as the salience of non-social stimuli can influence visual attention patterns in individuals with ASD [for a review, see Ref. (26)] and contribute to differences between studies. However, as Chita-Tegmark suggested in a review (13), tasks that display moving biological and geometric stimuli on opposite sides of the display, such as the task used in the current study and the one used by Pierce et al. (10, 16), have the advantage of clearly demarcating a visual preference for BIO-M or GEO-M in children with ASD.

A Preference for Biological Motion Predicts a Reduction in Symptom Severity 1 Year Later

To date, only a few research studies using limited samples have explored the evolution of Symptom Severity in young children with ASD. One such study demonstrated the idea that superior adaptive behavior is associated with better outcome in preschoolers with ASD (5). Also, better adaptive functioning has indeed been shown to be predictive of a small subset of young children who show "optimal progress" between ages 2 and 4, including those who no longer meet criteria for diagnosis at age 4 (7). In our study, we found that the best predictor of clinical improvement in Symptom Severity was a preference for BIO-M. Despite our small sample size, our results suggest that a preference for BIO-M may be a key to predicting early developmental trajectories and outcome.

Finally, our results point to specific criteria within the diagnostic assessment (ADOS-G) that were most improved in children

with a preference for BIO-M. These items are "Shared Enjoyment in Interaction," "Showing," and "Spontaneous Initiation of Joint Attention," Interestingly, these three items account for the "Social Affect" domain of the ADOS-G. According to a recent publication by Mundy et al. (27), these three criteria measure the initiation of joint attention behaviors, which also are described as "*infants' use of gestures and eye contact to direct others' attention to objects, to events, and to themselves*" [(28), p. 269]. The initiation of joint attention in early development of children with ASD is an example of a spontaneous and natural attention-sharing behavior that is intrinsically related to both social attention and social learning [for a review, see Ref. (28)]. Accordingly, increased social interest, as measured by a preference for BIO-M in the current study, may be related to early improvements in joint attention behaviors, thus resulting in improved early socio-communicative development (19, 20). These results corroborate the *social motivation hypothesis of autism* [for a review, see Ref. (18)], in that a preference for early social interest appears to support ensuing socio-communicative learning in young children with autism.

Limitations and Perspectives

The present study has several limitations. First, our sample size was relatively small. However, despite our small sample size, our results demonstrate that clinical differences can be detected during a 60-s standardized video, a quick and easy screening idea that warrants replication and further exploration. Second, this study explores clinical outcome of children with ASD during a 1-year period only. While future investigations should try to explore the influence of early social interest over a longer developmental period and with more participants, we are convinced that the strength of our statistical results reflects the importance of social orienting in ASD during preschool, a period that appears to be particularly ripe for developmental changes. Finally, subsequent studies may wish to explore the relationship between social interest and Symptom Severity taking into account baseline behavioral features of children with ASD, as well as specific therapeutic interventions, to better understand the factors moderating the longitudinal change.

CONCLUSION

Social attention plays an important role in early life, and may be a pivotal factor for individuals on the autism spectrum. Measures of social interest, as the one described in this study, offer the potential to better understand clinical features and outcome during early development in children with ASD. Our results provide support for this idea, by demonstrating a way of operationalizing social interest in young children to better understand and follow the heterogeneous phenotype between individuals on the spectrum. The resulting data are especially useful for the conceptualization of individualized early intervention in ASD and the assessment of progress during intervention.

ETHICS STATEMENT

The local ethical commission of Geneva approved this study.

AUTHOR CONTRIBUTIONS

MF, HW, BG, SE, and MS designed the study; MF, HW, and MS acquired the data; MF and MS analyzed the data and wrote the first draft of the article; MF, HW, BG, EG, SE, and MS contributed to the interpretation of the results and the writing of the manuscript. All authors have approved the final manuscript.

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CORRECTION
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Corrigendum: Brief Report: A Preference for Biological Motion Predicts a Reduction in Symptom Severity 1 Year Later in Preschoolers with Autism Spectrum Disorders

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A corrigendum on

Brief Report: A Preference for Biological Motion Predicts a Reduction in Symptom Severity 1 Year Later in Preschoolers with Autism Spectrum Disorders
by Franchini M, Wood de Wilde H, Glaser B, Gentaz E, Eliez S, Schaer M. Front Psychiatry (2016) 7:143. doi:10.3389/fpsyt.2016.00143

Text Correction

In the original article, there was an error on the details about the filter that we used during our analyses: [The software automatically counts a fixation point every time a participant spends at least 100 ms within a 30-pixel circle.].

A correction has been made to **[Measures]**, **[Paragraph Number 5]**. Details about the filter that we used during analyses have been correctly stated:

[A I-VT filter was enabled during analysis. (Classifier: 30°/s; Velocity calculator window length: 20 ms). The merge fixations option was further enabled (Max. time between fixations: 75 ms; Max angle between fixations: 0.5°).]

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

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

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Developmental Profile and Diagnoses in Children Presenting with Motor Stereotypies

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Introduction: Motor stereotypies represent a typical example of the difficulty in distinguishing non-clinical behaviors (physiological and transient) from symptoms or among different disorders ["primary stereotypies," associated with autistic spectrum disorder (ASD), intellectual disabilities, genetic syndromes, and sensory impairment]. The aim of this study was to obtain an accurate assessment on the relationship between stereotypies and neurodevelopmental disorders.

Methods: We studied 23 children (3 girls), aged 36–95 months, who requested a consultation due to the persistence or increased severity of motor stereotypies. None of the patients had a previous diagnosis of ASD. The assessment included the Motor Severity Stereotypy Scale (MSSS), the Repetitive Behavior Scale-Revised (RBS-R), the Raven's Colored Progressive Matrices, the Child Behavior CheckList for ages 1½–5 or 4–18 (CBCL), the Social Responsiveness Scale (SRS), and the Autism Diagnostic Observation Schedule-second edition (ADOS 2).

Results: All patients were showing motor stereotypies for periods of time varying from 6 to 77 months. The MSSS showed that each child had a limited number of stereotypies; their frequency and intensity were mild. The interference of stereotypies was variable; the impairment in daily life was mild. The RBS-R scores were positive for the subscale of "stereotypic behaviors" in all children. Moreover, several children presented other repetitive behaviors, mainly "ritualistic behavior" and "sameness behavior." All patients showed a normal cognitive level. The CBCL evidenced behavioral problems in 22% of the children: internalizing problems, attention, and withdrawn were the main complaints. On the SRS, all but one of the tested patients obtained clinical scores in the clinical range for at least one area. On the ADOS 2, 4 patients obtained scores indicating a moderate level of ASD symptoms, 4 had a mild level, and 15 showed no or minimal signs of ASD.

Discussion: Motor stereotypies in children with normal cognitive level represent a challenging diagnostic issue for which a finely tailored assessment is mandatory in order to define a precise developmental profile. Thus, careful and cautious use of standardized tests is warranted to avoid misdiagnosis. Furthermore, it is hard to consider motor stereotypies, even the primary ones, exclusively as a movement disorder.

Keywords: stereotypies, autistic spectrum disorder, children, repetitive behaviors, neurodevelopmental disorders, complex motor stereotypies, DSM 5

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INTRODUCTION

Stereotypies are a pattern of repetitive non-functional motor behavior that can interfere with the quality of social interactions, academic or other activities, or may result in injury (1, 2). Hand/arm flapping and waving, hand rotating, and finger wiggling are the stereotypies more frequently reported even if a wide range of other repetitive movements, sometimes accompanied by sounds or vocalizations, can be observed. Stereotypic movements generally last for seconds to minutes, tend to occur in clusters, and may appear many times throughout the day (3, 4). They are often triggered by periods of excitement, being engrossed in activities, stress, fatigue or boredom, and by daydreaming (5–7).

Stereotypies typically begin during the early developmental period, and they often represent a physiological and transient stage of development. Sixty percent of neurologically typical children show some stereotypic movements or behaviors between 2 and 5 years (8, 9). Besides the physiological ones, stereotypies are a main symptom of several developmental disorders, such as autistic spectrum disorder (ASD), intellectual disabilities (ID), genetic syndromes (Rett syndrome, Lesch–Nyhan syndrome, X-fragile syndrome, and others), and are also reported in sensory impaired individuals (10–12). In particular, stereotypies, together with other restricted, repetitive patterns of behavior, interest, or activities, represent a core symptom of ASD (13). Recent research suggests that restricted and repetitive behaviors can be subdivided into at least two subcategories, repetitive sensory motor and insistence on sameness behaviors, exhibiting different relationships with age and IQ (14). Moreover, stereotypic behaviors seem to be one of the four principal pathogenetic components in ASD, possibly differing in developmental trajectories and response to treatment (15).

If the above-mentioned conditions are ruled out, the stereotypies can be categorized as "primary" complex motor stereotypies (CMS) (16–18). Therefore, in the field of child neuropsychiatry, stereotypies represent a typical example of the difficulty in distinguishing non-clinical behaviors from symptoms or among different disorders.

In the DSM 5, the categorization into "primary" or "secondary" is not present: stereotypies are classified as stereotypic movement disorder (SMD) if they occur as a primary diagnosis or secondary to another disorder. It should be mentioned that the DSM 5 permits coding of SMD also in the presence of a neurodevelopmental disorder, adding the specifier of the associated condition (e.g., SMD associated with Lesch–Nyhan syndrome). On the other hand, when ASD is present, SMD is diagnosed only when there is self-injury or when the stereotypic behaviors are sufficiently severe to become a focus of treatment.

Some of the clinical conditions associated with stereotypies are relatively easy to diagnose. For instance, most genetic syndromes have evident phenotypic traits; moreover, in some of them, repetitive behaviors show specific profiles (19). In the same way, severe intellectual disability, sensory impairment, and low-functioning autism are conditions that can be ruled out without difficulty. On the other hand, major problems can be encountered in disentangling primary CMS from stereotypies occurring in high-functioning ASD. It should be mentioned that ASD was an exclusionary criterion in many studies on primary CMS: the children with a previous history of ASD diagnosis (based on a review of medical records) and those with overt risk of ASD (based on the Autism Spectrum Screening Questionnaire, carried out during a telephone screening) were excluded from the studies (20, 21).

From a differential diagnosis perspective, little help comes from previous studies aimed at comparing primary and secondary stereotypies. Previous studies have shown that individuals with autism had higher levels and intensity of stereotypy than individuals with mental retardation (22). These findings are consistent with other studies that have reported higher levels of stereotypy in individuals with disabilities than in age-matched individuals without disabilities (23). In a comparison study between typically developing children and patients with autism and PDD-NOS, the first ones showed constant and low levels of stereotypic behavior whereas levels increased with age in the children with autism and PDD-NOS (24). In a large cohort of 277 children, which included children with autism and non-autistic developmental disorders, Goldman and Greene (25) showed that the presence of stereotypies at preschool was more strongly linked with autism than with cognitive incompetence. Moreover, the number of stereotypies per child and the variety of stereotypies has been reported to be greater in low-functioning autism group, with head/trunk, hand/finger, and gait (e.g., spinning and pacing) stereotypies being the most frequent in this group (26). Finally, in a recent study comparing primary and secondary stereotypies, Ghosh et al. (27) found that the primary ones were simple, prevalently motor, of shorter duration, and of less frequency, whereas secondary had more vocalization, complexity, longer durations, and higher frequency; unexpectedly, worsening of stereotypies was noted in a higher percentage of the primary cases. Overall, these studies have limited usefulness when dealing with individual cases.

The aim of this study was to obtain an accurate analysis on the relationship between stereotypies and neurodevelopmental disorders. Therefore, we assessed a group of children requesting a consultation due to the presence, persistence, or increased severity of motor stereotypies.

MATERIALS AND METHODS

Participants

We studied 23 children, aged from 36 to 95 months, consecutively enrolled between September 2015 and June 2016 at the Outpatients Division of the Department of Pediatrics and Child Neuropsychiatry of "Sapienza" University of Rome. They were referred to our childhood movement disorder unit by their pediatrician. In all cases, the consultation was requested for the presence, persistence, or increased severity of motor stereotypies.

The only inclusion criterion was the presence of motor stereotypies, observed during the consultation, reported by parents or by home videos.

Exclusion criteria for the study were:

(1) known or suspected genetic syndromes;
(2) presence of other clinical neurological signs;
(3) presence of sensory impairment;
(4) previous diagnosis of ASD.

Procedures

After the first medical examination, eligible subjects were asked to participate in the study. Participants received a complete assessment with standardized tests, including an evaluation of cognitive profile [using Raven's Colored Progressive Matrices (CPM)]. Parents were asked to fill in questionnaires to check for other neuropsychiatric conditions: the Child Behavior CheckList for ages 1½–5 or 4–18 (CBCL), and the Social Responsiveness Scale (SRS) for children aged over 48 months. The Motor Severity Stereotypy Scale (MSSS) and the Repetitive Behavior Scale-Revised (RBS-R) were used to assess the stereotypies. The Autism Diagnostic Observation Schedule-Second edition (ADOS 2) was administered to the patients by fully qualified personnel, blinded to the clinical diagnosis. The study was approved by the "Sapienza – University of Rome" Ethics Committee (Ref. 3477). The parents gave their informed consent at the time of enrollment in the study.

Statistical Analysis

Quantitative data were summarized by means ± SD and categorical data by absolute and percent frequencies. Differences in total and subtotal scores of CBCL and SRS among groups based on the ADOS 2 calibrated severity score were assessed by the analysis of variance, followed by the Tukey test for multiple comparisons. The Pearson linear correlation coefficient r was computed to estimate the correlation between the CBCL pervasive developmental problem score and the ADOS 2 and SRS scores. To take into account possible violations of assumptions of parametric tests, non-parametric Kruskal–Wallis analysis of variance and Spearman's rank correlation coefficient were also applied. Since the results perfectly overlapped those of parametric analyses, only the latter are presented. Statistical analyses were performed by STATA Release 8.1.

RESULTS

The sample consisted of 23 children – 20 boys and 3 girls – aged between 36 and 95 months (mean = 53; SD = 15). The review of medical records showed that four patients had a history of motor delay (walking alone after 18 months of age), eight had motor coordination problems or immaturity in graphomotor skills, and seven had delay in expressive language. None of the children had a previous history of ASD diagnosis. At the time of consultation, all patients were showing motor stereotypies for periods of time varying from 6 to 77 months (mean = 33; SD = 16). The onset of stereotypies was reported between 4 and 51 months of age (mean = 19; SD = 14).

Their semiology had remained unchanged over time, mostly characterized by CMS: patients presented a single repetitive movement or complex sequences involving the entire body such as jumping, kicking, flapping hands, moving hands in front of the face or the eyes, or involving movements and "dystonic" postures of the trunk. Sounds or vocalizations accompanied the motor stereotypies in three patients. From the time of their onset, increasing frequency of stereotypies has been reported. Excitement or boredom was described as common triggers.

The MSSS showed that each child had a limited number of stereotypies, between 1 and 3 (mean = 1.6); their frequency and intensity were mild (range 1–4; mean = 2.8 for both). The interference of stereotypies was variable, from 0 to 4 (mean = 1.6). The mean MSSS final score was 21.1, suggestive of a mild impairment in daily life.

On the RBS-R, items in the subscale of "stereotypic behaviors" were scored by all children; moreover, the questionnaire revealed the presence of other repetitive behaviors in several children, mainly "ritualistic behavior" and "sameness behavior," even if at a lower degree (**Table 1**).

All patients showed a normal cognitive level: in particular, on the Raven's CPM, they obtained scores ranging between 32 and 95 percentiles (mean = 80, SD = 16), corresponding to IQ levels superior to 85.

The CBCL evidenced behavioral problems in 22% of the children (**Table 2**); internalizing problems, attention, and withdrawn were the main complaints reported by parents. Among the DSM-oriented scales, the pervasive developmental problems were the principal affected domain: indeed, three children obtained borderline and four clinical scores.

Symptoms of ASD were assessed by the SRS questionnaire (in the 15 children older than 48 months) and by the ADOS 2.

On the SRS, all but one of the 15 patients obtained clinical scores in the clinical range at least in one area (**Table 3**). Obviously, the more frequently affected domain was "restricted interests and repetitive behavior," which indicated the clinical range in 11 out of 15 children. Moreover, "social motivation" and "SRS total score" were affected in 56% of children.

For the ADOS 2, four patients obtained scores indicating a moderate level of ASD symptoms (calibrated severity score = 6–7), four had a mild level (calibrated severity score = 4–5), and 15 showed no or minimal signs of ASD (calibrated severity score = 0–3).

TABLE 1 | RBS-R: subscale scores and number endorsed.

	Subscale scores (mean ± SD)	Number endorsed[a] (mean ± SD)	Number of patients with number endorsed ≠ 0
I – stereotypic behavior	3.6 ± 2.3	2.0 ± 1.1	23
II – self-injurious behavior	0.4 ± 1.1	0.3 ± 0.6	4
III – compulsive behavior	1.1 ± 1.9	0.7 ± 1.2	9
IV – ritualistic behavior	1.4 ± 2	0.9 ± 1.1	12
V – sameness behavior	1.9 ± 2.5	1.6 ± 1.7	15
VI – restricted behavior	0.9 ± 1.5	0.5 ± 0.7	8

[a]*Number of items endorsed for each subscale (any rating other than 0).*

TABLE 2 | Number of patients obtaining borderline or clinical scores at the Child Behavior CheckList – ages 1½–5 or 4–18, according to chronological age.

	Borderline	Clinical
CBCL total	2	5
CBCL internalizing problems	3	5
CBCL externalizing problems	4	2
Symptoms scales		
Emotionally reactive	2	1
Anxious/depressed	0	1
Somatic complaints	0	2
Withdrawn	3	2
Sleep problems	0	2
Attention problems	3	3
Aggressive behavior	1	0
DSM-oriented scales		
Anxiety problems	1	0
Pervasive developmental problems	3	4
ADHD problems	2	1
Oppositional defiant problems	0	0

TABLE 3 | Number of patients obtaining clinical scores at the Social Responsiveness Scale (SRS).

	Mild range (*T* score: 60–65)	Moderate range (*T* score: 66–75)	Severe range (*T* score >76)
Total score	4	1	3
Social awareness	1	4	2
Social cognition	2	2	1
Social communication	3	2	1
Social motivation	5	3	1
Restricted interests and repetitive behavior	2	4	5

In the whole sample, no correlation was found between the ADOS 2 scores (calibrated severity score, total, and subtotal raw score) and the measures of stereotypic behavior (MSSS and RBS-R scores, subtotal, and total).

No statistical differences were found between subgroups of patients – divided on the basis of the ADOS 2 calibrated severity score – with regard to the total and subtotal scores of CBCL and SRS (**Table 4**). However, in the 15 patients aged above 48 months, a moderate correlation was found between the CBCL pervasive developmental problem scores and the following SRS scores: total ($r = 0.370$) and social communication ($r = 0.585$).

DISCUSSION

In this study, we focused on the assessment of a group of children seeking medical attention for the presence or the persistence of motor stereotypies. According to our data, the impairment due to the stereotypies was mild, without a clear interference in daily activities. The referral of children with mild stereotypies could be due to parents' concerns about a movement disorder that lasts over time and/or to an increased sensitivity to the ASD issue. Some of these children have a history of slight developmental delay in some areas (motor or language), but all had a normal cognitive level and no previous diagnosis of ASD.

Based on our assessment, most of them – 65% of the sample – can be easily diagnosed as having primary CMS. Namely, they showed a few stereotypies, with mild impairment; moreover, the ADOS 2 evidenced no or minimal risk of ASD. Notably, in many of these children, the RBS-R questionnaire disclosed the presence of other symptoms, mainly ritualistic behaviors, sameness, and restricted interests.

TABLE 4 | Comparison of CBCL and SRS *T* scores and number of patients with clinical scores in subgroups (following ADOS 2 score).

ADOS 2 – calibrated severity score	0–3	4–5	6–7
Number of patients	15	4	4
Mean age (range) (months)	57 (36–95)	37 (36–39)	57 (55–60)
CBCL			
Total score	Mean = 57 B = 1; C = 4	Mean = 53 B = 1; C = 0	Mean = 57 B = 0; C = 1
PDD subscale	Mean = 57 B = 0; C = 3	Mean = 60 B = 0; C = 1	Mean = 65 B = 3; C = 0
SRS			
Total score	Mean = 61 M = 3; Mo = 1; S = 2		Mean = 61 M = 1; Mo = 0; S = 1
Social awareness	Mean = 63 M = 1; Mo = 2; S = 2		Mean = 59 M = 0; Mo = 2; S = 0
Social cognition	Mean = 57 M = 1; Mo = 1; S = 1		Mean = 56 M = 1; Mo = 1; S = 0
Social communication	Mean = 59 M = 2; Mo = 1; S = 1		Mean = 54 M = 1; Mo = 1; S = 0
Social motivation	Mean = 58 M = 2; Mo = 2; S = 1		Mean = 65 M = 3; Mo = 1; S = 0
Restricted interests/repetitive behaviors	Mean = 71 M = 1; Mo = 3; S = 4		Mean = 70 M = 1; Mo = 1; S = 1

B, borderline; C, clinical; M, mild; Mo, moderate; S, severe.

These features constitute core symptoms of ASD and their presence, even if at a low level, in our "primary" patients, highlights the difficulty to define the boundaries between transient stereotypic movements in otherwise typically developing children, the primary motor stereotypies with or without comorbidity, and the stereotypic movements in the course of ASD.

Moreover, in some previous studies on children with primary CMS (4, 20), a series of comorbid conditions have already been reported; in particular, ADHD, tic disorders, developmental coordination disorder (DCD), and other neuropsychological problems were described in a significant percentage of cases. Thus, as the DSM 5 clearly states, the presence of stereotypic movements may indicate an undetected neurodevelopmental problem. Among these comorbidities, DCD seems to take on great interest, being present in school age (20) as well as preschool age patients (Baglioni et al., in preparation).

On the basis of all these observations, it is difficult to consider motor stereotypies, even the primary ones, exclusively as a movement disorder (28). A limited number of our patients showed behaviors compatible with a diagnosis of non-autistic ASD or autism, following the calibrated severity score of ADOS 2. This index is considered to be reliable and stable over time (29). These findings were unexpected mainly because, in the past, many of our patients underwent clinical consultations for developmental delay and the diagnosis of ASD was never suspected. A possible explanation for this could be that problems of communication or socialization were overlooked due to their normal cognitive level and/or that subsequent evaluations might be sensitive to developmental changes in social and communication goals that have to be progressively attained during development.

On the other hand, it should be noted that in the whole sample, important discrepancies between the results of different tests were disclosed. In particular, high rates of clinical scores on the SRS were also found in patients who showed no or minimal risk of ASD according to the ADOS 2.

The SRS is an instrument developed to measure social impairment, but many items also describe other core features of ASD, including communication deficits and repetitive behaviors (30), as well as symptoms not exclusively related to ASD diagnostic criteria (31). Moreover, when using the SRS as a quantitative phenotype measure, the effects of non-ASD-specific factors must be considered; if not, SRS scores are more appropriately interpreted as indicating general levels of impairment, rather than severity of ASD-specific symptoms or social impairment (32).

Our study has some limitations. First of all, a small number of children participated. Second, the wide age range of the subjects hampered gathering more homogeneous data (i.e., the SRS is validated from the age of 4 years, while the SRS-2, which can be administered from the age of 1.5 years, is not yet available in Italian). Third, we did not use the Autism Diagnostic Interview that, together with the ADOS 2 and the criteria of DSM 5, could have allowed us to gain more firm diagnostic conclusions within the study.

Nonetheless, our study highlights the challenge of establishing a categorical diagnosis in children with motor stereotypies. Obviously, beyond the classification, this issue is important in terms of treatment of stereotypies that is still a debated problem (33–36).

Waiting for the results of studies investigating the pathophysiological aspects of stereotypies in ASD subjects as in "primary" cases and possibly supporting current hypotheses (37–41), a dimensional approach to the diagnosis of stereotypic behaviors could be particularly suitable. In this perspective, the view of the DSM 5, that puts together the stereotypies (with the exceptions described above) and permits coding additional conditions, seems to be more useful than the rigid categorization of "primary" and "secondary."

In conclusion, complex stereotypies in children with normal cognitive level represent a challenging diagnostic issue for which a finely tailored assessment is mandatory in order to evaluate their peculiar developmental sentinel role. Notably, a careful and cautious use of standardized tests is warranted to avoid misdiagnosis.

AUTHOR CONTRIBUTIONS

FC made a substantial contribution to the conception of the work and in writing the paper; FV, DM, and VB contributed to the acquisition of data; CD contributed to the interpretation of data and literature analysis; FCh contributed to statistical analysis and critically reviewed the manuscript. All the authors read and approved the final version of the manuscript.

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Insulin-Like Growth Factor 1 and Related Compounds in the Treatment of Childhood-Onset Neurodevelopmental Disorders

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Insulin-Like Growth Factor 1 (IGF-1) is a neurotrophic polypeptide with crucial roles to play in Central Nervous System (CNS) growth, development and maturation. Following interrogation of the neurobiology underlying several neurodevelopmental disorders and Autism Spectrum Disorders (ASD), both recombinant IGF-1 (mecasermin) and related derivatives, such as (1-3)IGF-1, have emerged as potential therapeutic approaches. Clinical pilot studies and early reports have supported the safety/preliminary efficacy of IGF-1 and related compounds in the treatment of Rett Syndrome, with evidence mounting for its use in Phelan McDermid Syndrome and Fragile X Syndrome. In ASD, clinical trials are ongoing. Here, we review the role of IGF-1 in the molecular etiologies of these conditions in addition to the accumulating evidence from early clinical studies highlighting the possibility of IGF-1 and related compounds as potential treatments for these childhood-onset neurodevelopmental disorders.

Keywords: IGF-1, autism spectrum disorders, Rett Syndrome, Fragile X Syndrome, Phelan-Mcdermid Syndrome

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INTRODUCTION

Insulin-Like Growth Factor 1 (IGF1) is a polypeptide hormone and a member of a superfamily of related insulin like hormones termed Insulin Like Peptides (ILPs) (Fernandez and Torres-Alemán, 2012). IGF-1 is primarily released by hepatocytes in response to Growth Hormone, but is also produced in the Central Nervous System (CNS) where it has pleiotrophic effects on all major CNS cell types (Bach et al., 1991; O'Kusky and Ye, 2012). IGF-1 has crucial roles to play in the development, growth and maturation of the CNS and its synapses, roles which have been reviewed extensively elsewhere (Bach et al., 1991; Popken et al., 2004; Aberg et al., 2006; Llorens-Martín et al., 2009; Corvin et al., 2012; O'Kusky and Ye, 2012; Supeno et al., 2013; Huat et al., 2014; Dyer et al., 2016).

IGF-1 and IGF1 receptor (IGF1R) expression levels have a definite spatio-temporal patterns (Bach et al., 1991; Bartlett et al., 1992; Bondy and Lee, 1993; Zhang et al., 2007). Of note, the abundance of IGF-1R expression over IGF-1 hints at the importance of peripherally produced IGF-1 in mediating the effects of IGF-1 on the CNS (Fernandez and Torres-Alemán, 2012). IGF-1 acts on its glycoprotein receptor (IGF1R), a tyrosine kinase receptor, to activate canonical signaling pathways, including: (i) the PI3K (phosphatidylinositol-3 kinase)—AKT1 (serine-threonine-specific protein kinase)—FOXO (forkhead box protein O) and (ii) the RAS—MAPK (mitogen-activated protein kinases)—ERK (extracellular signal regulated kinases) pathway. Both of these pathways have important roles to play including cell cycle regulation, gene expression,

protein synthesis, autophagy, apoptosis and remodeling of the cytoskeleton (Fernandez and Torres-Alemán, 2012; Costales and Kolevzon, 2015).

Once released in the serum, IGF-1 can be cleaved to yield an amino terminal glycine-proline-glutamate (GPE tripeptide) and a truncated IGF-1 form called des-N-(1-3)-IGF-1, lacking the N-terminal GPE tripeptide, which has greatly reduced affinity for IGFBPs and is therefore, is more potent than IGF-1. (1-3)IGF-1 is an active metabolite with neuroprotective effects as well as effects on excitatory synaptic markers such as synapsin 1 and post-synaptic density 95 (PSD-95), recapitulating many of the effects of IGF-1 on synaptic maturation and plasticity (Guan et al., 1999; Corvin et al., 2012). Of note, this effect of (1-3) IGF-1 may be different in neuronal and non-neuronal cell populations. In one report studying the effects of IGF-1 acting on its canonical pathways in neuronal and non-neuronal cells, IGF-1 was found to increase pAkt statining in neurons but not glial cells, whilst (1-3) IGF-1 increased staining in glial cells but not neurons (Corvin et al., 2012). Thus, signaling by IGF-1 and its active tripeptide may demonstrate different effects in different CNS cell populations, adding further to the biological complexity of IGF-1 signaling. One mechanism of action of (1-3)IGF-1 may be to indirectly activate the IGF-1R via an increase in endogenous IGF-1 release (Corvin et al., 2012). Another important aspect of IGF-1 physiology is its binding to IGF Binding Proteins (IGFBP), which regulate its bioavailability, localization and activity (Ocrant et al., 1990; Clemmons, 1998; Hwa et al., 1999; Firth and Baxter, 2002).

Disruption of IGF-1 function has profound phenotypic consequences both in murine models and in humans, underscoring the important role of IGF-1 in CNS development and maturation (Beck et al., 1995; Woods et al., 1996; Netchine et al., 2011). In the present Mini-Review, we address the role of perturbed IGF-1 signaling and the therapeutic potential of IGF-1 and (1-3)IGF-1 in neurodevelopmental disorders such as Rett Syndrome (RTT), Fragile X Syndrome (FXS), Phelan McDermid Syndrome (PMDS) as well as broader Autism Spectrum Disorder (ASD).

RETT SYNDROME

Rett Syndrome (RTT) is a pervasive X-Linked neurodevelopmental disorder affecting 1:10,000 female (Percy and Lane, 2004). RTT is characterized by an apparently normal development, followed by a subsequent regression in psychomotor, social and cognitive abilities, deficits in social interaction and a loss of acquired fine motor skills and purposive hand movements. In the CNS, RTT is characterized by microcephaly, neuronal atrophy and leads to cardiorespiratory problems (Julu et al., 2001; Hagberg, 2002). At present, the treatment of RTT represents an unmet therapeutic need.

More than 85% of RTT is caused by a mutation in methyl CpG-binding protein 2 (*MeCP2*), encoding the protein MECP2, which has roles both inside and outside the nucleus (Kaufmann et al., 2005; Chahrour et al., 2008). Atypical cases of RTT may also be caused, in less than 10% cases, by mutations in cyclin-dependent kinase-like 5 (CDK-L5) and in the Forkead Box G1 (FOXG1) in 1% cases (Cahrour and Zoghbi, 2007).

The deletion of the *MeCP2* gene in mouse models recapitulates many of the autonomic, motor and cognitive features of the human RTT phenotype (Banerjee et al., 2012; Castro et al., 2014; Katz et al., 2016), together with reduced connectivity (Armstrong, 2005; Chapleau et al., 2009) and defects in neurotransmitter and receptor expression. *MeCP2* loss in mice results in an alteration in excitatory-inhibitory balance with reduced excitation/increased inhibition in cortical samples/tissue (Dani et al., 2005; Durand et al., 2012).

MeCP2 deficient brains demonstrate large numbers of modest transcriptional changes, both positive and negative (Katz et al., 2016). One well characterized target of MECP2 function is Brain Derived Neurotrophic Factor (BDNF), an important modulator of CNS growth, which shows synergy with IGF1 in the CNS (Ding et al., 2006). BDNF is down-regulated in murine models of RTT as well as patients with RTT (Chang et al., 2006; Zhou et al., 2006). Although studies in rats have shown that there is clear exchange of BDNF between the brain and the periphery (Pan et al., 1998) and *viceversa* (Poduslo and Curran, 1996), other studies have failed to raise BDNF in the brain to therapeutic levels, suggesting crossing through the blood-brain barrier (BBB) of this neurotrophin to be insufficient for clinical purposes (Pardridge et al., 1994). IGF-1 is a potential alternative, which on crossing the BBB acts on the same pathways as BDNF (such as the PI3K-Akt and MAP-ERK pathways) and appears important for BNDF effects on activity dependent plasticity (Pardridge et al., 1994; Ding et al., 2006).

In *Mecp2* mutant mice, administration of both IGF-1 and (1-3)IGF-1 reverses many of the features of the RTT phenotype (Chen and Russo-Neustadt, 2007; Castro et al., 2014). Castro et al. (2014) demonstrated reduced IGF-1 levels in *MeCP2* mutant mice, with subsequent daily administration of IGF-1 resulting in an improved lifespan, weight and autonomic parameters in the knockout mice. IGF-1 significantly improved abnormalities in activity dependent plasticity in *MeCP2* mutant mice (using a monocular deprivation paradigm). Similar effects are observed with (1-3) IGF-1 administration (Tropea et al., 2009), together with improved spine density, synaptic amplitude and increased excitatory synaptic markers (Tropea et al., 2009). Furthermore, in a mouse model of atypical RTT with mutations in CDLK5, IGF-1 was demonstrated to rescue deficits in dendritic spine instability and expression of PSD-95 adding further support to IGF-1 as a potential treatment in RTT (Della Sala et al., 2016).

One mechanism by which IGF-1 exerts its effects in RTT may be by acting on its canonical signaling pathways (such as PI3K and MAPK pathways as above). Interestingly, a recent study demonstrates that IGF-1 application may actually increase nuclear *MeCP2* transcript and protein (Tropea et al., 2016). Activity dependent plasticity was also shown to modulate MeCP2 expression and this additionally demonstrates the profound effects of IGF-1 on cellular neuroplasticity in the CNS. Restoration of these abnormalities in activity-dependent plasticity may be one important way in which IGF-1 may exert its effects in RTT. Interestingly, MeCP2 may affect IGF-1

levels by regulation of IGFBPs which has been demonstrated for IGFBP3 in both murine models and humans (Itoh et al., 2007).

IGF-1 is already indicated in the pediatric population for severe growth failure and IGF-1 deficiency. In RTT patients, two early studies have been performed demonstrating the tolerability and safety of IGF-1 as a potential treatment (Pini et al., 2012; Khwaja et al., 2014). Khwaja et al. (2014) demonstrated the safety of IGF-1 in 12 patients with *MeCP2* mutations (9 with RTT) with a 4 week multiple ascending dose (40–120 ug/kg bd) followed by a 20 week open label extension, without any serious adverse events or hypoglycamia. A previous clinical study had demonstrated safety in 6 patients with RTT receiving twice daily injections of IGF-1 for a 6 month period (Pini et al., 2012). This was followed by a single case study of one of the patients reporting the safety of repeated treatment with IGF-1 for a second 6 month cycle (Pini et al., 2014).

Preliminary efficacy analysis by Khwaja et al. (2014) demonstrated an improvement in cardiorespiratory parameters, some neurobehavioral parameters and EEG measures of mood and anxiety (reversed frontal alpha band asymmetry). Pini et al. analyzed 10 patients whom had received IGF-1 treatment in a clinical study and compared various parameters to age and disease severity matched controls (Pini et al., 2016). They reported a significant improvement in disease severity as assessed by clinicians as well as two independent and blinded observers using a novel video based scoring system. Whilst this evidence is preliminary, this early data demonstrating efficacy of IGF-1 in RTT is encouraging. The results of ongoing Phase 2 clinical trials using IGF-1 in RTT are eagerly awaited (NCT01777542). Furthermore, an analog of (1-3)IGF-1, NNZ-2566 (administered orally) has demonstrated efficacy in both clinician and caregiver assessments in an industry-led Phase 2 trial on patients with RTT aged 15-45 (NCT01703533), with a Phase 2 trial on younger patients in progress (NCT02715115). Taken together, there is encouraging evidence for the use of IGF-1 and (1-3) IGF-1 in the treatment of RTT (see **Tables 1**, **2**).

FRAGILE X SYNDROME

Fragile X Syndrome (FXS) results from a mutation in the *Fmr1* gene, encoding the protein Fragile X Mental Retardation Protein (FMRP1). The disorder is characterized by learning disability, social anxiety and attention deficit disorder, impaired social interactions and seizures as well as an abnormal physical phenotype with macro-orchidism and facial dysmorphism (Hagerman, 1997; Garber et al., 2008). The treatment of FXS represents a largely unmet clinical need.

FMRP, an mRNA binding protein, expressed in neuronal cell bodies and dendrites acts to regulate protein translation at the synapse, with important roles to play in activity-dependent plasticity (e.g., inhibition of translation triggered by mGluR1/5 in response to neuronal stimulation) (Bhakar et al., 2012). FMRP may also have important presynaptic effects on neuronal transmission, mediated via its effects on large conductance Ca^{2+} activated K^{+} channels (Deng et al., 2013).

Several studies have reported disruptions in MAPK/ERK signaling in FXS. Weng et al. (2008) have demonstrated delayed early-phase phosphorylation of ERK in mice deficient in FMRP whilst Curia et al. (2013) demonstrate a resistance to seizures with dephosphorylation of p-ERK in Fmr1 mice (Weng et al., 2008; Curia et al., 2013). In the PI3K pathway, the p110beta catalytic subunit can be regulated by FMRP, with p110beta and PI3K activity elevated in *Fmr1* knockout neurons. This suggests that dysregulation of PI3K signaling may be involved in the synaptic deficits seen in FXS. Indeed, inhibition of PI3K activity may correct dysregulated synaptic protein synthesis, AMPA internalization and spine density defects in knockout neurons (Gross et al., 2010). Thus, alterations in the same canonical pathways stimulated by IGF-1 and other neurotrophins may underlie a large part of the synaptic pathology seen in FXS.

Impressive preclinical evidence comes from a study by Deacon et al. (2015) using an analog of (1-3)IGF-1, NNZ-2566. In *Fmr1* knockout mice, NNZ-2566 demonstrated a significantly reduced brain phospho-ERK and phospho-Akt. Similarly, NNZ-2566 resulted in a significant reduction in spine numbers, which are increased in the *Fmr1* mice in comparison to controls. Improvement in hyperactivity and anxiety, learning and memory deficits was also observed (Deacon et al., 2015). In patients with FXS, a Phase 2 industry-led clinical trial has been completed using NNZ-2566, with clinical improvement in many of the core symptoms of FXS as disclosed by Neuren Pharmaceuticals (NCT01894958). The exact efficacy of IGF-1 related compounds such as NNZ-2566 awaits further clarification, but this early clinical evidence is encouraging.

PHELAN-MCDERMID SYNDROME

Phelan-McDermid Syndrome (PMDS) is another monogenic neurodevelopmental disorder and results from deletions in the SHANK3 gene on chromosome 22q13.3. Affected patients demonstrate global developmental delay, severe impairments in speech and intellectual disability (Phelan and McDermid, 2012). The protein product of SHANK3 is a key scaffolding protein present in the post-synaptic density of excitatory synapses, with key roles in activity-dependent plasticity and the functional maintenance of these synapses.

In 3-week old hippocampal neurons treated with siRNA to inhibit SHANK3 synthesis, there was a decreased number and an increased length of dendritic spines (Roussignol et al., 2005). Similarly, application of SHANK3 resulted in the formation of spine-like protrusions containing SHANK3 in aspiny neurons supporting the role of SHANK3 in spine formation and synaptic plasticity. Further, application of SHANK3 increased immunoreactivity for AMPAR subunits in cell body and dendritic spines (but not for GABA subunits), supporting its role in the function and maintenance of excitatory synapses (Roussignol et al., 2005). Using mice deficient in *Shank3*, Bozdagi et al. found reduced amplitude of miniature excitatory post-synaptic currents (mEPSCs) in the hippocampus and impaired Long Term Potentiation (LTP), with only transient spine expansion present (Bozdagi et al., 2010).

TABLE 1 | Previous studies and trials examining IGF-1 as a potential treatment in several childhood-onset neurodevelopmental disorders.

References	Disorder	Treatment	Dose	Sample SIze	Study type	Treatment duration	Findings	Conclusion	Company (if applicable)
Khwaja et al., 2014	RTT	rhIGF-1	40–120 ug/kg bd (MAD) followed by 12 weeks at max. dose	12	Clinical trial	6 months	Improvement in apnoea index neurobehavioural parameters, measures of mood and anxiety Reversal of alpha band desynchronization on EEG	Safety and preliminary efficacy supported	–
Pini et al., 2012	RTT	rhIGF-1	0.05 mg/kg bd first and last week; 0.1 mg/kg bd in between	6	Clinical study	6 months	No adverse events	Safety of IGF-1 supported	–
Pini et al., 2014	RTT	rhIGF-1	0.1 mg/kg bd	Single case study		6 months	No adverse events	Demonstrated safety of repeated doses in a single patient	–
Pini et al., 2016	RTT	rhIGF-1	0.05 mg/kg bd first and last week; 0.1 mg/kg bd in between	10 (incl. Pini et al., 2012)	Clinical study	6 months	Significant improvement in Rett Severity Score (RSS) and International Scoring System (RSS) Significant improvement in social/cognitive testing endurance	Prelimary efficacy of IGF-1 supported	–
NCT01703533	RTT	NNZ-2566	35 mg/kg or 70 mg/kg bd (note: oral administration)	56	Phase II Trial	28 days	No adverse events Significant improvement in Motor-Behavior Assessment Change Index, Clinical Global Impression of Improvement and Caregiver Top 3 Concerns	Safety and preliminary efficacy supported	Neuren Pharmaceuticals Ltd. see disclosure (Neuren Pharmaceuticals Ltd.)[a]
NCT01894958	FXS	NNZ-2566	35 mg/kg or 70 mg/kg bd (note: oral administration)	45	Phase II Trial	56 days	No serious adverse events Significant improvement in group and individual level analysis of specified core measures	Safety and preliminary efficacy supported	Neuren Pharmaceuticals Ltd.[b]
Kolevzon et al., 2014	PMDS	rhIGF-1	0.04 mg/kg bd to a maximum of 0.12 mg/kg bd	9	Phase II Trial	3 months	No serious adverse events Significant improvement on both the Aberrant Behavior Checklist and Repetitive Behavior Scale	Safety and preliminary efficacy supported	Neuren Pharmaceuticals Ltd.

RTT, Rett Syndrome; FXS, Fragile X Syndrome; PMDS, Phelan McDermid Syndrome, ASD, Autism Spectrum Disorder.

[a]*Neuren Pharmaceuticals Ltd. ASX Announcement 7th Dec 2015. Melbourne, Australia. Neuren's trofinetide successful in proof of concept Phase 2 clnical trial in Fragile X Syndrome. http://www.neurenpharma.com/IRM/PDF/1557/TrofinetidesuccessfulinPhase2trialinFragileX Last accessed 28th July 2016.*

[b]*Neuren Pharmaceuticals Ltd. Neuren (Neu) ASX Announcement 12th Nov 2014. Melbourne, Australia. Neuren's NZ-2566 successful in demonstrating clinical benefit in Rett syndrome Phase 2 trial. http://www.neurenpharma.com/IRM/PDF/1447/NeurensuccessfulinRettsyndromePhase2trial Last accessed 28th July 2016.*

TABLE 2 | Ongoing trials examining IGF-1 as a potential treatment in several childhood-onset neurodevelopmental disorders.

References	Disorder	Treatment	Dose	Sample size	Study type	Treatment duration	Findings	Company (if applicable)
NCT01777542	RTT	rhIGF-1	Unknown	In progress	Phase II Trial	10 months	In progress	–
NCT02715115	RTT	NNZ-2566	Various	In progress	Phase II Trial	11 weeks (avg.)	In progress	Neuren Pharmaceuticals Ltd.
NCT01970345	ASD	rhIGF-1	0.04 mg/kg bd to a maximum of 0.12 mg/kg bd	In progress	Phase II Trial	12 weeks	In progress	–

RTT, Rett Syndrome; FXS, Fragile X Syndrome; PMDS, Phelan McDermid Syndrome; ASD, Autism Spectrum Disorder.

In *Shank3* deficient mice, intraperitoneal injection of IGF-1, administered daily for a 2 week period reversed deficits in AMPAR receptors and LTP described above. Similar results were also seen for the active tripeptide (1-3) IGF-1 (Bozdagi et al., 2013). Recently, similar effects for IGF-1 on *Shank3* deficient human neurons has been demonstrated. By using induced pluripotent stem cells (iPSCs) from patients with PMDS and using them to produce functional neurons, Shcheglovitov et al. demonstrated that these neurons had a reduced expression of SHANK3 with accompanying defects in excitatory synaptic transmission as seen in the murine models above (Shcheglovitov et al., 2013). Treatment of the SHANK3 deficient neurons with IGF-1 increased the amplitude and frequency of EPSCs, restored the amplitude of evoked AMPA and NMDAR EPSCs and restored NMDA receptor currents on application of NMDA (Shcheglovitov et al., 2013). IGF-1 also caused a 340% increase in the fraction of puncta expressing PSD-95 in PMDS neurons (Shcheglovitov et al., 2013).

In a double blind, placebo controlled Phase 2 trial reported by Kolevzon et al., the safety and preliminary efficacy of IGF-1 treatment in PMDS were reported on 9 patients with PMDS aged 5–15 (Kolevzon et al., 2014). IGF-1 treatment was associated with significant improvements in social impairment and restrictive behaviors (Aberrant Behavior Checklist and Repetitive Behavior Scale). No serious adverse events occurred with the main side effects including sleep disturbance, hypoglycemia (<50 mg/dL), constipation, increased appetite and mood changes/irritability. This encouraging evidence awaits further confirmation and exploration in further clinical trials in the PMDS population.

AUTISM SPECTRUM DISORDER

Autism Spectrum Disorder is a heterogenous neurodevelopmental disorders characterized by deficits in social interaction and in speech and language with narrowed interests and repetitive behaviors (Wang and Doering, 2015). Autism affects about 3–6 per 1000 of the population, although recent estimates place its prevalence higher at 1 in 68 (CDC, 2012). The most substantial clue to ASD etiology is its substantial heritability (−90%) (Freitag, 2007). ASD demonstrates complex genetics, and whilst it is a genetically heterogenous disorder, evidence repeatedly implicates genes involved in synaptic development, function and activity-dependent plasticity by both common and rare variation (Wang et al., 2009; Hussman et al., 2011; De Rubeis et al., 2014). The potent effects of IGF-1 on synaptic function, maintenance and plasticity make it a potentially attractive target for the treatment of ASDs.

Vanhala et al. reported low levels of IGF-1 in children with autism, however sample sizes were small ($n = 11$) (Vanhala et al., 2006). In 25 young children with a diagnosis of autism, it was subsequently shown that IGF-1 levels were significantly reduced (Riikonen et al., 2001), and in those with a diagnosis of autism, Cerebrospinal Fluid (CSF) IGF-1 was correlated with head size (Mills et al., 2007). On measuring urinary IGF-1 excretion, Anlar et al. (2007) demonstrated that IGF-1 level was significantly lower in autistic children than in age matched controls. In contrast, a larger study has demonstrated significantly higher levels of IGF-1 in children with autism (Marchetto et al., 2016). The exact role of the IGF-1 axis in ASD awaits further clarification.

Interestingly, in a recent report using neurons derived from patients with ASD, Marchetto et al. found a partial rescue of deficits in neuronal networks (neuronal spike number and activity) on application of IGF-1 (Marchetto et al., 2016). At present, clinical trials of IGF-1 in Autism Spectrum Disorder are ongoing, with a phase 2 trial currently recruiting (NCT01970345). The trial aims to pilot the use of IGF-1 as a novel treatment for the core symptoms of ASD. The results of this trial are eagerly awaited. If successful, further trials will be needed in the ASD population to determine the exact efficacy of IGF-1 as a potential treatment, as well as studies to investigate the specific groups of patients with ASD which benefit the most from treatment with recombinant IGF-1.

POTENTIAL MOLECULAR MECHANISMS OF IGF1 AND DERIVATES IN DIFFERENT DISORDERS

There are three potential mechanisms by which IGF-1 may exert its effects in these neurodevelopmental disorders. The first is increased glutamatergic transmission, as seen in various preclinical studies (Tropea et al., 2009; Corvin et al., 2012; Castro et al., 2014; Marchetto et al., 2016). A potential consequence of this includes effects related to increased synaptic potentiation and plasticity (Bozdagi et al., 2013). A second potential action of IGF-1 in these neurodevelopmental disorders includes activation of molecular pathways involved in growth and connectivity (PI3K and MAPK). In RTT models, where these pathways are down-regulated, IGF1 induces an increase in the relative markers of

neuronal function (Tropea et al., 2009; Castro et al., 2014), whilst in FXS models, where they are up-regulated, an (1-3)IGF1 analog induces a decrease in the cellular pathways (Deacon et al., 2015). This may be the result of a homeostatic action of IGF-1, in re-establishing basal levels of activity in these canonical signaling pathways. The third potential mechanism relates to effects on transcription. This has been demonstrated in recent studies of IGF-1 on mecp2 transcript (Tropea et al., 2016). More work is needed to clarify the direct and indirect effects of IGF1 and derivates, and their action in different cell types.

CONCLUSION

Preliminary evidence is beginning to emerge from well validated murine models and early clinical studies, that treatment with recombinant human IGF-1 (rhIGF-1/mecasermin) and derived compounds may be of benefit in several childhood onset neurodevelopmental disorders. Whilst the evidence base is preliminary, further clinical trials and studies are needed in order to quantify the effects of IGF-1 on patients with these disorders, as well as identifying particular patients which may derive maximum benefit from treatment with IGF-1 and related compounds. The results of ongoing clinical trials are eagerly awaited.

AUTHOR CONTRIBUTIONS

CV, AD, and DT had substantial roles in the drafting, writing and editing of the review and final manuscript.

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A Developmental and Sequenced One-to-One Educational Intervention for Autism Spectrum Disorder: A Randomized Single-Blind Controlled Trial

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Introduction: Individuals with autism spectrum disorder (ASD) who also exhibit severe-to-moderate ranges of intellectual disability (ID) still face many challenges (i.e., less evidence-based trials, less inclusion in school with peers).

Methods: We implemented a novel model called the "Developmental and Sequenced One-to-One Educational Intervention" (DS1-EI) in 5- to 9-year-old children with co-occurring ASD and ID. The treatment protocol was adapted for school implementation by designing it using an educational agenda. The intervention was based on intensity, regular assessments, updating objectives, encouraging spontaneous communication, promoting skills through play with peers, supporting positive behaviors, providing supervision, capitalizing on teachers' unique skills, and providing developmental and sequenced learning. Developmental learning implies that the focus of training is what is close to the developmental expectations given a child's development in a specific domain. Sequenced learning means that the teacher changes the learning activities every 10–15 min to maintain the child's attention in the context of an anticipated time agenda. We selected 11 French institutions in which we implemented the model in small classrooms. Each institution recruited participants per dyads matched by age, sex, and developmental quotient. Patients from each dyad were then randomized to a DS1-EI group or a Treatment as usual (TAU) group for 36 months. The primary variables – the Childhood Autism Rating scale (CARS) and the psychoeducational profile (PEP-3) – will be blindly assessed by independent raters at the 18-month and 36-month follow-up.

Discussion and baseline description: We enrolled 75 participants: 38 were randomized to the DS1-EI and 37 to the TAU groups. At enrollment, we found no significant differences in participants' characteristics between groups. As expected, exposure to school was the only significant difference [9.4 (±4.1) h/week in the DS1-EI group vs. 3.4 (±4.5) h/week in the TAU group, Student's t-test, $t = 5.83$, $p < 0.001$].

Ethics and dissemination: The protocol was authorized by the competent national regulatory authority (*Agence nationale de sécurité du médicament et des produits de santé*) and approved by the local Ethics Committee (*Comité de Protection des Personnes*) at the University Hospital Saint-Antoine (May 7, 2013). The findings will be disseminated through peer-reviewed journals and national and international conferences.

Trial registration numbers: ANSM130282B-31 (April 16 2013) and ACTRN12616000592448 (May 6 2016).

Keywords: autism, intellectual disability, randomized controlled trial

BACKGROUND

Autism spectrum disorder (ASD) is characterized by the presence of atypical social communicative interaction and behaviors. The role of some genetic factors in ASD is known. However, there is a growing body of neurobiological research that indicates the presence of complex gene–environment interactions. Despite these findings, there is no approved biological treatment for this disorder and the first-line treatments pertain to psychosocial domains (1). Typically, ASD is diagnosed by means of a behavioral analysis during the 3- to 5-year-old age range; once diagnosed, the treatment is primarily delivered through behavioral interventions following different models. In essence, these models try to promote cognitive, communication, and behavioral skills that are considered essential to improve social skills in the long run (2, 3).

Several global interventions for core deficits in ASD have been proposed and assessed within clinical trials. The Treatment and Education of Autistic and Communication Handicapped Children (TEACCH) program uses many technical interventions to meet the individual needs of people with autism. The work program is tailored to some seminal aspects of ASD. First, it is centered on the individual. Individual needs are assessed through a comprehensive assessment of several developmental dimensions while taking into account emerging capacities. Second, it requires an understanding of autism, the adoption of appropriate adaptations and a broadly based intervention strategy (e.g., structured teaching, visual understanding, object manipulation, social communication skills) that builds on existing skills and interests. Third, the environment is organized to help children and adults understand and remember what to do (e.g., visual agendas, making expectations clear, and explicit, visual materials, structured architecture). The focus is on positive strategies to support behavioral and teaching strategies (4, 5).

Applied Behavioral Analysis (ABA) is a one-to-one intensive method that uses reinforcement of adaptative and acquired skills (6). The first structured attempts by Lovaas (7) were criticized (difficulties in generalization of learned behaviors; mechanical responses; lack of spontaneity) despite their encouraging first results. These criticisms led to the development of Pivotal Response Training [PRT], a more naturalistic behavioral treatment that has good documented effectiveness (8). PRT is a home-based intervention that includes parents in the routines. The method is based on choosing "pivotal" skills as the target of the treatment; following the child's choice of activities and games; reinforcing not only the correct answer expected by the professional but also all (meaning complete or incomplete) forms of attempts to respond; alternating between acquisition and maintenance; and using intrinsic reinforcers.

The Early Start Denver Model (ESDM) is an early and intensive intervention approach for young children. The interventions are based on the following: (i) a curriculum that evaluates the child's development across different developmental domains; (ii) specific procedures for learning and incorporating ABA principles, such as PRT; (iii) sessions focusing on interactions with children, interpersonal exchanges, and shared commitment with materials and activities of daily living; (iv) a positive affect, adults being responsive and sensitive to child cues; (v) verbal and non-verbal communication cues; (vi) proximal developmental windows, meaning that the focus of training is what is close to the developmental expectations given a child's development in the according domain; and (vii) parents' involvement. This program is implemented in small groups or individually at a specialized center or at home (3, 9).

The Developmental, Individual Differences, and Relationship-based (DIR) method is built on three axes: (i) the level of functional and emotional development reached by the child; (ii) the individual differences in information processing and motor planning; and (iii) the types of interactions that the child establishes with his/her partners (10). Floor Time is the core of the DIR method. It consists of sequences of guided play (15–20 min) that are repeated several times by parents throughout the day and are supervised by an expert. The DIR principles that should always be respected are to follow the child's lead and support his/her initiative; to focus on joint attention; to close circles of communication; to create semi-structured problem solving; to contrast repetitiveness with playful obstruction; to support visual attention; and to work on imitation (10, 11).

In an attempt to capture the common components among these models and what could be learned from evidence-based studies, Narzisi and colleagues (12) delineated the following principles: the first group regards timing: (1) starting as early as possible; (2) minimizing the gap between diagnosis and treatment; (3) being intensive (not less than 3–4 h of treatment per day); the second group is based on viewing parents as partners and involving family; the third group gathers principles related to treatment program: (1) providing regular assessments, supervision and updating the goals of treatment; (2) encouraging spontaneous communication; (3) promoting skills through play with peers; (4) finalizing the acquisition of new skills and their generalization

and maintenance in natural contexts; and (5) supporting positive behaviors rather than tackling challenging behaviors.

Why Should We Implement a School-Based Intervention?

Despite the encouraging results presented earlier, most of those programs (a notable exception being TEACCH) do not target school-aged children and are not proposed to occur in a school setting. This is unfortunate because schools are a favorable location for autism interventions (13). Additionally, many children with ASD do not receive a sufficient amount of treatment (14), even in countries with free access to health care (15). Because children with ASD benefit from being with other peers at school, the gap between education research and education practice (16) may be a missed opportunity to offer more support to these children. Additionally, larger doses of treatment could be offered in school contexts, especially when interventions are administered 1:1 (17). Several agencies have recommended conducting interventions in school-based settings (18, 19). Two objectives should be combined: school-based core deficit interventions and school-based social communication practice (20). There are already several studies that have shown that school-based interventions are able to reach larger numbers of children with ASD. This may improve challenges with generalization by using learned skills regarding communication in a natural environment, such as in the classroom (21, 22). Additionally, the preschool context seems to offer opportunities to develop communication skills (23, 24), and by offering opportunities to enter into play groups, teachers can supply reinforcements of the non-verbal ASD child requests (25).

However, there are very few school-based social communication interventions, and in many cases, teachers at school do their best without guided specific interventions for ASD children in the classroom. Consequently, there is a lack of response from teachers to the communicative acts produced by children with ASD (26). General educational teachers provide infrequent verbal prompting with ASD children (27), and they more frequently engage in functional play than symbolic play (28). They also lack supervision (20). Thus, there is a paradox between the need for appropriate intensive interventions for ASD and what is proposed in most school settings. For example, Mudford and colleagues (29) showed that the implementation of an evidence-based ABA program in preschoolers was not complete: 93% of the participants were not provided the dose of treatment (40 h/week). Additionally, from an efficiency perspective, although several programs support the concept of tailoring interventions to the child's needs and skills, to our knowledge, no one has questioned whether programs could be adapted according to teaching local skills.

Why Should We Study Children with ASD and Intellectual Disability?

As expressed in the dimensional approach of the new classifications in the DSM-5 (30), intellectual disability (ID) is a frequent challenge and comorbidity in ASD. According to studies, ID co-occurs in 50 to 75% of ASD cases (31). Risk factors of comorbid ID in ASD are gender (despite the high number of males with ASD, the male/female ratio decreases in ASD comorbid with ID) and the existence of seizures or of a neurodevelopmental or genetic syndrome (32, 33). The co-occurrence of ID also appears to be a prognostic factor for long-term outcomes of ASD (12, 34) and a risk factor of the incidence of challenging behaviors that provoke severe morbidity in some cases (35). To date, very few models have specifically addressed ASD comorbid with ID, in particular when ID is in the severe-to-moderate range. Therefore, the need to focus on this understudied population is warranted. Here, we wonder whether or not children with comorbid ASD and severe ID may be receptive to a pedagogical content? For such children over 5 years, could an adapted and one-to-one cognitive program in school be a road to improve non-verbal and verbal communication and to promote social skills?

METHODS/DESIGN

Objectives

In this paper, our aims are to describe a school-based intervention program (a developmental and sequenced one-to-one educational intervention, DS1-EI) that was adapted to the French health and education system; to justify the principles that were followed to implement the method and adapt it to a low-functioning population (i.e., ASD comorbid with ID); to describe the randomized controlled trial we began; and to present the sociodemographic and clinical characteristics of the participants at baseline.

Participants and Recruitment

All participants were recruited in outpatient French health care institutions that are specialized in treating children with autism and intellectual handicaps. At the request of the French national health regulatory authority [*Agence nationale de sécurité du médicament et des produits de santé* (ANSM)] and the main sponsor [*Caisse Nationale de Solidarité pour l'Autonomie* (CNSA)], we balanced day care hospitals and special education clinics to have a representative sample of French institutions. In each institution, we obtained a specific commitment to accept the implementation of a DS1-EI school-based program as described below and to recruit the same number of participants to be randomized into a DS1-EI exposed group (called the DS1-EI group) or a treatment as usual (TAU) group who would serve as controls. The commitment also entailed having the required resources from local school authorities to implement the DS1-EI and to have the leading teacher from the classroom be supervised. To avoid bias in the TAU group as a result of the diversity of institutions, we decided to randomize participants by site. **Figure 1** summarizes the list of institutions involved in the protocol and the number of patients by site. In total, we enrolled 75 participants.

Each parent provided informed written consent before inclusion. The inclusion criteria were a current diagnosis of ASD confirmed by a clinical assessment based on the International Classification of Diseases, 10th edition criteria and the Autism Diagnostic Interview-Revised (ADI-R) (36); an intellectual handicap with financial compensation from local agencies [*Maison Départementale du Handicap* (MDPH)]; being aged between 5 and 9 years; and having a communication developmental age of 24 months and under based on a Vineland assessment or a 3-year speech delay based on a Psycho-Educational Profile, third edition.

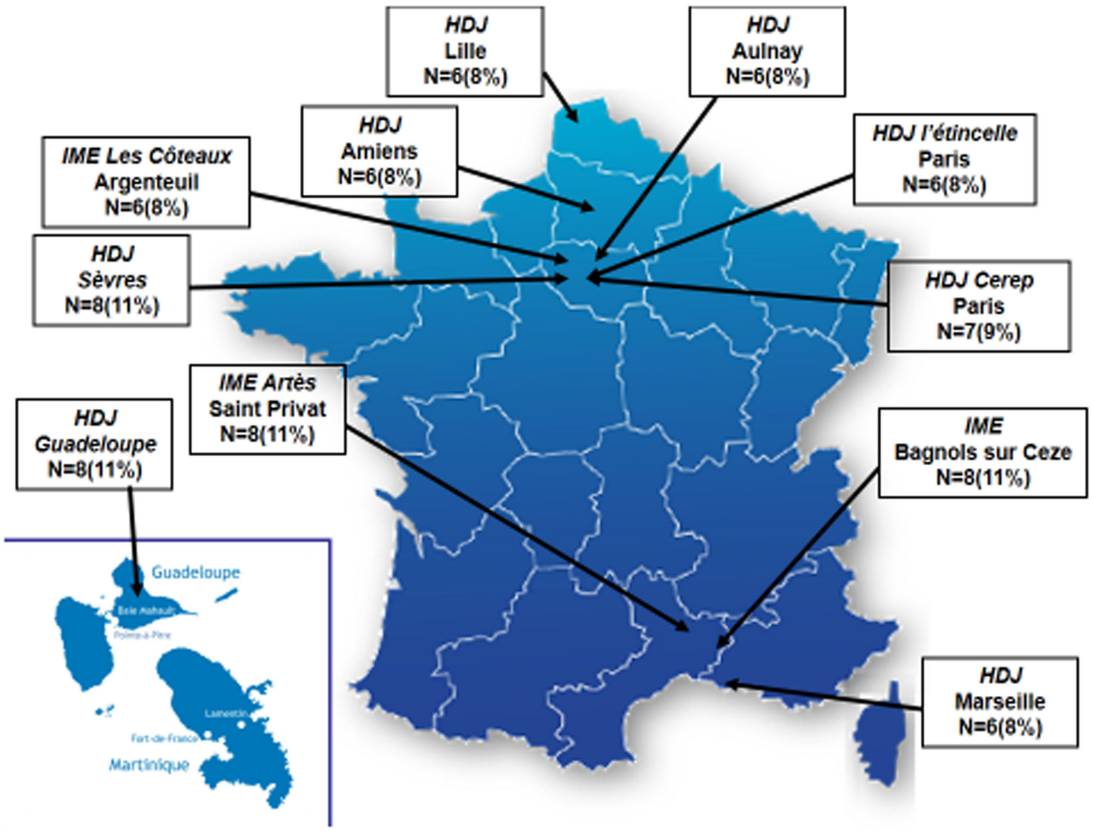

FIGURE 1 | **Institutions and participants' enrollment in the DS1-EI trial by site**. HDJ, *Hopital de Jour* (Day care medical center); IME, *Institut Medico-Educatif* (Special education center).

We did not exclude children with known organic syndromes and/or non-stabilized neuropaediatric (e.g., seizures) or medical (e.g., diabetes mellitus) comorbidities. However, during the medical assessment, we specifically listed comorbidities. The exclusion criteria were limited to parents' refusal to participate; family's plans to change institutions in the short term for any reason; and patient's severe behavioral impairments that would challenge treatment adherence. Of note, this last exclusion criterion was based on local institution staff decision. Before randomization, each site was requested to assess the IQ of the participants based on the Kaufman Assessment Battery for Children second edition (KABC-II) or, failing that, Vineland scores. Based on these results, dyads of participants matched for sex, age, and developmental quotient (DQ) were formed to limit the risk of bias between groups. Randomization before group allocation to TAU or DS1-EI group was performed by drawing lots in each dyad per site so that each site could have a TAU group and a DS1-EI group of three to four participants each. Randomization was performed by the methodological coordinating team at the Salpêtrière Hospital and was independent from local inclusion sites. TAU was defined as all therapeutic interventions given to a specific child. Given the study duration, we did not recommend not to change children's therapeutic protocol in the TAU group during the study period. The trial duration was defined as 36 months but included 12-, 18-, and 24-month intermediate assessments.

DS1-EI Treatment Principles

Participants randomized to the DS1-EI group received the specific experimental protocol four mornings per week (2 h 30 min); the rest of the week they continued to receive the usual protocol of each site. The treatment principles are summarized in **Table 1**. The setting was a small classroom with four pupils, but an adapted environment was proposed. Following the principles of TEACCH (5), each child was offered a desk, two chairs (one for the child, one for the adult working with the child), a screen where pictures of the child's schedule and activities were provided, and a locker with his or her picture. In contrast to TEACCH, the child sat with his back close to the wall where the screen was placed (see pictures in the supplementary material S1). The setting also included a large table for mid-session group collaboration and a place offering benches and carpets where group participants (both children and adults) met at the beginning and ending of a session. Although the program did not reach the 40 h per week recommended by some programs (37), it remained intensive, with 10 h of DS1-EI plus other therapeutic practices according to each institution (e.g., occupational therapy; speech therapy; social skill group activities). The program followed developmental rules, meaning the training was focused on the nearest expected activity/skill of a given child's development in a specific domain as recommended by the ESDM (9). In terms of timing, the program was sequenced in two ways. First, as in TEACCH,

TABLE 1 | A developmental and sequenced one-to-one educational intervention (DS1-EI) for autism spectrum disorder: main principles.

Characteristics	Brief definition	Justification
Setting	To be implemented in a small classroom with four pupils In an adapted environment	TEACCH, Barton et al. (13) TEACCH
Intensive	One-to-one support 10 h per week in addition to other treatment practices (e.g., occupational therapy, speech therapy, psychotherapy)	ABA, ESDM
Developmental	The focus of training is what is close to the developmental expectation given a child's development within a domain	ESDM
Sequenced	The 2 h 30 min sessions follow an anticipated and structured agenda Teachers change learning activities every 10–15 min to keep a child's attention	TEACCH Original
Curriculum based	A detailed assessment/curriculum is required to follow the developmental approach and to choose the appropriate cognitive/motor activity to be taught in each domain for preschoolers	ESDM, TEACCH
Educational objectives	Given the developmental quotient of the targeted children, the educational objectives are those of a second grade program for preschoolers (see **Table 2** below for details)	French Ministry for National Education
Reinforcers	Supporting positive behaviors rather than tackling challenging behaviors Using positive emotion engagement from teachers	ABA, ESDM ESDM
Group	Group activities are organized within the time schedule to encourage spontaneous communication and promote social skills through play with peers	Many programs
Supervision	Regular supervision of teachers with children's objectives being updated	ESDM, ABA, DIR
Exploiting teachers' unique skills	Implementation of the program will benefit from using teachers' individual skills, such as their knowledge of a specific method (e.g., the use of Picture Exchange Program) or of a particular child	COMVOOR

TEACCH, Treatment and Education of Autistic and Communication Handicapped Children; ABA, Applied Behavioral Analysis; ESDM, Early Start Denver Model; DIR, Developmental, Individual Differences and Relationship-based method; COMVOOR, Voorlopers in Communicatie.

the 2 h 30 min sessions followed an anticipated and structured agenda that was presented for each child on a screen. When a novel activity started, the corresponding pictogram was shown on the child's desk. Second, teachers were asked to change desk and activities every 10–15 min to maintain the child's attention and to help him improve by challenging patient's need of sameness. Thus, each 10–15 min, the child has a new activity and a new teacher. The program was also curriculum based and had specific educational objectives (see details below).

Academic Training

Because DS1-EI was a program implemented in classrooms, both the curriculum and the objectives followed academic recommendations from the French Ministry of National Education. The curriculum was adapted from the French program for nursery and primary schools and *handiscol* principles (http://eduscol.education.fr). This was decided based on the idea that these recommendations were part of a teacher's area of expertise and that it would promote participation in the program. Additionally, each classroom of N children was under the responsibility of one teacher helped by $(N - 1)$ assistants, according to the 1-to-1 design of the program. In the same vein, one of the principles of the program was capitalizing on teachers' individual skills. We believed that implementation of the program would benefit from using teachers' specific knowledge (e.g., the use of the Picture Exchange Program). A detailed assessment/curriculum was a prerequisite of each child's academic program because the DS1-EI was designed to follow a developmental approach, which required the selection of appropriate cognitive/motor activities for training within each domain. The curriculum is described in detail in the supplementary material S2. Regarding the academic/educational objectives, they were grouped into four domains: mathematics, language and communication, intermodality, and autonomy. **Table 2** provides some examples of the activities by domain and level of child's performance.

Teachers' Training and Supervision

Each teacher and each assistant were trained by Annik Hubert-Barthelemy during a 1-week session. They were provided with a method presentation and were trained to use positive affect, shared engagement, responsiveness, and sensitivity to child cues, to focus on both verbal and non-verbal communication, and to support positive behaviors rather than tackle challenging behaviors. The DS1-EI detailed assessment/curriculum was explained, including how to keep learning proposals close to a given child's developmental needs. The last 2 days of the training session was dedicated to define new objectives and adaptations. During the morning, the teacher with the help of his/her assistants had to fulfill children curriculums and to have related written observation. During the afternoon, curriculum was discussed and first learning activities for all domains were decided for each child.

Supervision was organized in three different steps: (i) daily sessions of verbal exchanges and written observations after the class about each child in each domain with all professionals (the teacher and the assistants); (ii) weekly supervisions by a psychologist; (iii) monthly supervisions by the main investigator to ensure the conformity of the program application and to help the teacher adapting the directives according to each child outcome.

Primary and Secondary Variables

Table 3 summarizes the variables that we planned to measure at enrollment and at several time points throughout the trial.

TABLE 2 | DS1-EI learning by domain and hierarchical proposals: examples of tasks.

	Level 1	Level 2
Domain 1: Mathematics		
Numeration	Nursery rhyme to 5	Nursery rhyme to 39
Problem solving	Organizing stickers	Constructing a logical paradigm
Domain 2: Language and communication		
Communication	Improving joint attention	Asking for help
Oral language	Naming five objects	Making sentences to express a wish
Written language	Knowing letters from own name	Knowing all letters of classroom names
Graphics	Using sticks	Copying words with a model
Domain 3: Intermodality		
Writing/drawing and listening	Using various tools (paint, pastels, markers…)	Using different techniques (cut, paste, stencils…)
Musical activities	Imitating a rhythm	Learning songs with body movements
Domain 4: Autonomy		
Motor activities	Walking/Running/Swimming	Walking on a beam
Drawing activities	Using felt	Cut/Paste
Discovering the world	Describing a tree	Drawing a tree
Social skills	Waiting one's turn	Improving autonomous work

TABLE 3 | DS1-EI study procedure and evaluation criteria.

	Selection	Inclusion	M12	M18	M24	M36
Inclusion criteria	X	–	–	–	–	–
Informed consent	X	–	–	–	–	–
CIM-10 diagnosis	–	X	–	X	–	X
Comorbidity	–	X	–	X	–	X
ADI-R	–	X	–	X	–	X
Vineland	–	X	X	X	X	X
CARS	–	X	–	X	–	X
CGAS	–	X	X	X	–	X
CGI	–	X	X	X	–	X
KABC	–	X	–	X	–	X
PEP-III	–	X	–	X	–	X
School assessment	–	X	X	X	X	X

ADI-R, Autism Diagnostic Interview-Revised; CARS, Childhood Autism Rating Scale; CGI, Clinical Global Impression; CGAS, Clinical Global Assessment Score; PEP-3, Psychoeducational Profile, 3rd Edition; DQ, developmental quotient; KABC, Kaufmann Assessment Battery for Children.

The primary outcome variables were (i) the Childhood Autism Rating scale (CARS), which measures autism severity (38); (ii) the psychoeducational profile, third edition (PEP-3), which measures the total DQ and 5-dimensional DQs related to cognition, receptive language, expressive language, fine motor skills, gross motor skills, and imitation, and (iii) the school's assessment (39). Secondary variables included the following measures: (i) the *Vineland Adaptive Behavior Scale II* (VABS-II) as a behavioral scale of independence; this scale is assessed through a parent/educator interview and is used to assess the ability of children to perform the daily activities required for personal and social sufficiency. The VABS-II examines four specific domains: Communication, Daily Living Skills, Socialization, and Motor Skills. The subscale scores are totaled to yield an Adaptive Behavior Composite score (40). (ii) The KABC-II standardized neuropsychological assessment to measure intelligence skills. This battery measures Verbal, Performance, Working Memory, Processing Speed and Total quotients (41). (iii) The *Clinical Global Impression* (CGI), which was used to assess global severity (42). (iv) Finally, the *Children Global Assessment Scale* (CGAS) (43). To assess clinical change during the 36-month study, we used a single-blind procedure (independent raters were blind to study group allocation) for all clinical assessments (PEP-3, KABC-II, CARS). Blind assessment was not possible for the measures that required 2-week observations of the participants (CGI, CGAS, school tests) or a parental interview (ADI-R, VABS-II).

Number of Participants

From previous studies that showed significant results in terms of efficacy it appears that the minimal number of patients in parallel design was 50 (2). This was the case for behavioral ABA approach [e.g., Ref. (6)] or for developmental ESDM approach [e.g., Ref. (3)]. The number of patients to enroll was based on the following theoretical statistics estimation: for a moderate effect size ($\alpha = 0.6$), a power fixed at 80%, and a level of significance for a p-value fixed at <0.05, 80 patients randomized into two groups are required for a student t-test. Given our choice to use linear mixed models (see below) to take into account participant's effect, we planned to recruit from 70 to 80 participants.

Statistical Analysis

Statistical analyses will be performed using R Software, Version 2.12.2. To assess whether improvement occurs in both primary and secondary variables, we will use linear mixed models with change in a given variable explained by group exposure (DS1-EI vs. TAU), time (baseline vs. 18 vs. 36 months) and their interaction (group exposure × time). We will also include a random effect and a site effect. This should account for individual heterogeneity, site heterogeneity, variable scores at inclusion, and change specific to DS1-EI within the same statistical regression. For missing data when available, we will use the last observation carried forward. In case of a non-Gaussian distribution, we will study the log transformation (or other transformation when appropriate) to achieve a normal distribution. Lost or drop-out patients will also be compared between groups using a separate non-parametric comparison.

TABLE 4 | Sociodemographic and clinical characteristics of the participants after group randomization.

	DS1-EI group (*N* = 38)	TAU group (*N* = 36)	Test, *p*
Sociodemographics			
Age, mean (± SD), year			
Male–Female	6.92 ± 1.57	7.34 ± 1.55	$t = -1.15$, $p = 0.254$
Socioeconomic status	32 (84%)/6 (16%)	30 (83%)/6 (17%)	Fisher, $p = 1$
Clinical characteristics			
ADI-R, current, mean (± SD)			
Social impairment score	21 ± 5.7	19.9 ± 5.8	$t = 0.81$, $p = 0.423$
Communication score	11.8 ± 4	10.9 ± 3.2	$t = 1.12$, $p = 0.266$
Repetitive interest score	6 ± 2.7	5.7 ± 3.1	$t = 0.44$, $p = 0.654$
Developmental score	4.2 ± 0.8	3.9 ± 1.1	$t = 1.29$, $p = 0.202$
CARS score	41.3 ± 7	40.2 ± 7.1	$t = 0.67$, $p = 0.504$
CGI score	5.8 ± 1	5.7 ± 0.9	$t = 0.44$, $p = 0.661$
CGAS score	27 ± 11.5	25.9 ± 11.1	$t = 0.38$, $p = 0.705$
PEP-3 (all scores in DQ): mean ± SD			
Cognition	22.3 ± 11.1	23.4 ± 11.1	$t = -0.44$, $p = 0.663$
Receptive language	14 ± 5.6	15.6 ± 8.6	$t = 0.85$, $p = 0.4$
Expressive language	16.2 ± 5.8	16.8 ± 6.6	$t = 0.4$, $p = 0.692$
Fine motor skills	26.6 ± 10.1	26.6 ± 9.7	$t = 0.0$, $p = 0.99$
Gross motor skills	23.7 ± 8	24.8 ± 6.2	$t = -0.62$, $p = 0.534$
Imitation	23.1 ± 7.8	25.8 ± 6.7	$t = -1.57$, $p = 0.12$
Vineland (all scores in DQ): mean ± SD			
Communication	13.3 ± 8.2	14.2 ± 7.6	$t = -0.49$, $p = 0.628$
Adaptation	26.5 ± 13.6	26.2 ± 12.5	$t = 0.07$, $p = 0.94$
Socialization	13.7 ± 9	13.3 ± 9.4	$t = 0.15$, $p = 0.88$
Daily living skills	30.9 ± 14.7	30 ± 12	$t = 0.26$, $p = 0.79$
Comorbidity			
Intellectual disability level (< or > 40 using KABC)	27 (90%)/3 (10%)	26 (90%)/3 (10%)	Fisher, $p = 1$
Known medical condition (no/yes)	34 (89%)/4 (11%)	27 (7589%)/9 (25%)	Fisher, $p = 0.183$

TAU, treatment as usual; ADI-R, Autism Diagnostic Interview-Revised; CARS, Childhood Autism Rating Scale; CGI, Clinical Global Impression; CGAS, Clinical Global Assessment Score; PEP-3, Psychoeducational Profile, 3rd Edition; DQ, developmental quotient; KABC, Kaufmann Assessment Battery for Children.

RESULTS

Participants at t0 after Randomization

Table 4 summarizes the participants' sociodemographic and clinical characteristics at enrollment. As expected, we found no significant differences at enrollment between the DS1-EI and TAU groups, indicating that the randomization by site did not introduce bias. As expected, exposure to school was the only significant difference found between the two groups: 9.4 (±4.1) hours per week in the DS1-EI group vs. 3.4 (±4.5) hours per week in the TAU group, Student's *t*-test, $t = 5.83$, $p < 0.001$.

DISCUSSION

We hope that the current trial will help demonstrate the feasibility of adapting and task-shifting a group of interventions used primarily as early interventions for autism to a school-based context and for use with older individuals with autism and intellectual disabilities. The school-based intervention program [the developmental and sequenced one-to-one educational intervention (DS1-EI)] was adapted from several methods including TEACCH (4, 5), ESDM (3), and ABA (6, 37). The key principles of the method are the intensity, the regular assessments and updating of objectives, the encouragement of spontaneous communication, promotion of skills through play with peers, support of positive behaviors instead of tackling challenging behaviors, regular team supervision, capitalization on teachers' unique skills, and developmental and sequenced learning (2). The use of sequenced learning (i.e., teacher and activity change every 10–15 min to keep the child's attention in the context of an anticipated time agenda) is likely the most original proposal. We are aware that treating children with autism and ID is very challenging. The effect sizes are typically small. To balance this risk of failure, we chose to have a rather long study duration with two single-blind assessments at 18 and 36 months. Given the number of sites, which may have introduced bias, we are satisfied that we did not find differences between the groups at baseline.

We are aware that there are several limitations to this trial. First, given the severity of the patients' conditions, the study duration and the nature of the intervention, only a single-blind for the primary variables was feasible. Second, the method used to randomize participants (to limit the bias of TAU) combined with the study duration and the supervision of local teams is likely to modify local staff practice and to lead to some DS1-EI principles being used in the TAU group as well. Third, because our public sponsors required us to balance hospital/academic/large city sites and non-hospital/remote rural area sites, we cannot assume that we will achieve a uniform level of adherence to the program from one site to another. Finally, given the study duration, we do not know whether we will retain a sufficient number of patients in the program to maintain the statistical power needed for per protocol analyses.

DECLARATIONS

Ethics Approval and Consent to Participate

The current trial protocol was authorized by the competent national health regulatory authority [*Agence nationale de sécurité du médicament et des produits de santé* (ANSM)]. The trial registration number (ANSM 130282B-31) was obtained on April 16, 2013. The protocol was approved by the local Ethics Committee (*Comité de Protection des Personnes*) of the University Hospital Saint-Antoine on May 7, 2013. Also, it was registered on the Australian New Zealand Clinical Trial Registry for public information availability (ACTRN12616000592448). Potential participants received oral and accessible information about the study given participants' cognitive profiles, and all parents/guardians were provided a written information leaflet about the trial. The information leaflet adhered to the current French guidelines for researchers on writing information sheets and consent forms. Only after written consent was obtained from the parents of potential participants did randomization occur.

Consent for Publication

Persons' data and images contained in this article are published with the consent of their parents/guardians.

Trial Committees

A pilot study committee has been formed and includes the principal investigator of the trial and all local investigators and study collaborators. They will meet three times a year. A scientific study committee has also been established and includes two administrative members of the promotor (*La Croix Rouge Française*), four members of the pilot study committee and four other independent members (two professionals, two family representatives). They will meet once a year.

AUTHOR NOTES

Antoine Tanet is neuropsychologist at the Child and Adolescent Psychiatry department of the Pitié-Salpêtrière Hospital and PhD Student at IMI2S group (Institut des Systèmes Intelligents et Robotiques, CNRS, UMR 7222, Pierre et Marie Curie University Paris 6). Annick Hubert-Barthélémy is clinical psychologist and supervisor of the DSI-E1 program at the Croix Rouge Française. Graciela Crespin in clinical psychologist and head of the Programme de Recherche et d'Etudes sur l'Autisme. Nicolas Bodeau is a statistician at the Child and Adolescent Psychiatry department of the Pitié-Salpêtrière Hospital. David Cohen is Professor and head of the Department of Child and Adolescent Psychiatry at Pitié-Salpêtrière Hospital, and senior member of IMI2S group (Institut des Systèmes Intelligents et Robotiques, CNRS, UMR 7222, Pierre et Marie Curie University Paris 6). Catherine Saint-George is a child psychiatrist, member of IMI2S group (Institut des Systèmes Intelligents et Robotiques, CNRS, UMR 7222, Pierre et Marie Curie University Paris 6).

AUTHOR CONTRIBUTIONS

DC, CS-G, and AH-B designed the study; AH-B and GC created the treatment program; AH-B and AT implemented the program in each study site; NB created the data base for implementation of the program; DC and NB performed the statistical analysis; AT, CS-G and DC did a first version of the manuscript; all authors critically revised the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fped.2016.00099

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REVIEW
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The Broad Autism (Endo)Phenotype: Neurostructural and Neurofunctional Correlates in Parents of Individuals with Autism Spectrum Disorders

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Autism Spectrum Disorders (ASD) are a set of neurodevelopmental disorders with an early-onset and a strong genetic component in their pathogenesis. According to genetic and epidemiological data, ASD relatives present personality traits similar to, but not as severe as the defining features of ASD, which have been indicated as the "Broader Autism Phenotype" (BAP). BAP features seem to be more prevalent in first-degree relatives of individuals with ASD than in the general population. Characterizing brain profiles of relatives of autistic probands may help to understand ASD endophenotype. The aim of this review was to provide an up-to-date overview of research findings on the neurostructural and neurofunctional substrates in parents of individuals with ASD (pASD). The primary hypothesis was that, like for the behavioral profile, the pASD express an intermediate neurobiological pattern between ASD individuals and healthy controls. The 13 reviewed studies evaluated structural magnetic resonance imaging (MRI) brain volumes, chemical signals using magnetic resonance spectroscopy (MRS), task-related functional activation by functional magnetic resonance imaging (fMRI), electroencephalography (EEG), or magnetoencephalography (MEG) in pASD.The studies showed that pASD are generally different from healthy controls at a structural and functional level despite often not behaviorally impaired. More atypicalities in neural patterns of pASD seem to be associated with higher scores at BAP assessment. Some of the observed atypicalities are the same of the ASD probands. In addition, the pattern of neural correlates in pASD resembles that of adult individuals with ASD, or it is specific, possibly due to a compensatory mechanism. Future studies should ideally include a group of pASD and HC with their ASD and non-ASD probands respectively. They should subgrouping the pASD according to the BAP scores, considering gender as a possible confounding factor, and correlating these scores to underlying brain structure and function. These types of studies may help to understand the genetic mechanisms involved in the various clinical dimension of ASD.

Keywords: Autism Spectrum Disorders, parents, Broader Autism Phenotype, magnetic resonance imaging, magnetic resonance spectroscopy, electroencephalography, magnetoenchephalography

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INTRODUCTION

Autism Spectrum Disorders (ASD) are a set of early-onset neurodevelopmental disorders that are characterized by a disrupted development of brain connectivity with several cascading effects on neuropsychological functions (Narzisi et al., 2013; Kana et al., 2014). A clinical dyad, comprising social communication difficulties and repetitive, stereotyped behavior must be present for a diagnosis of ASD (American Psychiatric Association, 2013). The exact cause of ASD is still unknown (Levy et al., 2009). Although, only 20% of ASD cases can be explained by a specific genetic cause, such as identifiable genetic syndromes, genetic mutations or de novo copy number variants (Jeste and Geschwind, 2014), recent twin studies estimate an heritability between 64 and 91% (Tick et al., 2016), suggesting an interaction between genetic vulnerability and environmental factors (Rossignol et al., 2014).

Genetic epidemiological data suggest that personality traits similar to, but not as severe as those of ASD, are also heritable (Freitag, 2007). This group of "sub-threshold" features, which are believed to be milder manifestations of ASD (Dell'Osso et al., 2016), have been indicated as the broader autism phenotype (BAP) (Piven et al., 1997). BAP includes peculiar social, communication, and cognitive processes, strong persistent interests, and rigid and aloof personality traits (Gerdts and Bernier, 2011; Sucksmith et al., 2011). Interestingly, it was shown that BAP traits are more prevalent in first-degree relatives of individuals with ASD than in other groups, supporting the hypothesis that ASD have a significant genetic component (Bailey et al., 1998; Losh et al., 2008).

Kanner and Asperger were the first to report behavioral features in parents that were similar in kind to those of their autistic offspring. In particular, Kanner (1943) observed that both first and second degree relatives of children with "early infantile autism" had common characteristics of late speech, mild obsessiveness and uninterest in people. Similarly, Asperger (1944) described a group of parents of children with autism as withdrawn, pedantic, eccentric, and loners, who had problems relating to the outside world. Later studies have shown that the expression of ASD traits in relatives concerns not only behavioral traits, but also social cognition abilities (e.g., Baron-Cohen and Hammer, 1997), neurocognitive functioning (e.g., Koczat et al., 2002) or biological dimensions (e.g., Lainhart et al., 2006) and that these aspects could relate to or explain the clinical presentation of the BAP.

The biological dimension of ASD has been largely investigated in the last decades, thanks to the growing availability of brain imaging techniques and analysis methods for *in vivo* examination of brain structure and function. All in all, these studies reported abnormal neuroanatomical and neurofunctional profiles in individuals with ASD, suggesting a dysfunction of key brain areas underlying the core impairments of ASD (Amaral et al., 2008; Bellani et al., 2013a,b; Billeci et al., 2013; Calderoni et al., 2014). As such, there has been great interest in evaluating whether these neurological profiles also characterize the relatives of autistic probands. Indeed, should the same brain abnormalities of ASD patients be present in their direct relatives, their heritable origin would be strongly supported together with their role as endophenotypes of the disorder (Sullivan et al., 2003; Palmen et al., 2005a). This is particularly true for studies exploring correlations in parents. In fact, while sibling and twin studies are suitable for detecting brain abnormalities under genetic control, studies on parents allow mitigating the role of the shared (pre- and perinatal) environment (Sullivan et al., 2003; Palmen et al., 2005a). Thus, if brain abnormalities are observed in parents, they are more likely to be of heritable origin and consequently reflect endophenotypes of the disorder. To assess the strength of this hypothesis, we provide here a critical revision of all studies exploring the neuroanatomical and neurofunctional profile of parents of individuals with ASD.

METHODS

To find papers concerning neuroimaging studies in parents of individuals with ASD, a sensitive search strategy was conducted in two relevant article databases: PubMed and ScienceDirect. Search terms included database subject headings for the concepts of pervasive developmental disorders (e.g. "autism," "autism spectrum disorder," "pervasive developmental disorders"), neuroimaging (e.g., "MRI," "MRS," "EEG," "MEG") and parents ("parents," "relatives," "fathers," "mothers," "broader phenotype"). The reference lists of the retrieved papers were searched to identify additional articles.

Studies adhering to the following criteria were incorporated in this review: (1) parents of individuals with ASD were the population under study; (2) Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Electroencephalography (EEG) and Magnetoencephalography (MEG) were used to investigate neurostructural and neurofunctional correlates in parents of individuals with ASD; (3) empirical findings about neural substrates were reported by the authors; (4) studies were published before March 30, 2016; (5) studies were published in an English peer-reviewed journal.

RESULTS

Thirteen published studies meeting the inclusion criteria were identified. **Table 1** summarizes the studies included in this review.

Structural MRI

Only three studies used sMRI to assess brain structure in parents of autistic probands.

Rojas et al. (2004) assessed total brain, hippocampus, and amygdala volumes in adults with ASD, parents of children with ASD (pASD) and healthy controls (HC), defined as adults with no personal or familial history of ASD. The left hyppocampus was found significantly larger in the ASD group in comparison to both the pASD and the HC group, and in the pASD group in comparison to the HC group. In the three groups, hyppocampus enlargement was more pronounced in males than in females. The right amygdala was smaller in the ASD group in comparison

TABLE 1 | Neuroanatomical and neurofunctional characteristics associated to the BAP in the parents of individuals with ASD.

Study (year)	Participants (nr, M/F, mean age ± SD in years)	BAP Questionnaires	Method	Results	Correlations with BAP scores and behavior
STRUCTURAL STUDIES					
Rojas et al., 2004	15 ASD (6/9) 30.3 ± 9.1	None	ROI manual tracing (HYP, AMY total brain)	ASD>pASD>HC left HYP	–
	17 pASD (15/2) 44.75 ± 4.4				
	17 HC (8/9) 43.6 ± 4.3			ASD<pASD, HC right AMY	
Palmen et al., 2005a	38 pASD (19/19) 50.3 ± 3.4	AQ	ROI semi-automatic tracing	No significant differences in volume	Positive correlations between AQ scores and intracranial and ventricular volume in pASD
	40 HC (20/20) 52.0 ± 4.1				
Peterson et al., 2006	23 pASD (8/15) 39.6 ± 6.0	None	VBM	pASD>HC in several GM regions (i.e. right precentral gyrus, right superior parietal lobule, and superior temporal gyri)	–
	23 HC (8/15) 38.3 ± 6.4			pASC<HC anterior portion of the left cerebellar hemisphere	
fMRI STUDIES					
Baron-Cohen et al., 2006	12 pASD (6/6) M: 39.1 ± 6.0	None	Visual Search Task (EFT) and Emotion Recognition Task (ET)	Females>Males>Fathers = Mothers in BA 19 in EFT task Females>Males>Fathers=Mothers in BA 21 e BA 44 in ET task	–
	F: 37.3 ± 5.9				
	12 HC (6/6) M: 23.1 ± 0.6				
	F: 21.6 ± 0.8				
Greimel et al., 2010	15 ASD (15/0) 14.9 ± 1.6	AQ	Empathy: other-task and self-task	pASD<pHC AMY other-task	No significant correlations between brain activity and AQ scores
				pASD<pHC FG other-task	
				ASD<HC FG other-task and self-task	
					Positive correlation between FG activation and GEM score in ASD
	15 HC (15/0) 15.0 ± 1.4			ASD<HC IFG self-task	
	11 pASD (11/0) 43.9 ± 5.1				
					Positive correlation between insula activation and BEES score in pASD and pHC
	9 pHC (9/0) 47.7 ± 5.3				
Wilson et al., 2013	16 pASD (6/10) 43.7 ± 8.1	AQ	Phonological processing: homophones vs pseudohomophone	pASD>HC pseudohomophone several regions (i.e. IC, STG, SMG, SMA, cerebellum)	Positive correlations between IFG activation and CTOPP scores in pASD and HC
	18 HC (6/12) 41.0 ± 8.1			pASD<HC left STG and left SMG phonological priming	
					Positive correlations between IC activation and CTOPP scores in pASD

(Continued)

TABLE 1 | Continued

Study (year)	Participants (nr, M/F, mean age ± SD in years)	BAP Questionnaires	Method	Results	Correlations with BAP scores and behavior
Yucel et al., 2014	40 pASD (20/20) 40.6 ± 0.7	BAPQ MPAS-R	Face processing	pASD>HC AMY	BAP+ >BAP−,HC LOC
				pASD>HC FG	
	15 BAP+ 40.9 ± 1.4			pASD<HC INS	
	25 BAP− 42.1 ± 1.28				
	20 HC (6/12) 39.8 ± 1.6				
MRS STUDIES					
Brown et al., 2013	13 ASD (9/4) 41.2 ± 6.9	AQ	Level of Glu, NAA, Cr in auditory cortex	ASD>HC Glu, NAA, Cr	Positive correlation, uncorrected for multiple comparisons, between left NAA and the SRS and left Glu and the AQ
		SRS		No differences between pASD and ASD or HC	
	15 pASD (11/4) 41.0 ± 8.1				
	15 HC (6/9) 41.1 ± 6.8				
EEG AND MEG STUDIES					
Dawson et al., 2005	21 pASD (10/11) 38.5 ± n.d.	None	Face processing ERPs	pASD<HC N170 right amplitude to faces	Positive correlation between N170 amplitude to faces and WMS Immediate and Delay task in HC
				pASD<HC N170 latency difference chairs-faces	
	21 HC (8/13) 38.9 ± n.d.				
Rojas et al., 2008	11 ASD (9/2) 42.6 ± 5.1	None	Auditory stimulation	pASD,ASD>HC induced tGBR	–
			Evoked, induced and total power tGBR	pASD,ASD<HC evoked tGBR, PLF, anterior-posterior asymmetry	
	16 pASD (9/7) 31.5 ± 9.3				
			PLF tGBR	No differences between pASD and ASD	
			Source Localization		
	16 HC (7/9) 43.1 ± 6.7				
Rojas et al., 2011	21 pASD (7/13) 43.7 ± 7.3	AQ	Auditory stimulation	pASD<HC total and evoked power, PLF ASSR	Negative correlation between ASSR PLF and AQ communication subscale
	20 HC (6/15) 43.8 ± 6.9	SRS	Evoked, induced and total power tGBR	No differences in tGBR	
			PLF tGBR		Negative correlation between tGBR and ASSR evoked power and SRS scores
			Evoked, induced and total power ASSR		
			PLF ASSR		
McFadden et al., 2012	23 pASD (8/15) 35.8 ± 10.0	None	Language auditory stimulation	pASD>HC evoked and total gamma SMG, LOC	Significant but different correlations between gamma or beta activity and language measures (expressive, receptive, figurative language and phonological processing) in pASD and HC

(Continued)

TABLE 1 | Continued

Study (year)	Participants (nr, M/F, mean age ± SD in years)	BAP Questionnaires	Method	Results	Correlations with BAP scores and behavior
			Evoked, induced and total power gamma and beta	pASD>HC evoked and total gamma SMG, LOC	
	28 HC (12/16) 38.7 ± 6.3				
			PLF gamma and beta	pASD>HC left lateralization	
Buard et al., 2013	12 ASD (?/?) 28.3 ± 13.3	None	Picture-naming task	ASD<HC high-gamma in right STG, evoked high-beta/low-gamma in left IFG and PLF beta in OCC	No significant correlation between MEG measures and language scores
	14 pASD (?/?) 37.9 ± 5.9				
			Evoked, induced and total power gamma and beta	pASD>HC high-gamma in left STG and evoked high-beta/low-gamma in left FG	
	35 HC (?/?) 34.2 ± 11.9				
			PLF gamma and beta	ASD>HC connectivity between IFG and FG and between STG and OCC in both gamma and beta band	
			Granger Causality		

BAP, Broader Autism Phenotype; ASD, Autism Spectrum Disorders; HC, healthy controls; pASD, parents of individuals with ASD; SD, standard deviation.
AQ, Autism Quotient; BEES, Balanced Emotional Empathy Scale; BAPQ, Broad Autism Phenotype Questionnaire; MPAS-R, Modified Personality Assessment Schedule —Revised; SRS, Social Responsiveness Scale.
ROI, region of interest; VBM, voxel-based morphometry; EFT, "Adult Embedded Figures" test; ET, "Reading the Mind in the Eyes" (or Eyes) test; Glu, glutamate; NAA, n-acetyl aspartate + n-acetyl aspartyl; Cr, phosphocreatine and creatine; ERPs, evoked response potentials; tGBR, transient gamma-band response; ASSR, auditory steady-state response; PLF, phase locking factor; CTOOP, Comprehensive Test of Phonological Processing; WMS, Wechsler Memory Scale.
HYP, hippocampus; AMY, amygdala; GM, gray matter; BA, Broadmann area; FG, fusiform gyrus; IFG, inferior frontal gyrus; IC, insular cortex, STG, superior temporal gyrus, SMG, supramarginal gyrus; SMA, supplementary motor area; INS, insula; LOC, lateral occipital cortex; OCC, occipital lobe.

to both the pASD and the HC group, while no significant differences were found between pASD and HC. No differences were detected in the total brain volume among the three groups.

Palmen et al. (2005a) compared couples of pASD with known increased brain volumes with HC couples for volume differences in total brain, cortical lobes, cerebral and cortical gray matter (GM) and white matter (WM), cerebellum, and ventricles. The overt aim of the study was to investigate whether the cerebral enlargement observed in ASD probands (Palmen et al., 2005b) extended also to parents, and in this case whether fathers and mothers were equally affected and if the same regions, as those of the autistic probands, were interested in the enlargement. The authors found no group or gender differences in any of the brain volumes, including the volume of intracranium, total brain, GM and WM of the cerebrum, frontal, temporal, parietal, and occipital GM and WM, cerebellum, third and lateral ventricle. Nevertheless, within the pASD group significant positive correlations were found between the Autism Quotient (AQ) (Baron-Cohen et al., 2001a) scores and intracranial and ventricular volumes, suggesting that autistic traits might be associated to an enlargement in these structures.

In the third study, Peterson et al. (2006) compared regional GM volume in pASD and in HC, reporting an increase in several GM regions in pASD (e.g., superior temporal gyri, inferior and middle frontal gyri, superior parietal lobule, anterior cingulate). A single large relative decrease was observed in the anterior portion of the left cerebellar hemisphere in pASD compared with HC. Males showed increased GM compared with females in both groups, while no between-group differences respect to gender emerged.

It is worth noting that in the three above mentioned studies three different procedures were applied for data analysis. Specifically, Rojas et al. (2004) used manual tracing for selecting hippocampus and amygdala, Palmen et al. (2005a) applied a semi-automatic procedure to obtain a segmentation of the brain in the structure of interest and Peterson et al. (2006) applied an approach based on voxel-based morphometry (VBM).

Magnetic Resonance Spectroscopy

Only one study used Magnetic Resonance Spectroscopy (MRS) to assess brain chemistry in parents of individuals with ASD (Brown et al., 2013). The aim of the study was therefore to determine whether the parents of ASD patients show higher

levels of Glutamate (Hyperglutamate Theory) as compared to controls (Fatemi, 2008). The level of Glutamate (Glu), together with other potentially interesting molecules, including n-acetyl-aspartate (NAA), choline (Cho), myoinositol (mI) and creatine (Cr), was measured in the auditory cortex of subjects with ASD, pASD and HC. BAP traits in pASD were assessed by AQ and by Social Responsiveness Scale (SRS) (Constantino, 2002). While ASD subjects had increased levels of Glu compared with both pASD and HC, no differences were found between pASD and HC. Although not significantly different, the mean levels of the explored molecules in the pASD group were found to be intermediate between the HC and the ASD group. A significant positive correlation between left NAA and the SRS as well as between left Glu and the AQ was observed, but these correlations did not remain significant after multiple comparison correction. Both ASD and pASD did not exhibit sex differences in any of the MRS measures.

Functional MRI (fMRI)

The first study that evaluated the BAP in pASD through fMRI technique was performed by Baron-Cohen et al. (2006). In this investigation, the authors used the visual search task "Adult Embedded Figures Test" (EFT) (Witkin et al., 1962), and the advanced emotion recognition task test "Reading the Mind in the Eyes" (or Eyes) (ET) (Baron-Cohen et al., 2001b) in order to see if the parents showed the same atypical brain function observed in the autistic children (Baron-Cohen et al., 1999; Ring et al., 1999). They also preliminarily explored the influence of sex on brain functioning during these two tasks in a small sample of six males and six females. Results indicated that pASD showed atypical brain activity compared with HC; moreover sex differences in neural underpinnings of both tests were found. As far as the EFT task is concerned, pASD showed less activity in the visual cortex while a reduced activity in the mid-temporal gyrus, and the inferior frontal gyrus was observed using the ET task.

As regards sex differences in the EFT, female controls displayed increased activity in middle occipital gyrus than male controls while both mothers and fathers showed even less activity in this area than sex-matched controls. In the ET, female controls exhibited more activity in the left medial temporal gyrus and left dorsolateral prefrontal cortex than male controls, while both mothers and fathers of children with ASD showed a brain activity similar to that of male controls. Mothers and fathers had comparable brain activation. One of the region identified as atypically activated in the ET task (B44) overlaps with a region previously identified as involved in "theory of mind" (Frith and Frith, 1999).

Greimel et al. (2010) explored in ASD boys and in their fathers (pASD) aspects related to the social domain of ASD, and in particular to the mechanism of empathy. Two aspects of empathy were evaluated related to (1) inferring how another person feels (other-task), and (2) responding appropriately to emotions of others (self-task). Comparison groups consist of age-matched typically developing boys (HC) and their fathers (pHC). Brain activation was analyzed in three predefined ROIs, the fusiform gyrus (FG), the inferior frontal gyrus (IFG) and the AMY and correlations with behavioral traits were evaluated. Empathic abilities were assessed by the Griffith Empathy Measure (GEM) in ASD and by the Balanced Emotional Empathy Scale (BEES) in pASD.

Despite a normal performance in reference to the number of correct/incorrect responses and even a faster response than pHC, pASD showed an abnormal brain activation. Specifically, both boys with ASD and their fathers obtained reduced anterior FG activation during the other-task, and boys with ASD additionally exhibited reduced FG activation during the self-task compared to HC. Interestingly, the activation within the FG occurred outside the well-known fusiform face area leading to exclude that differences of activation detected in this area were ascribable to a deficit in face processing. This hypothesis was corroborated also by the recording of the gaze during the fMRI task that showed an intact gaze pattern in scanning faces both in the adolescents with ASD and in their fathers. A diminished activation was also found in AMY in fathers of boys with ASD compared to control fathers when inferring others' emotions from weak cues, while in the ASD group this result was only obtained at an uncorrected threshold. The author hypothesized that fathers activated strategies to compensate for FG and AMY dysfunction. An involvement of the mirror neuron system (MNS) was also observed mainly in the ASD adolescents who showed a reduced activation of the IFG during the self-task. In both pASD and ASD groups a significant correlation between behavioral measures of empathy and brain activation was detected: specifically, in the ASD group the correlation was significant with activation of FG while in the pASD group with activation of the insula. However, no significant correlation was found between brain activity and AQ scores in pASD.

Together with social impairments, language dysfunction is another well-known hallmark of ASD. Extending the boundaries, language ability, specifically phonological processing ability, has been proposed to be one of six candidate BAP traits (Dawson et al., 2002).

Wilson et al. (2013) explored the neural correlates of phonological processing ability in a group of parents of children with ASD and in a group of age-matched controls. The task proposed consisted of prime-target word pairs differing in terms of their phonological relatedness including both word-word homophone and pseudoword-word pseudohomophone. Brain activation was also correlated with a behavioral measure of phonological processing ability obtained by the non-word repetition subtest of the Comprehensive Test of Phonological Processing (CTOPP) (Wagner et al., 1999).

Despite non-significant differences in terms of task performances and CTOPP scores and low AQ scores, pASD showed significantly higher hemodynamic responses than controls for pseudohomophone compared with homophone priming. Several cortical regions were involved in this abnormal activation, including the left anterior insular cortex (IC), the bilateral cerebellum and thalamus, left postcentral gyrus, precentral gyrus, and supplementary motor area (SMA), right superior temporal gyrus (STG) and supramarginal gyrus (SMG): interestingly, most of these regions had been previously implicated in language processing (Baddeley, 1992; Ackermann and Riecker, 2004; Hickok and Poeppel, 2007; Ghosh et al.,

2008). Significant positive correlations were also observed between greater hemodynamic response and CTOPP in right STG, left IFG and IC in pASD and in several regions in controls (i.e. bilateral occipital gyrus, parietal lobule, postcentral gyrus, lingual gyrus, and IFG).

Moreover, parents of boys with ASD exhibited increased hemodynamic suppression in response to phonological priming compared with controls in several cortical regions including both the left lateralized STG and SMG. Both groups expressed a significant left lateralization in the ROI selected for the analysis.

The more recent fMRI study conducted in parents of individuals with ASD investigated neural substrates of face processing (Yucel et al., 2014). This is the only study which subset the parents on BAP traits. Specifically, in order to investigate the characteristics of a specific endophenotype linked to social behavior, the parents were classified in a group having "aloof personality" (BAP+) and a group having "non-aloof personality" (BAP−). The classification was based on the Broad Autism Phenotype Questionnaire (BAPQ) and the Modified Personality Assessment Schedule—Revised (MPAS-R) specifically designed to determine the presence or absence of "aloof personality." Using two face activation paradigms, one based on face memory and the other based on emotional matching, the authors found that pASD had a higher activation of AMY and FG and a lower activation of right insula compared with HC, while no significant difference in activation was observed between BAP+ and BAP− in these regions. Conversely, BAP+ and BAP− parents significantly differ in terms of activation of the lateral occipital cortex (LOC). Indeed, BAP+ parents showed a bilateral hyper-activation in the LOC compared with both BAP− and HC.

Neurophysiology (Electroencephalography and Magnetoencephalography)

The first electrophysiological study in pASD was performed by Dawson et al. (2005) who evaluated event-related brain potentials to face and non-face stimuli. Specifically, upright and inverted faces or chairs were presented to a group of pASD and HC and N170 amplitude and latency was measured at the inferior right and left posterior temporal regions. While HC showed the typical pattern of higher right than left N170 amplitude in response to faces (Bentin et al., 1996), pASD exhibited reduced right N170 amplitude resulting in bilaterally distributed brain activity to faces. In addition, HC had the expected faster N170 response to upright faces compared to upright chairs (Itier et al., 2006), while pASD showed no differences in latency in response to the two types of stimuli. Abnormalities in brain activity in pASD compared to controls were also associated to lower performances in behavioral tests (face recognition and object memory).

Subsequent studies explored brain activity in pASD in response to different stimuli using magnetoencephalography (MEG), focusing on high-frequency bands.

First, Rojas et al. (2008) investigated both evoked and induced components of the *transient gamma-band response (tGBR)*, elicited by auditory stimulation in subjects with ASD, in pASD and in a comparison group of healthy subjects. Source localization of the data was performed on MRI data acquired on the subjects enrolled in the study (Peterson et al., 2006). In addition to evoked and induced power, the authors also computed the *phase locking factor (PLF)* as a measure of phase consistency across trials.

Both pASD and the ASD groups showed bilaterally higher induced tGBR response compared with controls, while evoked tGBR was found bilaterally reduced in the same comparison. The PLF was also bilaterally reduced in both the pASD and the ASD group compared with HC. Moreover, both the pASD and the ASD group had a reduced anterior-posterior asymmetry of the magnetic sources compared with controls. In this study, no differences between pASD and ASD were found: such findings could be attributable to the low statistical power, but could also suggest that parents had the familial liability relevant to gamma-band disturbances.

Later, Rojas et al. (2011) extended the results of their previous work analyzing not only the tGBR component of gamma-band power, but also the *auditory steady-state response (ASSR)*, in response to auditory stimulation. A group of pASD was compared with a control group of HC. In this study, authors also correlated MEG results with scores indicative of BAP−traits (AQ and SRS). The group of pASD exhibited reduced evoked power, total power (left hemisphere) and PLF (left hemisphere) of the ASSR component relative to the HC group. However, the authors were not able to replicate their previous findings relative to tGBR (Rojas et al., 2008), as they did not found any significant differences between pASD and HC.

Interestingly, an inverse correlation between ASSR PLF and the AQ communication subscale was found in pASD, confirming an association of gamma-band activity to perception of speech sounds and lexicality (Kaiser, 2004; Basirat et al., 2008). An inverse correlation was also observed in pASD between SRS scores and tGBR and ASSR gamma-band evoked power suggesting an indirect relationship between auditory gamma-band dysfunction and social traits of ASD.

In another investigation (McFadden et al., 2012), gamma-band response was analyzed in pASD and in HC in response to auditory language stimuli, rather than to simple auditory stimuli. In this contest, beta band activity was also examined since it has been suggested to be involved in language processing (Shahin et al., 2009). While in the previous two investigations (Rojas et al., 2008, 2011) pASD showed decreased evoked gamma-band response compared with HC, in this study pASD exhibited increased evoked power. In addition, there was an increase in pASD of total gamma power compared with controls. Source localization analysis showed that this increase was mainly localized in the SMG, in the lateral occipital cortex (LOC), and in the FG.

Beta evoked activity was also found increased in pASD compared with controls mainly in SMG, but also in LOC and FFG possibly reflecting differences in cognitive function during language processing. While in both groups the task generally elicited left lateralized responses, pASD showed greater left lateralization than controls, confirming also in this case an atypical lateralization of the brain in pASD. Significant but different correlations were found between gamma or beta band activity and language measures.

Gamma and beta band responses were also assessed in pASD compared with HC during a picture-naming task (Buard et al., 2013). Subjects were instructed to sub-vocalize (to reduce motion artifacts) the name of the object depicted in the image they were shown. Due to their involvement in language function and in visual processing, FG, STG, IFG and occipital lobe (OCC) were considered as the regions of interest. As in the three previous studies (Rojas et al., 2008, 2011; McFadden et al., 2012), evoked and induced power together with PLF were computed. In addition, Granger causality function, as a measure of effective connectivity among the activated regions, was measured.

Interestingly, the ASD group and the pASD showed different patterns of activation both in gamma and beta bands. While the ASD group exhibited reduced evoked high-gamma activity in the right STG, increased evoked high-beta/low-gamma in the left IFG and reduced PLF beta in the OCC, the pASD group showed increased evoked high-gamma in the left STG and evoked high-beta/low-gamma in the left FG.

Functional connectivity abnormalities were only observed in the ASD group compared with the control group: specifically, over-connectivity was found in the left hemisphere between IFG and FG and between STG and OCC in both gamma and beta band. This altered functional connectivity from anterior to posterior language and visual areas may partially explain the impaired activation of these regions in the ASD group, ascribable to alterations in long-range neural synchronization.

DISCUSSION

The main leading hypothesis tested in this review is that pASD present with a number of neuroanatomical and neurofunctional characteristics observed in individuals with ASD, but to a lesser extent. This hypothesis is supported by previous studies demonstrating intermediate levels of biochemical, immunological, morphological and neuropsychological endophenotypes/biomarkers in pASD (Ruggeri et al., 2014).

In some cases, the results of the studies, using different methodological approaches, have supported the primary hypothesis, while in other cases different results have emerged.

Are pASD Different from HC?

All of the 13 reviewed studies compared the pASD with a sample of HC.

(a) No differences in total brain volumes between pASD and controls were found in any of the three sMRI studies (Rojas et al., 2004; Palmen et al., 2005a; Peterson et al., 2006). This finding is not surprising when considering that even within the ASD population many/most adults, unlike children, do not differ from controls in overall brain volume. Indeed, there is increasing evidence that brain growth trajectory is abnormal in subjects with ASD and that they have differences in the timing of both initiation and cessation of overall brain growth, resulting in larger brain volumes during childhood followed by later normalization (Courchesne et al., 2001, 2003; Dawson et al., 2007).

(b) More inconsistent findings were reported for the single brain structures. While Palmen et al. (2005a) found no differences between pASD and HC groups in any of the volumes considered, including cortical lobes, cerebral GM and WM, cerebellum, and ventricles, increased volumes were found in the left hippocampus (Rojas et al., 2004) or in a number of GM regions (Peterson et al., 2006). The different approach in analyzing brain regions (global brain structures—Palmen et al., 2005a—vs. focal structures—Rojas et al., 2004—vs. whole brain approach—Peterson et al., 2006) prevents a comparison among studies. Overall, the inconsistency of these results reflects that of the studies on subjects with ASD (Ameis and Catani, 2015). Methodological differences between investigations and the potential for heterogeneity of underlying brain alterations in ASD likely contribute to the inconsistency of these results.

(c) Functional studies showed some atypicalities in face processing, empathy and language/auditory processing in pASD compared with HC.

Face

The study by Dawson et al. (2005) support the social motivation impairment showing an abnormal N170 response to faces both in its latency and amplitude with a pattern resembling that observed for subject with ASD (Apicella et al., 2013). Yucel et al. (2014) observed an increased activation in pASD compared with controls during an emotion recognition task in regions that are specialized for face processing, i.e., the fusiform gyrus and the amygdala.

Empathy

An opposite pattern was found by Greimel et al. (2010) who explored empathy during the presentation of emotional stimuli in pASD and found a decreased activation in the same regions. It is possible that the two different types of task lead to different brain activations. Moreover, the different results could be explained by the fact that the sample of Greimel study is composed of males only who are generally less empathic than females (Klein and Hodges, 2001), and therefore process emotion to a lesser extent than females. In addition it is worth noting that Yucel et al. (2014) found a decreased activation of the insula, which is known to be linked to empathic abilities (Carr et al., 2003) as also suggested by the positive correlation found in Greimel et al. (2010) between insula activation and the BEES. Emotion recognition impairments in pASD also emerged from the study of Baron-Cohen et al. (2006) who observed a decreased activation in the left IFG of pASD compared with controls during an emotion recognition test.

Language

Wilson et al. (2013) showed that pASD compared with HC exhibit a greater hemodynamic response to pseudohomophones respect to homophones and an enhanced hemodynamic suppression in response to phonological priming. Interestingly, Peterson et al. (2006) observed both cerebellar enhancements and reductions, although in different cerebellar regions than those differently activated in the phonological task, and larger left STG

and SMG GM volumes in pASD relative to HC. These regions are known to be involved in language and phonological processing (Turkeltaub and Coslett, 2010) and to be functionally impaired in ASD (Mostofsky et al., 2009).

Abnormalities associated to language processing have been shown also by MEG studies, mainly associated to gamma-band response. In particular, gamma-band deficit, which has been suggested as a biomarker of ASD (Jamal et al., 2013; Rojas and Wilson, 2014), exists also in pASD, and abnormalities seem to extend also to the beta band. In ASD individuals, dysfunctional gamma-band response has been associated with GABAergic inhibitory deficits (Hussman, 2001; Fatemi et al., 2009). Conversely, multiple evidences suggest an increased neuronal excitability in ASD, involving a higher than normal serum glutamate (Shinohe et al., 2006), and increased metabotropic glutamate receptor expression (Fatemi et al., 2011). Overall, these evidences have been summarized in the excitation/inhibition imbalance (EI) theory of ASD (Rubenstein and Merzenich, 2003).

Rojas et al. (2008, 2011) explored gamma band response to auditory stimulation in pASD. Induced response has been found increased in pASD (Rojas et al., 2008), while evoked response and PLF were decreased (Rojas et al., 2011) compared with HC in response to simple auditory stimulus. However, more complex stimuli activate a different pattern as observed in subsequent investigations (McFadden et al., 2012; Buard et al., 2013). In both these studies, pASD showed an increased evoked gamma band response compared with HC, which extended also to beta band in Buard et al. (2013). The different findings among these studies might be explained by the different level of complexity of the tasks: specifically, subjects were requested to be engaged in higher order cognitive processes including language and sustained attention (McFadden et al., 2012; Buard et al., 2013), or only passive listening to a simple auditory stimulus was required (Rojas et al., 2008, 2011).

The studies exploring auditory/language processing suggest that when pASD are involved in higher cognitive function they activate a higher brain response compared to that of controls, possibly as a compensatory mechanism in absence of behavioral impairment. In the study by Wilson et al. (2013) the greater hemodynamic responses in the parent group might reflect the heavier demands requested by the pseudohomophone primes on phonological recoding and working memory skills compared with homophone primes, and it can be interpreted as an index of more effortful processing during this task. Analogously, in the studies by McFadden et al. (2012) and Buard et al. (2013) the increase in gamma and/or beta could reflect a greater cognitive effort in phonology and receptive language tasks, which determine an abnormal synchronous activation of language networks (Jerbi et al., 2009).

It is worth noting that functional abnormalities at a neural level in pASD are not always associated to behavioral impairments. For example Greimel et al. (2010) found a non-compromised empathic ability in an emotion recognition task while Wilson et al. (2013) found no difference in terms of phonological processing (CTOPP scores) between pASD and HC. Conversely in the study by Dawson et al. (2005) the authors found that neurofunctional abnormalities and neuropsychological performances in pASD were associated, suggesting that pASD are more compromised at a neural level than at a behavioral level.

How Are Parents of Individuals with ASD Compared to Other Individuals with ASD?

Only five of the 13 studies addressed the question of the overlap between pASD and other individuals with ASD (Rojas et al., 2004, 2008; Greimel et al., 2010; Brown et al., 2013; Buard et al., 2013). All reported similarities in some aspects of brain structure and function consistent with the hypothesis of a continuum of some ASD features expressed in pASD, with milder but qualitatively similar brain alterations to those detected in ASD.

In particular, structural (Rojas et al., 2004) and spectroscopy (Brown et al., 2013) studies revealed a brain endophenotype in pASD intermediate between ASD patients and HC. Specifically, Rojas et al. (2004) found that hippocampus enlargement interested also pASD, but to a lesser extent than ASD individuals. Vice-versa, the amygdala was smaller in ASD patients compared to pASD. Brown et al. (2013) showed that the mean levels of the explored molecules in the parent group were intermediate between ASD individuals and HC. However, these findings were not statistically significant possibly due to the small sample size and/or to the low scores at the AQ and the SRS of the pASD subjects.

The fMRI study exploring the neural correlates of empathy (Greimel et al., 2010) reported a reduced activation in the right anterior fusiform gyrus in both adolescents with ASD and pASD compared to age and IQ matched controls. Finally, using EEG a reduced early auditory gamma-band response shared by both adults with ASD and pASD in comparison to HC was detected (Rojas et al., 2008).

Several other patterns of brain activity were not shared by pASD and ASD patients, potentially suggesting a lesser role of these aspects as endophenotypes of the disorder. For example, in the same fMRI study exploring empathy (Greimel et al., 2010), reduced amygdala activity was found in pASD but not in ASD. Also, using MEG during a picture naming task, Buard et al. (2013) found that gamma-band activity showed opposite profiles in pASD and in ASD subjects relative to controls, being increased in the former and reduced in the latter.

All in all, the limited number of studies addressing this question does not allow for definitive conclusions although it seems to be conceivable that some aspects of brain structure and function are shared by pASD and ASD patients, supporting their possible role as endophenotype of the disorder.

How Are Parents of Individuals with ASD Compared to Their Probands?

The study by Greimel et al. (2010) was the only one that enrolled both the probands and their fathers to explore the transmission of neural substrates. The results confirmed the primary hypothesis of a neurofunctional pattern in pASD intermediate between HC and ASD. In particular, pASD showed an abnormal neural activation during the other-task similar to their probands,

expressed by a reduced hemodynamic response in FG – a tempo-occipital brain region primarily involved in face processing-. Conversely, unlike their probands, pASD showed a normal response during the self-task. It is of interest that a reduced activation of regions previously associated to the MNS, namely IFG, was found in ASD probands but not in their fathers. These results support the hypothesis that FG dysfunction in ASD is genetically influenced (Polk et al., 2007).

Are the Neurostructural and Neurofunctional Alterations Reported in Parents of Individuals with ASD Specifically Related to BAP Features?

Six of the reviewed studies (Palmen et al., 2005a; Greimel et al., 2010; Rojas et al., 2011; Brown et al., 2013; Wilson et al., 2013; Yucel et al., 2014) assessed the BAP characteristics of the pASD applying instruments such as the AQ, the BAPQ or the SRS. Significant correlations between scores at the questionnaires and the brain structural and functional indexes were found in almost all these studies. In particular, Palmen et al. (2005a) found a significant positive correlation between AQ scores and intracranial and ventricular volume in pASD, while Brown et al. (2013) found a significant, uncorrected, positive correlation between left NAA and the SRS and left Glu and the AQ. Both studies did not report significant differences in pASD compared to HC (Palmen et al., 2005a; Brown et al., 2013); however, the fact that pASD scored very low at questionnaires assessing BAP could represent a possible bias leading to negative findings. Interestingly, in both studies a positive correlation between neurostructural results and BAP features was found, suggesting that enlarged brain volume or increased Glu level respectively could still be associated to the autistic phenotype.

Notably, Wilson et al. (2013) found significant differences in brain activation during a language task in the pASD sample, despite low scores at the AQ. This result could suggest that deficits in neural substrates of language processing could be associated with autistic traits, confirming it as one of the core impairment of ASD.

This is confirmed by the investigation of Rojas et al. (2011) in which a negative correlation between ASSR PLF and AQ communication subscale as well as between SRS scores and tGBR/ASSR evoked power was observed. ASSR PLF and evoked power were found decreased in pASD compared to HC: therefore, it can be argued that deficits in auditory gamma band are correlated to problems in communications (AQ) and socials skills (SRS). Despite the authors did not find significant differences regarding tGBR, a significant correlation between SRS scores and this feature was observed in pASD suggesting a possible association with the autistic phenotype, as proposed in their previous study (Rojas et al., 2008).

Greimel et al. (2010) did not find any significant correlation between brain activation and AQ in pASD, while a brain-behavior relationship was detected with empathic scores (BEES).

The paper by Yucel et al. (2014) was the only one subgrouping the parent sample according to BAP traits. Interestingly, the authors observed that while an atypical activation of face processing regions was common to both groups of parents, BAP+, but not BAP− parents showed an hyper-activation of lateral occipital cortex. The hyper-activation of LOC in BAP+ could reflect an aberrant "compensatory" activation of these regions in BAP+ parents. These data suggest that while neural circuitry abnormalities in the regions specific for face processing are necessary for the occurrence of the BAP, they are not sufficient to result in autism-related social behavior.

Overall, these findings suggest a possible link between the subclinical dimension of BAP and neurobiological expression of brain function and structure.

Does Gender Influence Neurostructural and Neurofunctional Results in Parents of Individuals with ASD?

Previous studies have reported sex differences in brain in healthy populations and these processes have shown to differ in people with ASD. In particular, sexual dimorphism in brain regions that are crucial to language and social abilities has been proposed (Lai et al., 2013; Retico et al., 2016).

Understanding cerebral gender differences is important, among other reasons, to explain the increased vulnerability of males to ASD. Few studies have explored gender differences in pASD in order to investigate the heritability of sex differences in brain structures and function.

From a structural point of view, it was observed that males, both pASD and HC had increased total, hippocampal and amygdala volumes (Rojas et al., 2004), as well as GM (Peterson et al., 2006) compared to pASD and HC females, but this difference did not contribute to between-groups differences. Brown et al. (2013) investigated gender differences in MRS measures both in ASD and pASD and did not find any differences as well. Conversely, Baron-Cohen et al. (2006) found significant differences in brain function related to gender. In particular, their results support the hypothesis of the "Extreme Male Brain Theory" of ASD, according to which "the male brain is programmed to systemize and the female brain to empathize" (Benenson, 2003). Indeed, both mothers and fathers showed an activation even lower than that of male controls in regions were female controls had a higher activity. The results of this study may suggest a genetic component of the hyper-masculinization of the brain.

Do Parents of Individuals with ASD Express an Atypical Lateralization of the Brain?

In typical development, lateralization of brain function underlies specialized cognitive and behavioral processes (Mesulam, 1990). In particular, several pieces of evidence exist about a left lateralization in language regions (Knecht et al., 2000), and right lateralization in attentional regions (Corbetta and Shulman, 2011) in the majority of individuals with typical development. Atypical lateralization in brain structure and function has been associated with ASD (Conti et al., 2016); more specifically, reduced left lateralization or reversed lateralization of brain structure and function in core language regions and in the WM tracts that connect them has been shown in ASD using different

techniques (Kleinhans et al., 2008; Lange et al., 2010; Seery et al., 2013).

Whether the pattern of lateralization related to language processing observed in subjects with ASD is the same in their parents is not clear from the reviewed studies.

In Wilson et al. (2013), hemispheric lateralization analysis did not indicate greater right hemispheric language dominance in the pASD: in fact, both pASD and HC showed left lateralization across the selected ROIs. McFadden et al. (2012) found that pASD showed even an increased left lateralization than controls. Rojas et al. (2011) showed that differences in ASSR response in pASD was restricted to the left hemisphere, however across groups the tGBR and ASSR evoked power was increased in the right hemisphere. Rojas et al. (2008) highlighted a peculiar pattern of asymmetric activation in control subjects in which the activation of the right hemisphere was anterior to that of the left hemisphere. This pattern was not observed in ASD and was mild in pASD.

Yucel et al. (2014), observed in the face processing task a significant effect of hemisphere. Specifically, FG showed greater activation in right than left hemisphere in BAP+ compared with BAP− and HC, and right amygdala was more active than left in BAP+ compared with BAP−. Since right lateralization was observed specifically in BAP+ pASD, a compensatory mechanism of activation in these regions could be hypothesized for this subgroup of parents. Additional support for this interpretation can be found in the investigation of Rojas et al. (2004) on the basis of which pASD has reduced right amygdala volume compared with HC: it may be possible that to compensate the reduction of volume an abnormal high activation is required.

CONCLUSIONS AND FUTURE DIRECTIONS

Although, results are often unclear and contradictory, some general considerations can be done:

(i) pASD differ from HC both at a structural and functional level and these neural abnormalities are not always associated with behavioral impairments;
(ii) The neural pattern in pASD seems to be intermediate between HC and ASD probands;
(iii) More atypicalities in neural patterns of pASD seem to be associated with higher autistic traits;
(iv) The pattern of neural correlates in pASD resembles that of adult individuals with ASD or it is specific to pASD, possibly due to a compensatory mechanism;
(v) The gender might influence the results.

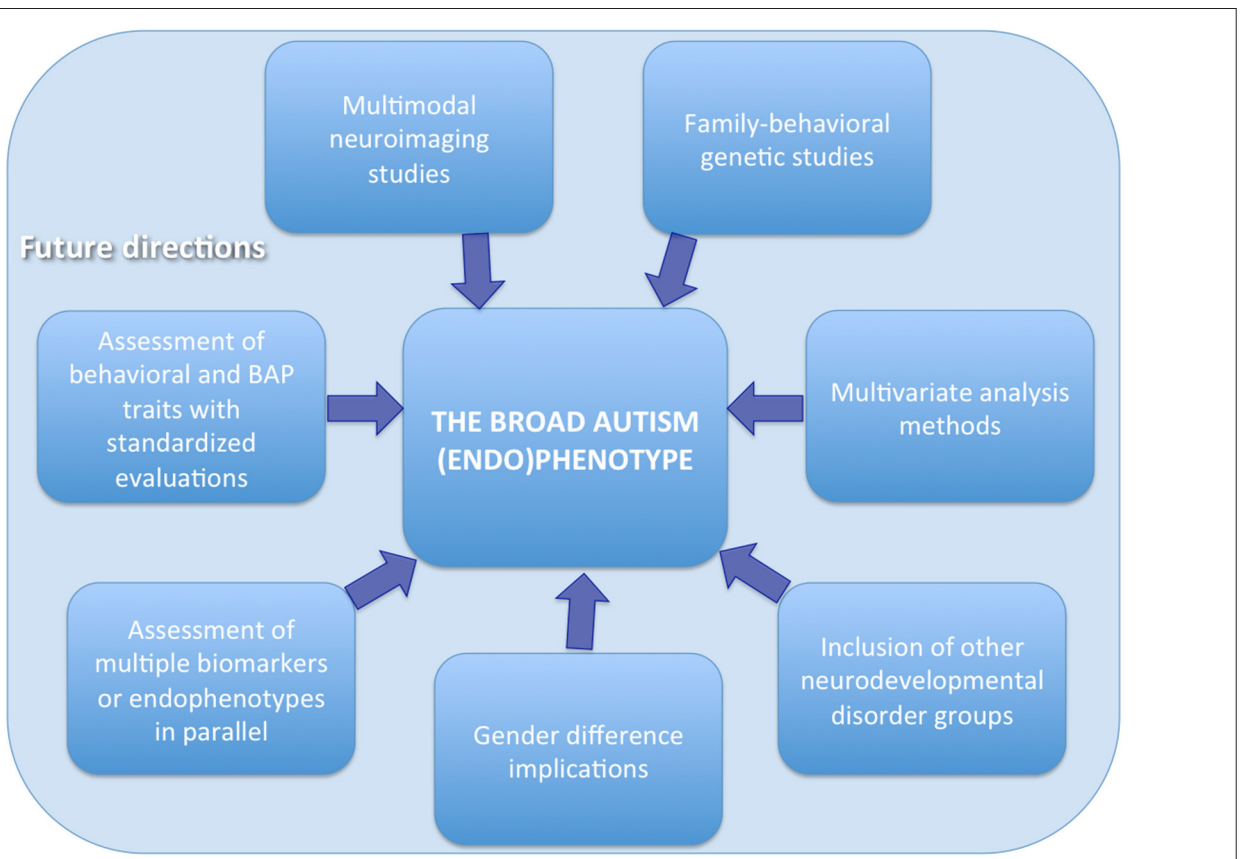

FIGURE 1 | Recommendations for future studies assessing the broader autism (endo)phenotype.

In conclusion, our review reports findings that are often non-replicated, preventing a univocal interpretation of the results.

In order to elucidate the brain structural and functional underpinnings in pASD and their potential role as endophenotype, several aspects should be considered when planning future studies (**Figure 1**).

First, neuroimaging studies should be ideally include a group of pASD and HC with their ASD and non-ASD probands, respectively. Second, a family-behavioral genetic design should be adopted in order to analyze the behavioral features as well as the genetics not only of the probands, but also of the parents and the siblings, and to link these data to underlying brain structure and function. Third, behavioral assessment as well as BAP traits evaluation should be performed using standardized questionnaires and tests in order to subgroups the probands and their relatives according to the obtained scores and to investigate a possible correlation between brain abnormalities and BAP traits and/or behavioral impairments. Fourth, multimodal imaging techniques could also be adopted to better elucidate brain correlates of BAP. For example, the integration of neuroimaging data with neurophysiological signals (EEG and MEG) offers advantages of both high spatial and temporal resolution (Ingalhalikar et al., 2012; Berman et al., 2016). The application of these methods also in pASD probands could provide new insights into the endophenotype of ASD. Fifth, since gender can influence neural substrates, this factor needs to be carefully taken into account when grouping samples and interpreting the results. Indeed, brain endophenotypes could be related to differences in the developmental, psychiatric, and medical endophenotypes between males and females with ASD. These research findings may in turn help the clinical assessment and treatment of ASD and the search for possible etiologies (Rubenstein et al., 2015). Sixth, studies on the BAP could also benefit of the assessment of multiple endophenotypes/biomarkers in parallel by collecting, in addition to neuroimaging data, immunological, biochemical, or neuropsychological data and evaluating the cross talk among the different modalities (Ruggeri et al., 2014). Seventh, the inclusion of samples with other neurodevelopmental disorders rather than ASD can help to disentangle the specific from the non-specific endophenotypes associated to each condition. Indeed brain alterations have been found in relatives of probands with Attention Deficit/Hyperactivity Disorder—ADHD—(Casey et al., 2007; Hale et al., 2010; Poissant et al., 2014; Rapin et al., 2014), language impairments (Plante, 1991; Ors et al., 2002) and learning or intellectual disabilities (Mannerkoski et al., 2009). In particular, previous literature suggests that there are cognitive and brain endophenotypes common to ASD and ADHD and that studying the similarities and differences between these two disorders might be a powerful research approach to increase our understanding of their pathophysiology (Rommelse et al., 2011). Finally the use of multivariate approaches, based for example on machine learning (Retico et al., 2014; Segovia et al., 2014), can provide more insightful results than the traditional statistical analysis methods.

In conclusion, these types of implementations may help to better elucidate the hereditary mechanisms involved in the various clinical dimension of ASD.

AUTHOR CONTRIBUTIONS

LB performed the literature-search, analyzed the data and drafted the paper, SC, EC made substantial contribution in the literature analysis and in writing the paper, CG, CC collaborated during the literature analysis, LD, GC participated in the design of the paper and supervised the wiring, FM, AG made substantial contributions to the conception of the review, and revisited the manuscript critically. All the authors read and approved the final version of the manuscript.

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METHODS
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GOLIAH: A Gaming Platform for Home-Based Intervention in Autism – Principles and Design

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Children with Autism need intensive intervention and this is challenging in terms of manpower, costs, and time. Advances in Information Communication Technology and computer gaming may help in this respect by creating a nomadically deployable closed-loop intervention system involving the child and active participation of parents and therapists. An automated serious gaming platform enabling intensive intervention in nomadic settings has been developed by mapping two pivotal skills in autism spectrum disorder: Imitation and Joint Attention (JA). Eleven games – seven Imitations and four JA – were derived from the Early Start Denver Model. The games involved application of visual and audio stimuli with multiple difficulty levels and a wide variety of tasks and actions pertaining to the Imitation and JA. The platform runs on mobile devices and allows the therapist to (1) characterize the child's initial difficulties/strengths, ensuring tailored and adapted intervention by choosing appropriate games and (2) investigate and track the temporal evolution of the child's progress through a set of automatically extracted quantitative performance metrics. The platform allows the therapist to change the game or its difficulty levels during the intervention depending on the child's progress. Performance of the platform was assessed in a 3-month open trial with 10 children with autism (Trial ID: NCT02560415, Clinicaltrials.gov). The children and the parents participated in 80% of the sessions both at home (77.5%) and at the hospital (90%). All children went through all the games but, given the diversity of the games and the heterogeneity of children profiles and abilities, for a given game the number of sessions dedicated to the game varied and could be tailored through automatic scoring. Parents (N = 10) highlighted enhancement in the child's concentration, flexibility, and self-esteem in 78, 89, and 44% of the cases, respectively, and 56% observed an enhanced parents–child relationship. This pilot study shows the feasibility of using the developed gaming platform for home-based intensive intervention. However, the overall capability of the platform in delivering intervention needs to be assessed in a bigger open trial.

Keywords: autism spectrum disorder, Early Start Denver Model, serious game, intensive intervention, Imitation, Joint Attention, nomadic settings

INTRODUCTION

Autism spectrum disorder (ASD) is a spectrum of neurodevelopmental disorders characterized by the presence of atypical social communicative interaction and behaviors (1). Typically, ASD is diagnosed through behavioral analysis in the 3–5 years age range and, once diagnosed, its treatment is mainly delivered through behavioral intervention following different intervention models. In essence, these models try to teach a child cognitive, social, and behavioral skills that are considered essential for independent living in the long run and various techniques have been developed over the years (2–7). However, two major problems associated with such interventions are as follows: (1) a person's specific development intervention protocol, accounting for the actual difficulties and strengths of a child, needs to be designed to achieve maximal effects – ASD is a broad spectrum with significant inter-child variability, and it has already been established that tailor-made personalized intervention may be more effective compared to any generic type of intervention (8) and (2) at least 20 h/week are supposed to be needed for an intensive intervention (9, 10).

Characterization of a child is typically done through behavioral assessment by a trained therapist in clinical settings but such an approach is often prone to have subjective biases. To avoid such biases, one needs to employ a set of stimuli multiple times ensuring their repeatability and then extracting a set of objective measures for characterizing the outcomes. Repeatability is an essential criterion in this case so that an average performance measure in a stimulus-specific way could be obtained reflecting the child's actual ability for responding to the stimuli in question. Such repeatability and the 20 h/week intensive intervention are difficult to achieve (10). In fact, its implementation needs a trained therapist and, given the prevalence of ASD, the workload of a therapist could make the effective implementation of this strategy impractical. Moreover, the involvement of trained parents/caregivers to be part of intervention also in home setting seems to be an effective strategy in order to increase the learning opportunity for children with ASD (11, 12). This requires parent training and regular monitoring to check whether the parents are implementing and properly adhering to the intervention protocol outlined by the therapist. However, the economic implication of such process is quite substantial.

In recent years, computer-based approaches have been shown to be effective in improving the learning cognitive and social skills of children with various learning disability conditions (13–15). In these methods, the target intervention is mapped into a set of computer games and is thereby training the children since children enjoy playing games rather than going through the conventional learning process (16–18). Most of these computer applications designed for people with autism focus on the relationship between one user and one computer and aim to help with specific behavioral problems associated with autism. Computers are motivating for children with autism due to their predictability and consistency, compared with the unpredictable nature of human responses. In regard to social interaction, the computer does not send confusing social messages. Research on the use of computers (19) for students with autism revealed increase in (1) focused attention, (2) overall attention span, (3) sitting behavior, (4) fine motor skills, (5) generalization skills (from computer to related non-computer activities); and decrease in (6) agitation, (7) self-stimulatory behaviors, and (8) perseverative responses. The importance of assistive technology for children with autism has been established by the fact that this technology can be used in rehabilitation for daily activities.

Motivated by these facts, we conceived a closed-loop system with computer gaming at its center that allows the interaction between subjects with autism and a partner. This approach may help in mitigating the effect of isolation that could affect the traditional computer applications mentioned above. The solution we developed is innovative because it seeks to go over the actual lacuna in various computer games for children with ASD. In fact, in most computer games for ASD, the children are engaged only with a computer screen. In our protocol children are engaged with another person (therapist/caregiver) who has a computer and share the activity with the child.

The intensity of intervention for ASD plays a crucial role in terms of clinical outcome. However, the hours of intervention assigned to children with ASD are usually less than the real need of the children. To mitigate this problem, the gaming platform is an interesting solution to increase (1) the hours of treatment for children with ASD and (2) involve caregivers in the intervention. The intensity of the treatment and the involvement of caregivers are two important requirements of the intervention in ASD. In this sense, the gaming platform is in line with the recent recommendation about the intervention proposed by Ref. (11, 12).

The conceptual view of a closed-loop system that may enable effective intervention integrating both the home and clinical settings is shown in **Figure 1**.

At the heart of the system is a computerized gaming library [Gaming Open Library Intervention for Autism at Home (GOLIAH)] that consists of a set of computer games created by mapping the desired intervention stimuli, Imitation, and Joint Attention (JA) in this case, into the games. In theory, the library could be divided into two parts – assessment games and intervention games – although they could be used interchangeably without loss of any generality. At the beginning, the child would be asked to play a set of games carefully selected from the library by the therapist for characterizing the child's difficulties/strengths. Since a particular type of stimulus could be mapped in different ways in multiple games, this will allow using different games for ascertaining the child's difficulties/strengths pertaining to a type of stimulus in a repeatable way without inflicting boredom on the child and thereby obtaining a much more precise average assessment of the child. Once characterized, the therapist could choose appropriate games (designated as the intervention games for convenience) from the gaming library that the child needs to play at his/her home setting on a regular basis adhering to a protocol outlined by the therapist. The aim here is to enhance the cognitive performance of the child through playing these games at home so that the effective intervention hours could be increased. The games could be made flexible enough so that the child may play the games with his/her parents (actively involving the parents without requiring an extensive training process) on a regular basis and with the therapist remotely connected through the internet at pre-scheduled times. The gaming system could

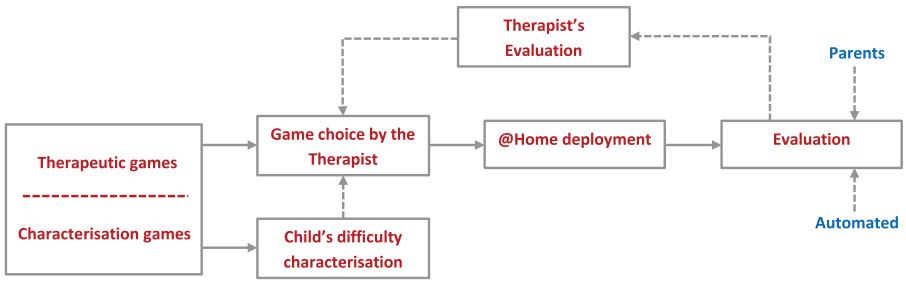

FIGURE 1 | The conceptual closed-loop intervention system. The games contained in the platform are used for assessing the child, first, and for intervention purposes later. The first aim is to characterize the starting cognitive skills of a child by playing games at different levels of difficulty. After identifying the current level of ability of the child, a series of games and difficulties will be planned by the therapist and employed at home. According to the evaluation, both automated and manual, a new set of games will be planned by the therapist.

have an automated evaluation process embedded in it that would extract a set of quantitative evaluation metrics, characterizing the child's performance with each game and thereby providing the temporal evolution characteristics of the child's performance. On the other hand, the parents could also assign a score manually according to a scoring criterion suggested by the therapist to signify how the child's performance has evolved against each stimulus according to their own perception. All the automated and manually evaluated scores could be transmitted to the therapist who may compare them to check, on the one hand, how the child is improving and, on the other hand, whether the parents are adhering to the prescribed protocol truthfully. This could act as the basis of the evaluation by the therapist when he/she plays the game remotely with the child at a pre-scheduled time. Depending on this final evaluation, the therapist may choose a set of different intervention games from the gaming library once the child achieves the target set by the therapist and the whole process may continue. This closed-loop approach may help in alleviating several problems currently encountered by the autism therapists and have many advantages as described below in **Table 1**.

Improving social interaction skills of children with autism is a difficult task for their families as well as for well-trained therapists (20, 21). Although ASD remains a devastating disorder with a poor outcome in adult life (22, 23), there have been important improvements in the condition with the development of various therapeutic approaches. The literature on interventions in ASD has become quite extensive, with increasing convergence between behavioral and developmental methods (24, 25). The focus of many interventions is directed toward the development of skills that are considered to be "pivotal," such as Imitation and JA (26–28).

Imitation plays a critical role in the development of every child. Among the several definitions of imitation, no definition is universally agreed upon: (1) Thorndike (26) offered a definition based on visual aspects: "learning to do an action by watching someone doing it." However, a full definition of imitation must consider multi-sensory aspects. (2) Wallon (28) defined imitation as a learning technique without reward (or reinforcement). (3) Whiten and Ham (29) defined imitation as the process by which the imitator learns some behavioral characteristics of the model. Imitation fulfils two essential functions for adaptation: it is used for learning and it serves to communicate without words (30).

TABLE 1 | Advantages of the closed-loop GOLIAH approach.

Tailoring intervention through careful assessment of the child

- Being computer based, the stimuli for assessment can be programed in an exact reproducible way
- The same type of stimulus could be mapped into different games giving the child the feeling that he/she plays different games. This is particularly important for assessing the child's difficulties since repetition of the same game may force them not to respond to his/her capability level out of boredom. This fact is also true during the intervention stage
- Different difficulty levels could be incorporated within the games to ascertain the child's performance even for a specific type of stimulus
- The whole process could be run automatically without incurring extra load on the therapist at the assessment phase
- A set of quantitative measures could be extracted in an automated way assessing the child objectively

Nomadic intervention

- The process could be deployed in nomadic environments where the child may play the game either with his/her parents or remotely with the therapist through internet connections
- Parents will need minimal training
- Automated measurements could give an objective idea about how the child's performance changes over time in stimulus-specific way
- The therapist can adjust the intervention remotely and dynamically by adding/removing games from the pre-stored library
- It also opens up the possibility of a batch-mode intervention where the therapist may deliver intervention to multiple children located at various locations in one session

Two children involved in imitation are temporally synchronized; they respond to the perception of movements or actions to produce a similar behavior. Compared to imitation, JA introduces a third partner during interaction. Emery defined JA as a triadic interaction that showed that both agents focus on a single object (31). Some authors (32) have argued that JA implies viewing the behavior of other agents as intentionally driven. In that sense, JA is much more than gaze following or simultaneous looking (33).

Lack of Imitation and JA are the main problems when interacting with children with ASD. While playing a game or conducting other activities with a social partner, these children tend to not concentrate on what others are actually doing, switching to repetitive and stereotypical behaviors that are of interest for the child but that usually have no or few relations with the actual social context. Imitation is possible but the communicative value of early imitation seems poorly understood (30). Also, children with ASD can display

concerted attention to toys or objects that they like, but they have difficulties in sharing attention or interests with others (34). For example, maintaining eye contact with the caregiver is especially complicated (35, 36) and the lack of JA is the consequence (37, 38).

Owing to the importance of Imitation and JA as core difficulties in ASD, we mapped a subset of related stimuli from the Early Start Denver Model (ESDM) protocol into the gaming platform containing a set of games with varying levels of difficulties that could be dynamically adjusted by the therapists. This program aims to meet the socio-emotional needs of children and their families, to identify and use validated and effective intervention techniques that are based on developmental needs (39). The ESDM recently received strong evidence of its efficacy at the level of clinical outcome (40) and brain plasticity (2).

Motivated by these facts, the purpose of the work is to design a novel computerized gaming platform that would allow: (1) delivering intensive intervention in nomadic environments for Imitation and JA tasks in children with autism, (2) tailoring and adapting intervention through child-specific assessment of difficulties, (3) enhancing effective intervention hours, and (4) without increasing the cost of delivery. The major point to note here is that GOLIAH is not intended to replace one of the state-of-the-art interventions for ASD but to supplement and expand it for achieving its maximal benefit.

TABLE 2 | Socio-demographic and clinical characteristics of the participants.

	ASD (*N* = 10)
Age, mean (±SD)	6.8 (±1.4)
Male – female	10 – 0
ADI-R, current, mean (±SD)	
Social impairment score	14.14 (±4.58)
Communication score	10 (±5.82)
Repetitive interest score	4 (±2.91)
Cognitive Level (WISC3/WPPSI)	
VIQ	103.1 (±14)
PIQ	96.1 (±24.8)
Vineland: mean (±SD)	
Communication score	88.2 (±16.7)
Daily living score	84.3 (±13.4)
Socialization	79.5 (±10.3)

ASD: Autistic Spectrum Disorder; ADI-R: Autism Diagnostic Interview-Revised; WISC 3: Wechsler Intelligence Scale for Children 3; WPPSI: Wechsler Preschool and Primary Scale of Intelligence; VIQ: Verbal Intelligent Quotient; PIQ: Performance Intelligent Quotient.

METHODS

Participants

We tested the software in a 3-month open trial with 10 children with ASD (all boys, aged 5–9 years) to assess the performance of the software itself. All children were recruited in the Department of Child and Adolescent Psychiatry, Hôpital Pitié-Salpêtrière, Paris and in the Department of Child Neuropsychiatry, IRCCS Stella Maris Foundation, Calambrone, Pisa. The study was approved by the local ethics committees of each institution (*Comité de Protection des Personnes* Ile De France VI under agreement number CCP 21-14, and Comitato Etico of the Stella Maris under agreement number 05/2011) and was in accordance with the declaration of Helsinki. Each parent gave informed written consent before inclusion for participation and for publication of the individual clinical data. Clinical characteristics of the children are given in **Table 2**.

Procedures

The intervention protocol used with children included six sessions per week (from Monday to Friday) of training with GOLIAH; five sessions per week were at home with the parents (mother or father) playing with their children in the afternoon; and one session per week was planned at the hospital. The duration of each session, both at home and at hospital, was equal to 20 min. The sessions at home and at hospital were the same in terms of tasks. The only differences were the different setting (i.e., home or hospital) and the partner (therapist or parent). Each child's plan was tailored on the basis of functional profile and adapted during the 3-months protocol according to children progress in playing the games. This open-trial aimed at assessing (1) the usefulness of the gaming platform with children–therapist interactions as well as with children–parents, (2) whether tailored intervention was useful when used at home and with non-professional therapist/parents, and (3) whether children performed as expected when using the different Imitation and JA games. To do so, we used both objective data computed from the platform and clinical annotations produced by therapists during weekly sessions at hospital. (4) Finally, subjective views from users were also explored through a questionnaire.

At the beginning of the study, a 3-month open trial was planned with 60 sessions (four sessions at home per week + one session at the hospital per week = five sessions per week × 12 weeks = 60 sessions). To assess in detail the usability of the gaming platform, we planned a systematic recording of the number of times each game was played in each session by each of the 10 children included in the 3-month study period. Details are shown individually in **Table 3**.

Instruments

Software Design

The game software has been developed in Microsoft Visual Studio 10 Platform in C# language. The platform has as many classes as the number of included mini-games; thus, creation of new games will not alter the existing ones. Real-time communication between two devices is performed through a multi-threading process that includes: (1) game flow thread in which all the game tasks are performed (including sending objects to the other user) and (2) receiving thread in which the objects sent by the other user are received and fire the semaphore in the game flow thread. The two players are connected to a server, developed in C#, which acts as a bridge between them. In fact, the objects exchange occurs through a Socket connection based on a TCP/IP protocol that ensures that the information exchange will not be lost during the transmission.

Choice of Stimuli

The ESDM is a comprehensive behavioral early intervention protocol for children with autism. It uses a combination of developmental and behavioral techniques in both therapist and parent-implemented early intervention models (41, 42). It is an intervention for infants with ASD aged 12–48 months that combines applied behavior analysis (ABA) with developmental

TABLE 3 | Number of sessions per game and per child during the 3-month study period.

Child	1	2	3	4	5	6	7	8	9	10	N of sessions per game for all children: mean (range)
Imitation games											
Imitate free drawing	11	4	4	6	3	19	16	19	15	16	11 (3–19)
Imitate step by step draw	17	13	24	10	5	20	11	18	13	9	14 (5–24)
Imitate speech	17	13	15	9	11	15	11	19	12	6	13 (6–19)
Imitate sounds	2	19	10	13	11	10	17	9	11	8	11 (2–19)
Imitate actions	15	23	7	6	10	14	11	14	4	16	12 (4–23)
Imitate actions and build	12	11	19	13	12	12	14	11	12	13	13 (11–19)
Guess the instrument	4	3	11	10	9	2	1	7	6	5	6 (1–11)
Joint attention games											
Follow the therapist's pointing	15	19	20	17	12	14	13	16	21	12	16 (12–21)
Cooperative drawing	2	19	15	11	13	9	11	11	18	18	13 (2–19)
Bake a cake	11	14	16	15	12	18	9	12	19	7	13 (7–19)
Receptive communication	21	25	31	20	17	16	15	25	9	12	19 (9–31)
No. of sessions per child for all games: mean (range)	12 (2–21)	15 (3–25)	16 (4–31)	12 (6–20)	10 (3–17)	14 (2–20)	12 (1–17)	15 (7–25)	13 (4–21)	11 (5–18)	

and relationship-based approaches. The intervention is provided by trained therapists (Antonio Narzisi is a certified therapist from MIND Institute, University of California Davis, Davis, CA, USA) and parents.

Each child's treatment program includes models based on development, functional profile, relational patterns, and modification of behaviors. The curriculum includes, among others, systematic activities on receptive and expressive communication, as well as social, play, cognitive, self-care, and fine and gross motor skills. Particular attention is devoted to specific tasks regarding Imitation and JA. ESDM considers JA as an activity in which two subjects are engaged with each other in the same cooperative activity, attending to the same objects, or playing or working together on a common activity. A JA routine is made up of several phases: (1) the opening or set-up phase that involves the acts that precede the establishment of the first shared play activity based on the theme of the play. (2) The child and adult are engaged in a definable play activity, either object centered, such as building blocks, pouring water, marking with crayons, or involving a social game, such as singing a song, dancing to music, or playing hide and seek. (3) The elaboration phase involves variation on the theme to keep it interesting or to highlight different aspects of the activity. This preserves the play from becoming repetitious and allows more skill areas to be addressed. (4) The closing is the fourth and final phase when attention is waning or the teaching value of the activity is all used up. It is a time to put materials away and to transit to something else. Closing allows nice transitions in changing activity, location, and time.

Regarding imitation, in the ESDM different tasks may be proposed to the children: (a) imitation of actions on objects, (b) imitation of gestures, and (c) vocal imitation of sounds and words. During intervention sessions, children are asked to imitate conventional or unconventional actions with and/or without objects using or not the vocalizations.

Mapping ESDM Stimuli for Imitation and JA into a Computerized Gaming Platform

The Imitation and JA stimuli are mapped into 11 games: seven Imitation and four JA games. Although currently the proposed platform consists of 11 games, it is flexible enough for developing/adding new games according to the need. A list of the games and the ESDM stimuli they address is depicted in **Table 4**. In developing the games, special attention has been devoted to their realistic resemblance to the real-life scenario, more importantly emulating human–human interactions during the game playing phase. Each of the games incorporates different levels of difficulty ranging from the application of one stimulus (e.g., the sound of a train), to a combination of different stimuli (e.g., the sound and the image of a train).

The seven Imitation-based games comprise of tasks involving the imitation of drawing, speech, sounds, and building actions. For instance, the one related to the sound imitation (Imitation game 4) requires the child to repeat the sound played on the device, either a tablet or a computer. Whereas in the building action game (Imitation game 6), the child would build an object, starting from simple cubes, in a similar way to a normal session with Lego toys. The other four games are based on JA stimuli, including the identification of objects (such as fruits, home furniture, and vehicles), described or pointed to by the therapist/parent.

The Gaming Platform

The multi-player gaming platform developed here requires two computers or tablets with an active internet connection. One computer/tablet is operated by the therapist or parent (depending upon the application scenario) acting as the *therapist/parent* and the other by the child designated as the *player*. Currently, the platform is available in three different languages (Italian, English, and French) for providing instructions to the child and the therapist/parent.

The choice of the language, the game to play as well as the goal setting is made by the therapist/parent. As instance, when playing the musical instrument game, the *therapist/parent* can select between two different goal settings: listen and recognize a sequence of (a) three or (b) six musical instruments. The role of the player is to achieve the goal set by the therapist/parent at the end of the game. In the game described above, the child will listen to a sequence of instruments and, depending on the goal selected, he will listen and recognize the sequence of three or six instruments.

TABLE 4 | Mapping of ESDM stimuli for JA and imitation into the games.

Game type	Description	ESDM stimuli
Imitation game 1: imitate free drawing	Imitation of the drawing done by the online therapist/parent	(lev.4) FM 4
Imitation game 2: imitate step by step drawing	Imitation of a drawing created step by step from the online therapist/parent (three difficulties)	(lev.4) FM 4
Imitation game 3: imitate speech	Imitation of words or phrases from the library (three difficulties)	(lev.2) IM 3, 9
Imitation game 4: imitate sounds	Imitation of sounds chosen from the library (four difficulties and two categories of stimuli)	(lev.2) IM 2
Imitation game 5: imitate actions	Imitation of the actions with balls made by the online therapist/parent (three difficulties and two types of task)	(lev.2) IM 6
Imitation game 6: imitate actions and build	Imitation of the actions with cubes made by the online therapist/parent (three difficulties and two types of task)	(lev.3) FM 3
Imitation game 7: guess the instrument	Identification of the musical instruments played and chosen by the therapist/parent from the library (two difficulties)	(lev. 1, 2) IM
Joint attention game 1: follow the therapist's pointing (both audio and visual)	Identification of the object indicated (verbally, visually or pointed) by the therapist on the video and chosen from the library (six difficulties and eight categories of stimuli)	(lev.1) RC 1, 4 (lev.2) JA 2, 4, 6
Joint attention game 2: cooperative drawing – connect dots	The therapist and the child cooperate to complete a figure shown on the right, by clicking on the corners of the figure itself (two difficulties and four categories of stimuli)	JA
Joint attention game 3: bake a cake	The child cooks a recipe by clicking and dragging into a bowl the ingredients chosen by the therapist/parent from the library of recipes (11 categories of stimuli)	JA
Joint attention game 4: receptive communication	The child identifies the objects described by the therapist/parent and chosen from the library (three difficulties and five categories of stimuli)	(lev.2) RC 5, (lev.1) RC 6, (lev.1) RC 4

FM, fine motor subset; IM, imitation subset; RC, receptive communication subset; JA, Joint Attention subset.

The games can also be categorized in (a) stand-alone operation game and (b) game requiring active co-operation between the therapist and the child. (a) The stand-alone operation games contain pre-developed libraries containing the stimuli and the instruction to achieve the goal. The imitation game 4 – Imitate Sound is an example of stand-alone game; the therapist/parent selects a list of animal's sounds to imitate: the player will listen to each sound and imitate it. (b) In the second category of games, the therapist/parent has an active role: he/she needs to cooperate with the child to achieve the goal of the game and can also create new stimuli. An example of this category is the JA game 2 – Cooperative drawing-connect dots: both therapist/parent and the child have to cooperate to connect the dots and create the final figure. Details and figures of these games can be found in the supplementary material.

All the games have different levels of difficulty allowing the therapist/parent to adjust the initial level of difficulty according to the cognitive skills identified by the therapist at the beginning of the treatment process or dynamically adjusting it as the player's performance progresses with time.

The performance of the player could be assessed mainly in two different ways: through an (a) automated evaluation based on a predefined scoring convention and through a (b) manual evaluation by the therapist/parent. (a) The automated evaluation does not require any action to the therapist/parent: the game will automatically assign a score to the performance of the child. For example, the game will assign a positive score if the child has selected the right musical instruments. (b) The manual evaluation requires to the therapist/parent to select among three different buttons: score 0 if the player did not achieve the goal, 1 for partial achievement, and 2 for successfully satisfying the goal. As instance, at the end of the imitation game 4 – Imitate Sound, the therapist/parent has to click among three buttons indicating score 0, 1, or 2. Without loss of generality, a more complicated scoring system could be programed easily according to the need of granularity to assess the achievement of the player.

Apart from the simple scores describing whether the player has achieved the goal, a set of objective metrics and an array of possible events are also extracted by the platform in an automated way. A list of such objective measurements is given in **Table 5** along with their definitions.

This set of objective metrics allows the therapist to analyze quantitatively the performance of the player in a stimulus-specific way not only at a particular time point but also the progression of the child's performance over a time window (hours, days, months, etc.) giving a holistic picture of the child's development. For example, the therapist might want to analyze if the child recognize a particular musical instruments and if this recognition becomes quicker throughout the sessions. In addition, the objective metrics allow the therapist to ascertain the appropriateness of scoring and adherence to the prescribed protocol by the parents. Such analysis could be done both online and offline by the therapist as the metrics are stored each time the player plays the game. For example, in the imitation game 1 – Free drawing, both the therapist/parent and the player's drawing are saved as well as the scores given by the parent. The therapist could then check if the parent's scores adhere to the scoring guidelines suggested by the therapist.

The gaming platform provides a flexible means for giving a reward to the player on successful completion of the goal capturing the essence of reward-based intervention. In the current version, a smiley face is shown at the end of each game in the player's device, regardless of the score obtained as a positive reinforcement that also gives an impression of feedback to the player. Such feedback is once again programmable and an appropriate reward could be set by the therapist depending on the player's motivation factors (such as playing music that the child likes, etc.).

Descriptions of the Games

At the start of the game, the main window, shown in **Figure 2** will appear on the therapist/parent's device. He/she will first

TABLE 5 | The objective metrics extracted by the gaming platform.

Measurement type	Measured metrics	Description
Automated	Name of stimulus	Type of the stimulus embedded within the game and the name of the object the player has to click or drag or draw
	Time of the stimulus	Defined by the difference $\Delta T_s = T_{ss} - T_{es}$ between a start time T_{ss} variable (the time instant the stimulus starts to be shown or played on the child's device) and an end time T_{es} variable (the time instant the stimulus is finished)
	Time of response	Defined by the difference $\Delta T_r = T_{sr} - T_{er}$ between a start time T_{sr} variable (the time instant the child starts to respond) and an end time T_{er} variable (the time instant the child complete his/her response)
	Type of response	Defined by the correctness of the child's response depending on whether the child performs action as intended by the therapist/parent (only Correct or Incorrect)
	Score of response	Assigned score to the response of the child, either 1 or 0 signifying whether the intended response has been achieved or not respectively – a more complicated scoring system could be programed
	Image of the stimulus and the response	A screenshot of the child's device obtained during imitation drawing and the action games – assisting the therapist to analyze the response further offline to ascertain the quality of response.
	Sound recording	The audio response of the player recorded during the sound and speech imitation games – allowing the therapist to check the quality of response
Manual	Therapist/parent evaluation	Defined as Complete/Partially complete/Incomplete response of the child according to the therapist/parent judgment
	Manual score	Assigned to 0/1/2 corresponding to the therapist/parent evaluation of the child's action – a more complicated scoring system could be programed

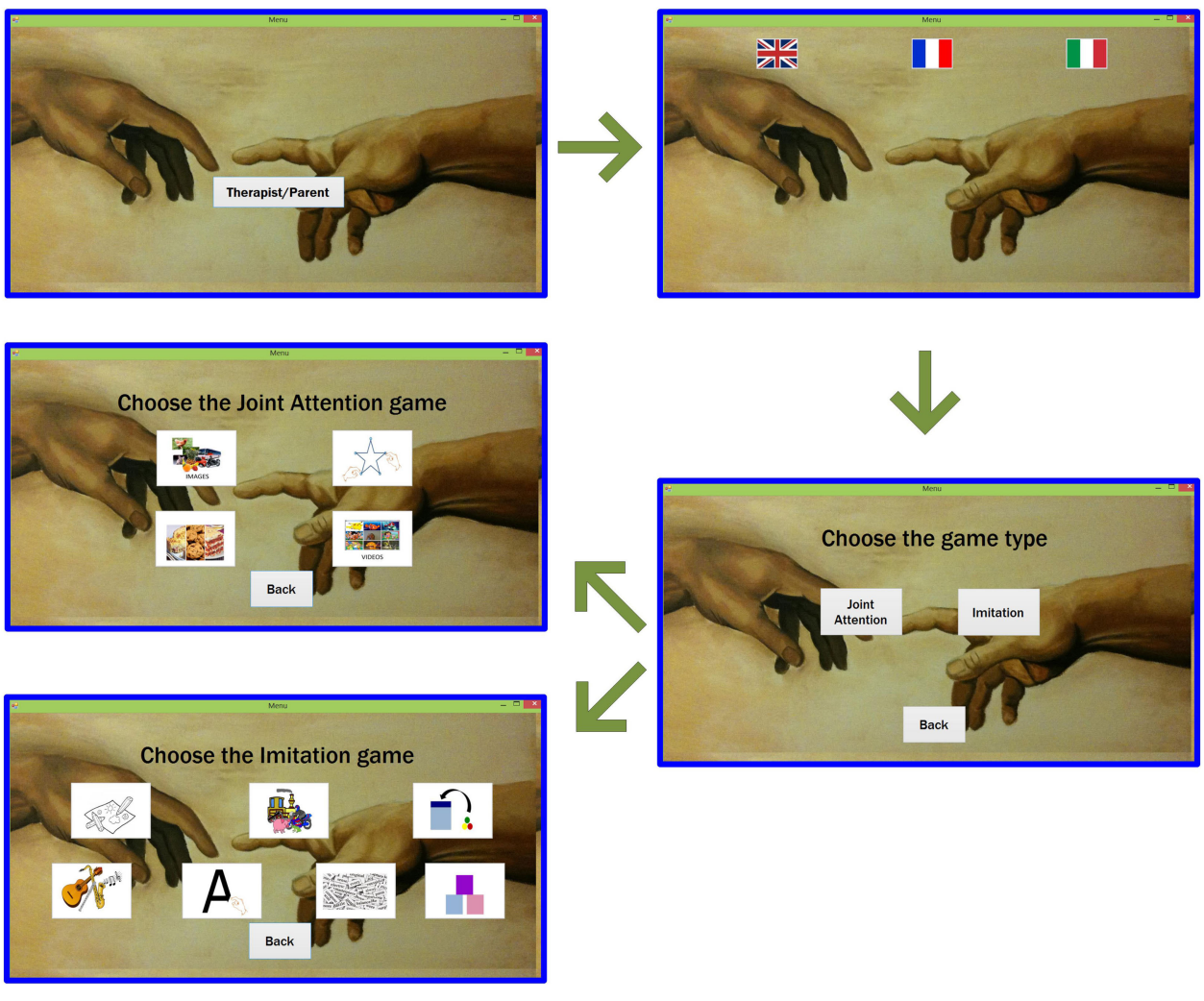

FIGURE 2 | Main windows of the therapist/parent during the beginning of the game. The therapist/parent (blue windows) will select the language, the category of the game (whether Joint Attention or Imitation), and the game, according to the category chosen.

choose the language in which the stimuli and instructions will be played. Thereafter, the therapist/parent selects the desired game that will automatically be launched on both devices.

Here, we report only the description of two games (Free drawing and Bake a recipe) and we use it to illustrate the children's performances through sessions of both Imitation and JA (a detailed description of all other games is reported on Supplementary Materials GamesDescription.doc).

Joint Attention Game 3 – Bake a Recipe

This game is targeted to cook a recipe by mixing six ingredients in a bowl, as shown in **Figure 3**. The therapist/parent selects the recipe to cook among 11 dishes from a standardized library, which includes pizza, tiramisu', lasagne, omelet, roasted chicken, pasta, etc. For each of the six ingredients, as soon as the therapist/parent clicks on it, an arrow connecting this ingredient to the bowl appears on the player's device, as shown in **Figure 3**. The player needs to drag the ingredients into the bowl. When all the ingredients have been dragged into the bowl, the player has to click on the Mix button and, finally, he/she has to choose the recipe they cooked among seven dishes.

As before, an event with positive or negative score is generated each time the player clicks on an ingredient and drags it into the bowl, as well as when the correct recipe is recognized.

Imitation Game 1 – Free Drawing

This imitation game is intended for examining the player's ability to imitate several objects drawn by the therapist/parent, starting from very basic drawings, such as scribbles and dots, to very complicated, such as letters and numbers. The whole process of this game is shown in **Figure 4**, where the blue window indicates the therapist/parent's window and the red window indicates the player's window. Once launched, a window will appear on both therapist/parent and player's device with clearly marked separate drawing panels. The therapist/parent can draw any object of any shape in the panel dedicated to him/her (on the right). Once completed, the therapist/parent's drawing appears on the player's device and the player needs to imitate that drawing in his/her dedicated panel (on the left). The live outline of the player's drawing will appear on the therapist/parent's device. Depending on whether the drawing is correct or not, the therapist/parent can decide to finish the game (by clicking on the tick button) or encourage the player to have another try (by clicking on the cross button). The quality of the imitation will be evaluated by the therapist/parent among three possibilities: correct, incorrect, or partially correct. To avoid discrepancies and to create normalization, the therapists involved in this study have reached an agreement, according to the ESDM, on how to evaluate the drawings and sounds imitation and train the parents to adhere to it.

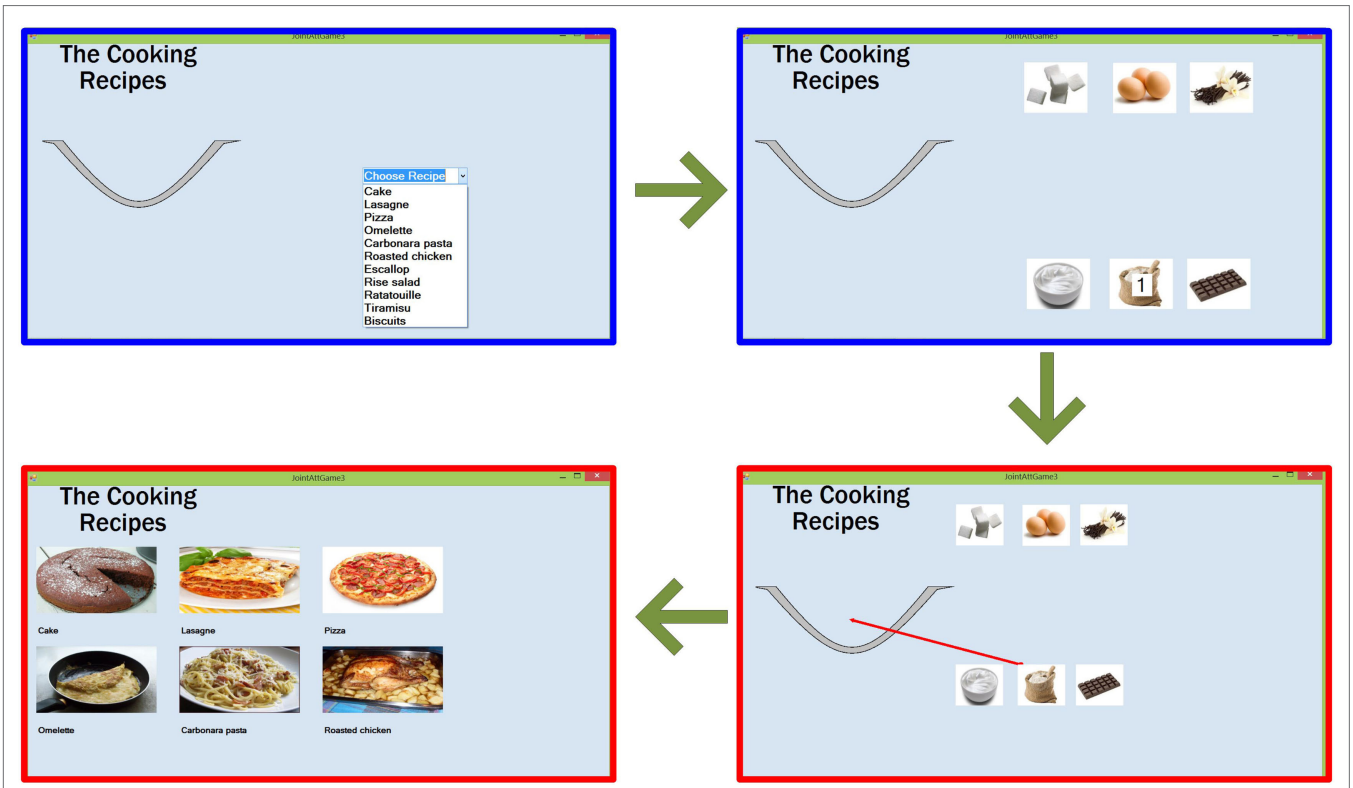

FIGURE 3 | Flow of the Joint Attention game 3 – Bake a recipe. The therapist/parent (blue windows), after selecting the recipe, will select each ingredient to be dragged into the bowl. The red arrow on the player's device (red window) will indicate the ingredient selected by the therapist/parent. After dragging all the ingredients, the player's will click on the recipe cooked.

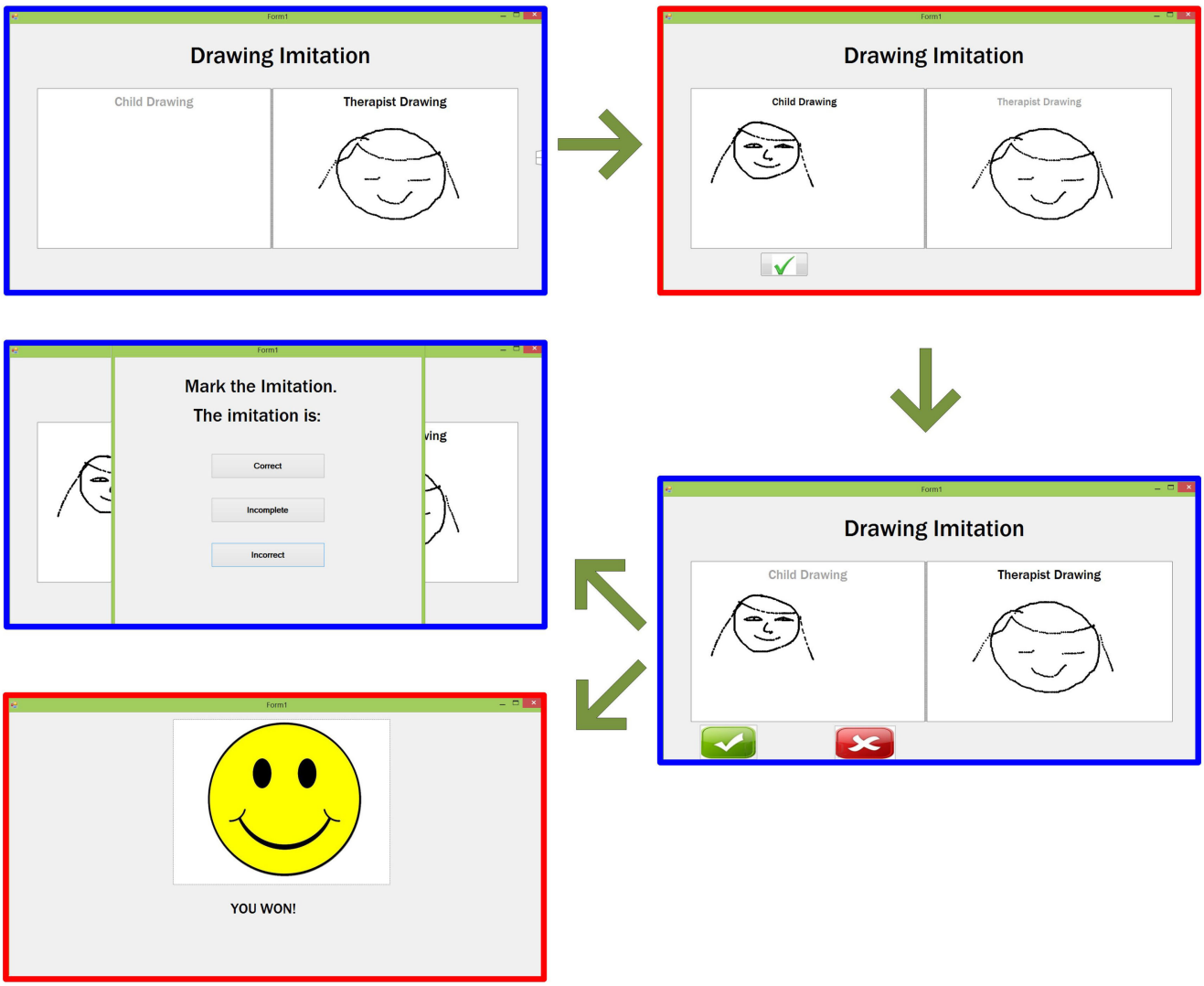

FIGURE 4 | Flow of the Imitation game 1 – Free drawing. The therapist/parent's drawing (blue window) appears on the player's window (red window) who will then imitate the drawing and send it to the therapist/parent. After the therapist/parent's feedback, the smiley will appear on the player's device, while the therapist/parent will evaluate the imitation as Correct/Incomplete/Incorrect.

RESULTS AND DISCUSSION

Validation by Testing with Children

Overall, during the study period, the children and the parents participated in 77.5% of the planned sessions at home and in 90% of the hospital sessions. All children went through all games (seven Imitation games and four JA games). Given the diversity of the games and the heterogeneity of children profile and abilities, for a given game the number of sessions dedicated to the game varied. Also given the levels of difficulty within a game, all the conditions of the games have not been exploited by the children at the end of the 3 months. All games were well tolerated and followed both by children and parents showing the robustness of the gaming platform and the feasibility of the course of the games. One family initially had troubles in using the two tablets system related to Wi-Fi connecting problems that could be easily corrected. Tailoring treatment during the hospital session and data transfer from home was easily achieved.

Children's Performance through Sessions and Games

We selected two games to illustrate the children's performances through sessions of both Imitation and JA by using either quantitative or qualitative scoring. Our goal here was to verify how meaningful the extracted scores were from each game session to follow the child's progress or difficulties.

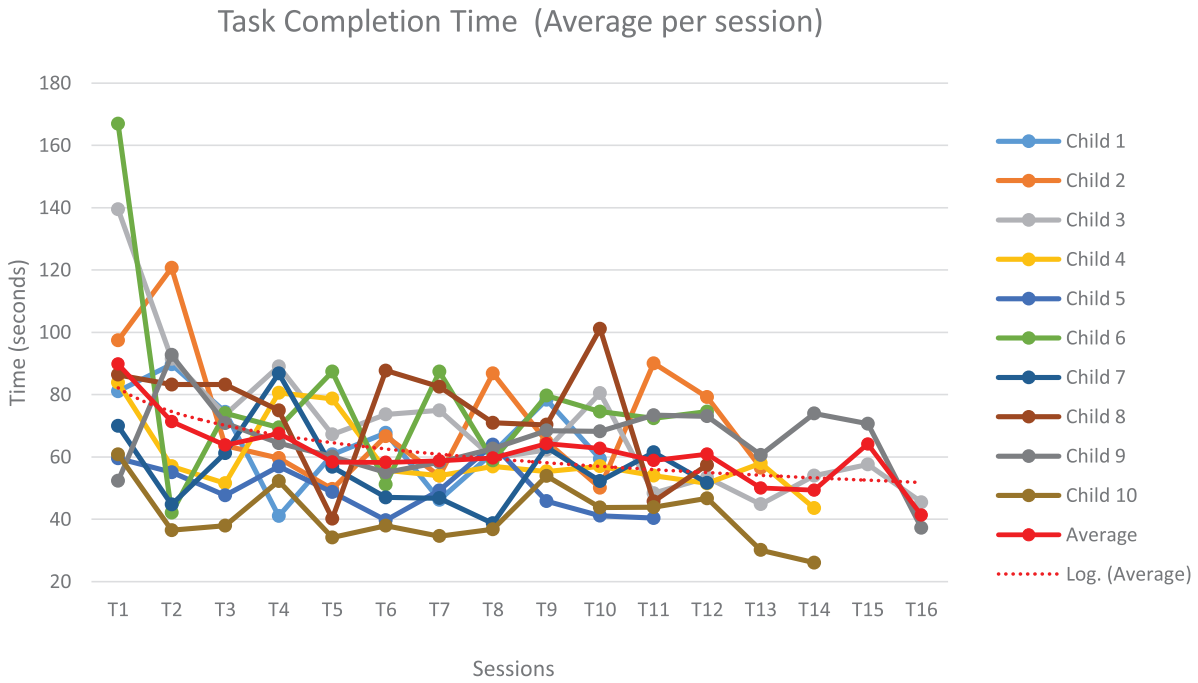

FIGURE 5 | Evolution of the time (in seconds) to complete the task for the Joint Attention game 3 – Bake a recipe. Evolution of the time occurred to complete the Joint Attention game 3 for each child (each color represents a child) across different sessions. The average across children, in dotted red, shows a decreasing trend across sessions.

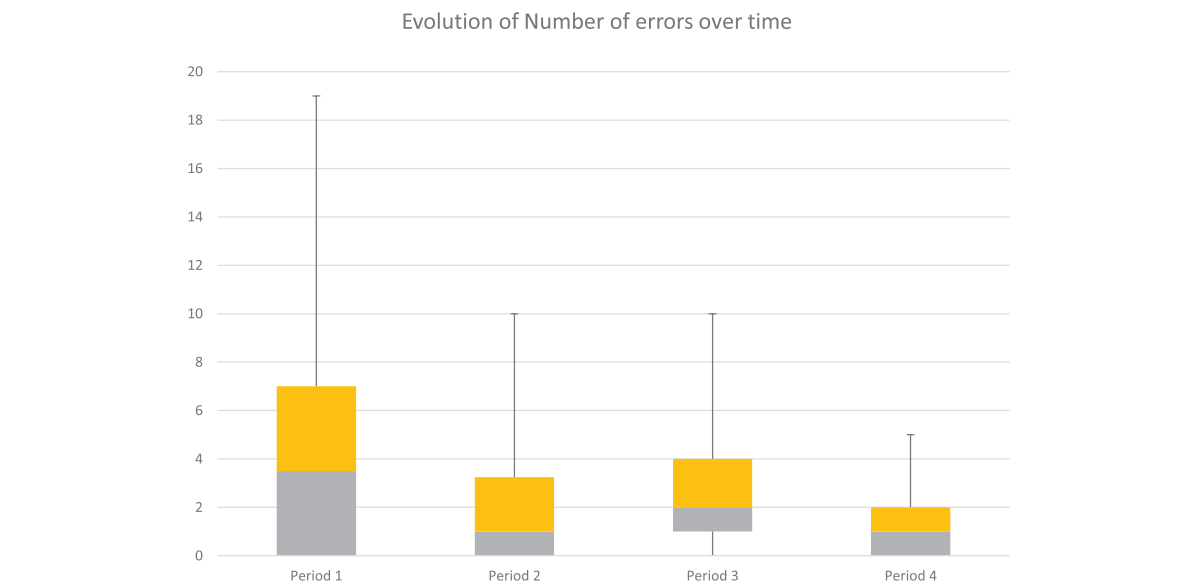

FIGURE 6 | Number of errors performed to complete the task for the Joint Attention game 3 during different periods. The figure contains the number of mistakes committed by the 10 children during the Joint Attention game 3. The total number of errors decreases across the different periods, as shown by the variability (from 19 to 5).

Bake a Recipe (Joint Attention Game 3 – Quantitative Scoring)

Figures 5 and **6** show children's performances for the JA game 3 – Bake a recipe. **Figure 5** represents the evolution of the time (in seconds) to complete the task for the JA game 3. For one session (T_i, T_{i+1} …), completion time is averaged, as the children practice the game several times during one session. As sessions progressed over time, children become faster to achieve the task. Each line corresponds to the evolution of the task completion time across different sessions for a given child.

The red dot curve represents the evolution of task completion time averaged for all children ($N = 10$): a common overall decrease was observed in all subjects. To assess whether the task completion time significantly decreased over the sessions, we used a linear mixed model with the Log (time to complete the task) to be explained by the number of sessions as a continuous variable. The Log function was required to have a normal distribution. We found that the time to complete the task significantly decreased along sessions ($\beta = -0.021$, t-value $= -5.53$, $p < 0.001$).

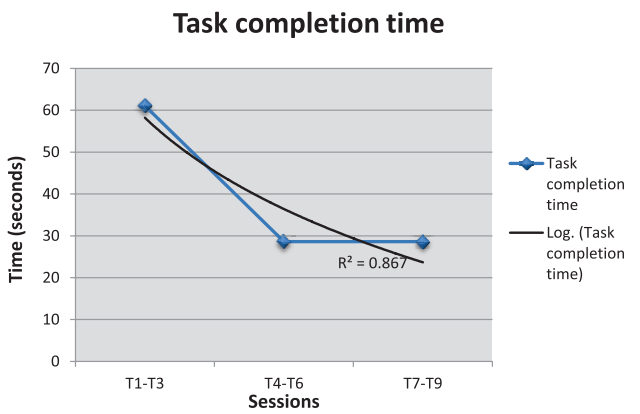

FIGURE 7 | Evolution of the time (in seconds) to complete the drawing in Imitation game 1. Evolution of the time occurred to complete the Imitation game 1 for one child across different sessions. Figure shows that the child becomes faster at reproducing the drawing model.

In parallel, the number of errors decreased also over time (**Figure 6**). For this game, the mistakes that have been taken into account are as follows: wrong and fake answers during the first "mixing ingredients" phase of the game (when the child selects the wrong ingredient or when he presses one or several wrong ingredients after selecting the correct one) and wrong answers during the "choose recipe" phase of the game (when he/she has to guess the cooked recipe). For reasons of readability of the boxplot type graph (**Figure 6**), the sessions have been grouped into four periods (period 1 = T_1, T_2, T_3, T_4; period 2 = T_5, T_6, T_7, T_8; period 3 = T_9, T_{10}, T_{11}, T_{12}; and period 4 = T_{13}, T_{14}, T_{15}, T_{16}).

According to our data, the children who had already good performances at the beginning (Period 1), kept their performances constant all along. But there is an important decrease of the number of errors per child across the four periods, particularly for the children who committed several mistakes initially. At the end (Period 4), the number of mistakes is very low for all children. To assess whether the number of errors significantly decreased over the number of sessions, we used a linear mixed model with a binomial variable (the probability of correct answers) to be explained by the number of sessions as a continuous variable. We found that the probability of correct answers significantly increased with the number of sessions ($\beta = 0.039$, z-value $= 2.78$, $p = 0.005$). In sum, for this game, the results after 3-month training are promising.

Free Drawing (Imitation Game 1 – Qualitative Scoring)

For the second game (Imitation game 1 – Free drawing), the evolution of performances is illustrated from the results of one

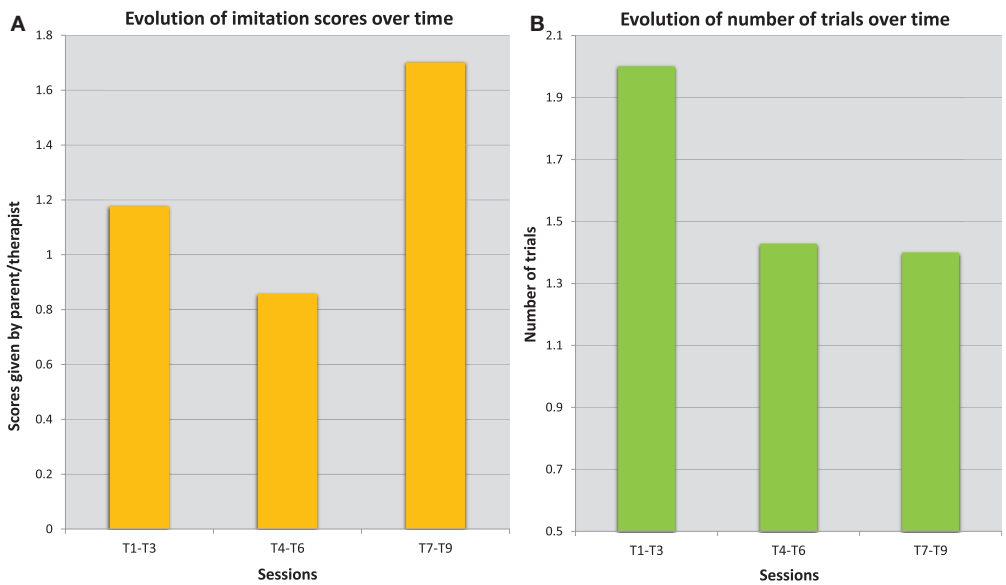

FIGURE 8 | Evolution of the performances of one child during the Imitation game 1. The error bars **(A)** describes the variations of the scores given by the therapist at hospital for different sessions. The quality of imitation improved throughout the sessions: the average score (av = 1.7) during the third period (T7–T9) is closer to the maximum score (score 2) and higher than the initial scores for the periods T1–T3 (av = 1.2), and T4–T6 (av = 0.9). The average number of trials required to complete the imitation, shown on the right **(B)**, has decreased as well across different sessions from the first period (T1–T3 with av = 2) to the next periods (T4–T6 and T7-T9 with av = 1.4).

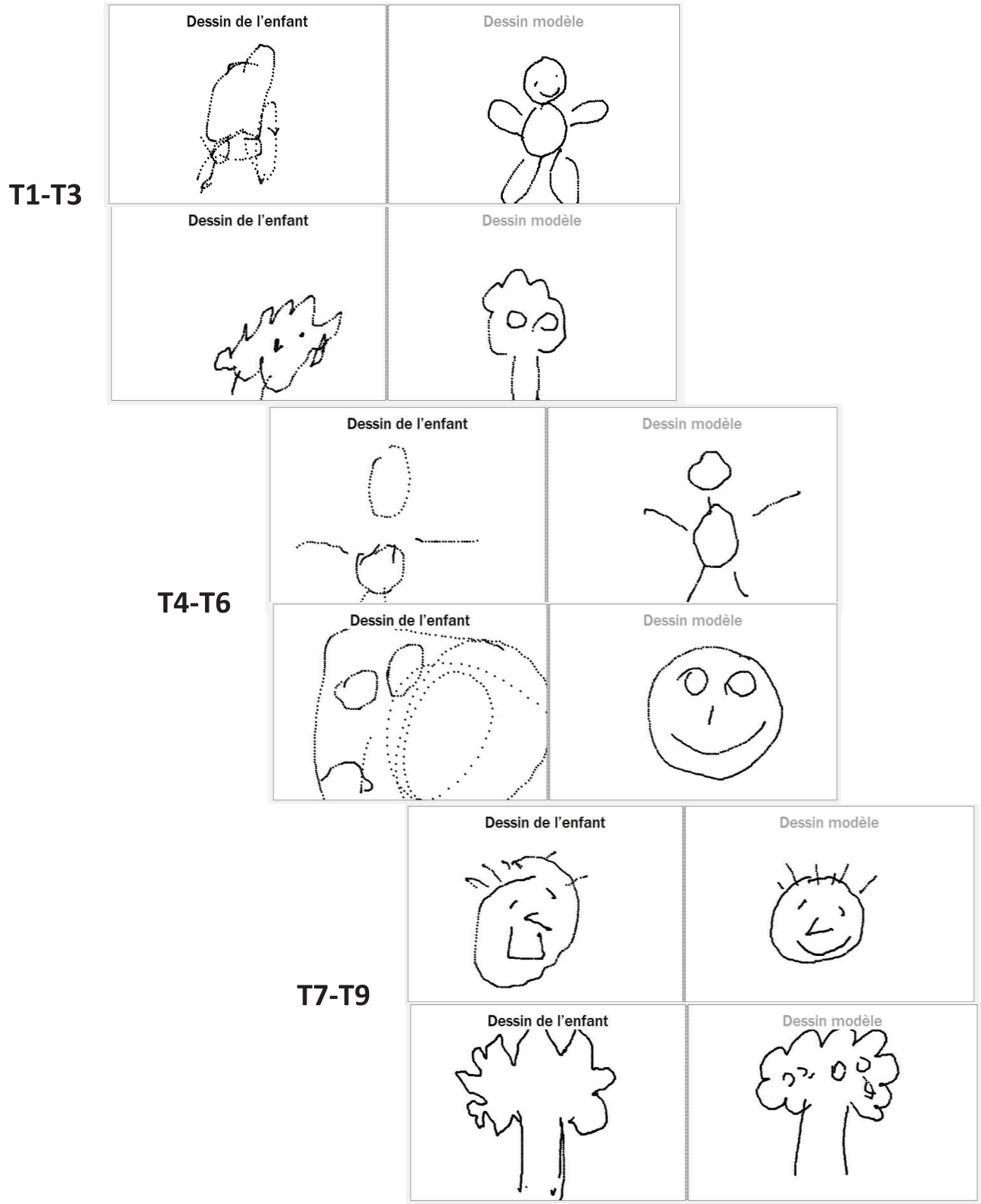

FIGURE 9 | Evolution of the imitation skills of a child across three periods. Example of the evolution of the imitation skill for one of the children across different periods.

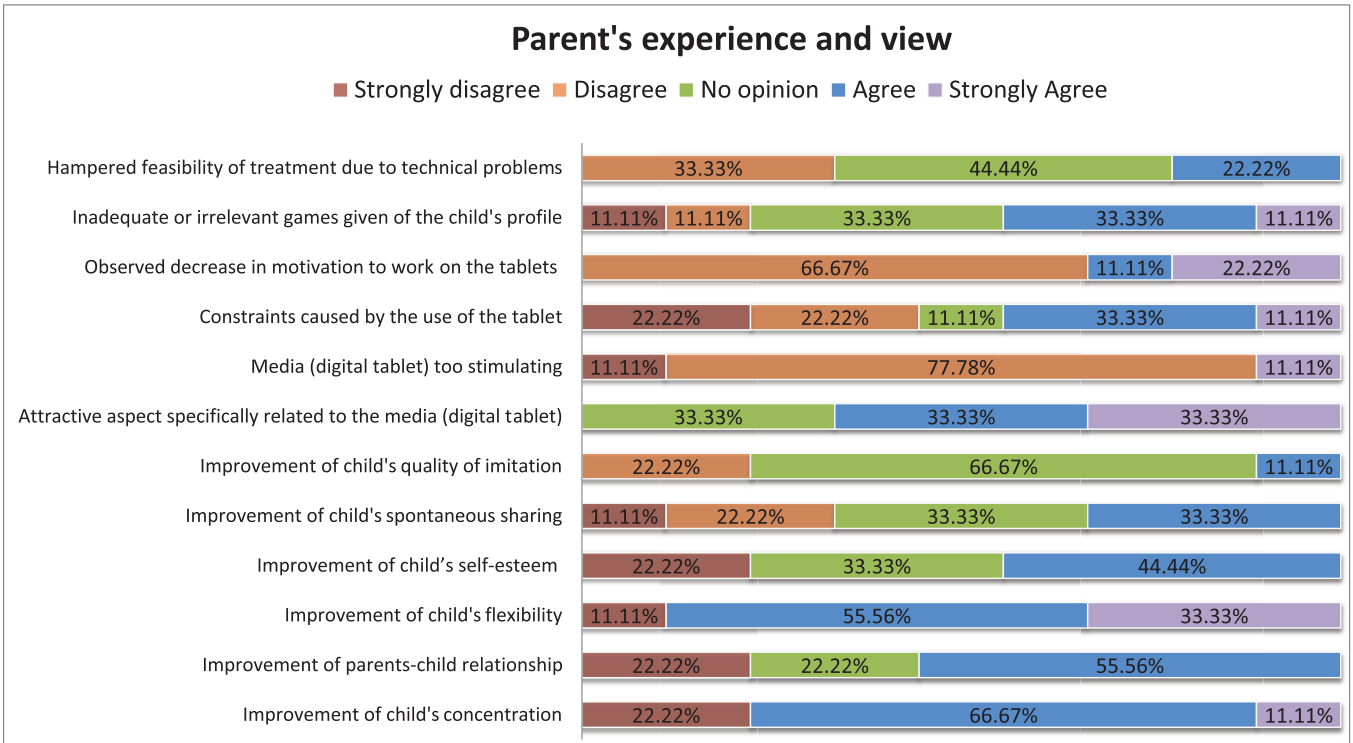

FIGURE 10 | Results related to the questionnaire proposed to the parents. Answers given by the parents of the children recruited for the study to the questionnaire containing the questions related to the use of the GOLIAH platform.

child, since the results are mainly qualitative and it is difficult to compare the drawing performances of one child with another (complexity of pictures, differences in drawing time, differences in fine motor skills, etc.).

Figure 7 shows that the child becomes faster at reproducing the drawing model ($R^2 = 0.867$). In addition, the quality of imitation improved throughout the sessions as shown by the evolution of the imitation scores (given by the therapist/parent) in **Figure 8**. The quality of the imitation is evaluated by the therapist/parent among three possibilities: correct (score 2), partially correct (score 1), and incorrect (score 0). **Figure 8A** shows that the average score (av = 1.7) during the third period (T_7–T_9) is closer to the maximum score (score 2) and different from the initial scores for the periods T_1–T_3 (av = 1.2) and T_4–T_6 (av = 0.9). Furthermore, as shown in **Figure 8B**, the child needed fewer trials to reproduce the therapist/parent's drawing. As an illustration, **Figure 9** represents the evolution of child's imitation skills in drawing across the three periods.

Parents Experience and View

At the end of the 3-month open trial, a web questionnaire was sent to the parents of children who participated in the open-trial (10 parents). The questionnaire contained 12 questions with a positive or negative orientation toward the serious game (see details at https://goo.gl/foMpPI). The questions asked about the use of the game (ease of use for parents, chosen media, technical problems, etc.) and the improvement in the child's skills (concentration, attention, imitation, self-esteem, etc.). The parents had to answer through a Likert scale from 1 to 5 (1 = strongly disagree, 2 = disagree, 3 = no opinion, 4 = agree, 5 = strongly agree). Results are summarized in **Figure 10** and show that parents have positively assessed the use of the serious game as a treatment. Sixty-seven percent of interrogated families did not observe a decrease in the child's motivation to work on tablets; 44% of them were not particularly disturbed by the constraints on daily activities caused by the use of the serious game on tablets, and 33% judged that the feasibility of treatment was not seriously hampered due to technical problems. The media (digital tablet) was not considered as too stimulating by 89% of the families and more than 67% of them thought that there was a specifically attractive aspect related to the media itself. Only one negative point was noted: 44% of the parents found that the games were inadequate given their children's profile. At the beginning of our pilot study, we were aware of this possible limitation. However, since our focus was to assess the feasibility and usability of the game, older participants were preferred because they could be more willing to collaborate and test the game.

Concerning progress on the children's skills, it seems that there is not so much progress on Imitation since the majority of the parents (67%) had no specific opinion on this topic. On the contrary, JA (spontaneous sharing) seemed to be slightly ameliorated (33% agreement). Interestingly, some skills that were not directly trained by the games strongly evolved during the course of the 3-month open trial according to parents: child's self-esteem, child's concentration, and child's flexibility. Moreover, the quality of parents–child relationship was qualified as enhanced for 56% of the parents. We could hypothesize that the interactive nature of GOLIAH and its

pleasantness for the child had the effect of improving parent–child interaction also in other contexts, which is a generalization effect that often is lacking in treatments for autism.

CONCLUSION

In the current paper, we described a gaming platform for home-based intervention in ASD. Within the context of a pilot open trial, we showed the feasibility of the intervention. We found that (1) the gaming platform was useful during both children–therapist interaction at hospital as well as children–parents interaction at home, (2) tailored intervention was compatible with at home use and non-professional therapist/parents, (3) children performed as expected when using the different Imitation and JA games and no game appeared inaccurate, (4) data computed from the platform and clinical annotations produced by parents and therapists allowed session-to-session monitoring and helped therapists to dynamically reconfigure treatment, and (5) subjective views from users (mainly parents here) were overall positive. From the clinical point of view, the most important benefits of this novel method of intervention for children with autism are: (a) the rapid performance amelioration on tasks based on Imitation and JA that are considered pivotal for children with autism; (b) to create a scenario where the spontaneous, and usually lone, activity with video games is easily pushed to become a shared activity; (c) a general amelioration of attention and availability to discuss the results of a performance. Nevertheless, some limitations must be considered. First, the lack of more precise and external evaluation of improvements in Imitation and JA with specific methodology; second, a deeper analysis of the minority of parents who have signaled difficulties in applying GOLIAH is needed to individuate for which child and for which family it could be more indicated; third, in a future study, it will be important to study the gender differences than the current GOLIAH tasks and to evaluate the appropriateness of the GOLIAH tasks also with girls with ASD. Given the promising preliminary results, we are moving now within the context of FP7 MICHELANGELO project to further ascertain the efficacy of the gaming platform in the context of a bigger ($N = 30$) and longer (6 months) clinical trial, including a control group. Besides Imitation and JA, two cognitive skills directly targeted within the gaming platform, we plan to use external primary variables (i.e., Vineland scores and Social Communication Questionnaire) to assess generalization.

AUTHOR CONTRIBUTIONS

VB created the entire gaming platform with help from WJ and SH. KM and MW first conceptualized the gaming platform and KM coordinated the work. AN made critical evaluations of the stimuli to be selected from ESDM protocol, recruited and evaluated the children in Pisa, and ran the open trial; A-LJ adapted ESDM stimuli in serious game stimuli; JX recruited the children in Paris; ET recruited and evaluated children in Paris and ran the open trial; MC, DC, and FM provided supervision and reviewed the paper. The MICHELANGELO study group contributed to the overall project and study design, help in managing computational data and engineering issues.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpsyt.2016.00070

PRESENTATION 1 | This file contains the description of the other nine games of the gaming platform which have not been described in the paper.

DATA SHEET 1 | This file contains the data used for the analysis of the games Bake a recipe and Free Drawing presented in the Results and Discussion section.

Availability of Data and Materials

The data used to represent the results of the games Bake a recipe and Free Drawing can be found in the Supplementary material as Data Sheet 1.

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

APPENDIX A

MICHELANGELO Study Group

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Dynamic Akt/mTOR Signaling in Children with Autism Spectrum Disorder

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Autism spectrum disorder (ASD) is a behaviorally defined disorder affecting 1 in 68 children. Currently, there is no known cause for the majority of ASD cases nor are there physiological diagnostic tools or biomarkers to aid behavioral diagnosis. Whole-genome linkage studies, genome-wide association studies, copy number variation screening, and SNP analyses have identified several ASD candidate genes, but which vary greatly among individuals and family clusters, suggesting that a variety of genetic mutations may result in a common pathology or alter a common mechanistic pathway. The Akt/mammalian target of rapamycin (mTOR) pathway is involved in many cellular processes including synaptic plasticity and immune function that can alter neurodevelopment. In this study, we examined the activity of the Akt/mTOR pathway in cells isolated from children with ASD and typically developing controls. We observed higher activity of mTOR, extracellular receptor kinase, and p70S6 kinase and lower activity of glycogen synthase kinase 3 (GSK3)α and tuberin (TSC2) in cells from children with ASD. These data suggest a phosphorylation pattern indicative of higher activity in the Akt/mTOR pathway in children with general/idiopathic ASD and may suggest a common pathological pathway of interest for ASD.

Keywords: autism, signaling, phosphorylation, T cells, mTOR

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INTRODUCTION

Autism spectrum disorder (ASD) is characterized by severe and pervasive impairment in reciprocal social interaction skills, communication skills, or the presence of stereotyped behavior, interests, and activities (1). According to the most recent studies by the US Centers for Disease Control and Prevention, ASD is estimated to affect 1 in 68 children younger than 8 years (2). ASD remains a behaviorally defined disorder with no current physiological diagnostic tools or biological signatures. The cause(s) for the majority of cases of ASD remain unknown. In genetically identical monozygotic twins, there is a concordance rate of 44–91%; in dizygotic twins, the concordance rate for ASD is 0–37%; and in non-twin siblings, the rate is 0–24%; data that suggest a strong heritable component for this disorder (3–9). Although there is evidence to suggest that the disorder is highly heritable, no single genetic cause for all ASD has been identified. Heritability of ASD may suggest a genetic component in the disorder's etiology; however, the genes involved vary greatly among individuals and family clusters and therefore suggest a more likely model that a variety of genetic mutations and/or environmental contributors may result in a common pathology or disruption of a common pathway.

Whole-genome linkage studies, genome-wide association studies, copy number variation screening, and SNP analyses have identified several ASD candidate genes (10). Associations between candidate genetic mechanisms and ASD have implicated a diverse range of functions including metabolism, immune function, neuronal migration, synapse formation, neuronal growth, and neurotransmission. Among some of the notable associations are mutations in *RELN* (11), *SHANK3* (12), *NLGN3*, *NLGN4X* (13), *MET* (14), *GABRB3* (15), *OXTR* (16), and *SLC6A4* (16). Furthermore, several single-gene mutation syndromic disorders incur increased risk of developing ASD including Rett syndrome (*MeCP2*), Fragile X (*FMR1*), Tuberous sclerosis (either *TSC1* or *TSC2*), Cowden syndrome (*PTEN*), Timothy syndrome (*CACNA1C*), and Angelman syndrome (*UBE3A*) (17–19). Even with the recent advancements in identifying candidate genes involved in ASD, all identified genetic risk factors combined account for only 10–20% of the total ASD population (10). The genetic mechanisms or mutations are clearly diverse and heterogeneous and may be influenced by environmental factors.

Among the potential candidate genes identified in ASD to date, those involved in Akt/mammalian target of rapamycin (mTOR) signaling and the downstream effects of this pathway are highly represented including *FMR1*, *PTEN*, *TSC1*, and *TSC2* (20). In addition, microarray analysis of peripheral blood suggests that there is abnormal activity in this pathway (21). The Akt/mTOR pathway is involved in many cellular processes that may impact neurodevelopment and have relevance to ASD symptoms. For example, in neurons, this pathway is believed to be important in the process of learning and memory formation by augmenting long-term potentiation (LTP) of synapses (22). The process of LTP involves the strengthening of synapses as a result of sustained signaling in the Akt/mTOR pathway (23).

The Akt/mTOR pathway is complex, and a defect in any of the proteins involved can lead to aberrant signaling. Increased Akt/mTOR activity is consistent with deficiencies of FMR1, TSC1/2, or PTEN found in Fragile X, TSC, and Cowden syndrome and suggests that increased Akt/mTOR activity may have a role in the pathophysiology of the general ASD population and not limited to single ASD genetic mutations. We hypothesized that there are a diverse collection of physiological abnormalities including genetic mutation or environment factors that converge to dysregulate the Akt/mTOR pathway in individuals with ASD.

To probe directly for dysregulation in the Akt/mTOR pathway, we examined the phosphorylation activity of several proteins in the Akt/mTOR pathway in children with ASD and typically developing (TD) controls. Protein phosphorylation was examined in freshly isolated T cells (24) and following stimulation from both ASD and TD control children of the same age. Phosphorylation levels of insulin receptor substrate-1 (IRS1), phosphatase and tensin homolog (PTEN), tuberin (*TSC2*), Akt, glycogen synthase kinase 3 (GSK3)α, GSK3β, mTOR, p70S6 kinase (p70S6K), ribosomal protein S6 (RPS6), and extracellular receptor kinase (ERK) were measured in T cells collected from peripheral blood of all subjects to cast a wide net over the entirety of the Akt/mTOR pathway. In this study, we describe a dysregulation of Akt/mTOR signaling in T cells isolated from children with ASD compared with TD control children, data that might help point to possible etiological mechanisms in ASD.

MATERIALS AND METHODS

Subjects and Behavioral Assessments

Study participants were recruited as part of the Autism Phenome Project (25, 26). The study protocol was approved and carried out in accordance with the recommendations of the Institutional Review Board for the UC Davis School of Medicine, and parents of each subject provided written informed consent for their child to participate in accordance with the Declaration of Helsinki. Participants consisted of 41 children with ASD [mean age (SD) = 6.13 (1.23) years, 32 males] and 31 TD controls [mean age (SD) = 5.95 (1.27) years, 22 males] (**Table 1**). Age and male:female ratios were not statistically different. Diagnostic instruments for ASD included the Autism Diagnostic Observation Schedule-Generic (ADOS-G) (27) and the Autism Diagnostic Interview—Revised (28). ADOS scores [mean (SD)] were 12.6 (3.8) for social affect, 4.6 (1.7) for restricted/repetitive behaviors, and 7.4 (1.9) for severity. All diagnostic assessments were conducted or directly observed by trained, licensed clinical psychologists who specialize in autism and had been trained according to research standards for these tools. Inclusion criteria for ASD were taken from the diagnostic definition of ASD in young children formulated and agreed upon by the Collaborative Programs of Excellence in Autism. Inclusion criteria for TD controls included developmental scores within two SDs of the mean on all subscales of the Mullen's scale of early learning.

TABLE 1 | Study population demographics.

Group	Autism spectrum disorder (ASD) (*N* = 41)	Controls (*N* = 32)
Age (years), median (range)	5.7 (4.6–9.3)	5.5 (4.5–9.9)
Gender		
Male	32 (72%)	22 (55%)
Female	9 (28%)	10 (45%)
Race/ethnicity		
Caucasian, non-Hispanic	26 (64%)	24 (75%)
African American, non-Hispanic	1 (2%)	–
Hispanic	8 (20%)	5 (16%)
Asian, non-hispanic	1 (2%)	–
Other, non-hispanic	5 (12%)	3 (9%)
Mullen, median [interquartile range (IQR)]		
Differential quotient (DQ)	56.1 (35.4–77.1)	105.8 (98.6–114.4)
Verbal differential quotient (VDQ)	69.1 (60.1–83.6)	105.8 (98.3–116)
Non-verbal differential quotient (NVDQ)	66.2 (48.4–78.9)	105.5 (99.2–112.1)
Autism Diagnostic Observation Schedule (ADOS), median (IQR)		
Social affect	12 (9–16)	N/A
Restricted and repetitive behavior	5 (3–6)	N/A
Severity	7 (6–9)	N/A

Controls with a sibling or first-degree relative with ASD were excluded from the study. Behavioral data include Mullen's Scales of Early Learning (DQ, VDQ, NVDQ) scores with median and interquartile range and ADOS (social affect, restricted and repetitive behavior, severity) scores with median and interquartile range.

For ASD differential quotient (DQ) [mean (SD)] = 59.8 (27.5), verbal differential quotient (VDQ) = 72.3 (18.5), and non-verbal differential quotient (NVDQ) = 66.2 (21.7); for TD, DQ = 105.8 (11.4), VDQ = 105.8 (12.7), and NVDQ = 105.5 (10.1). Exclusion criteria for TD controls included any known developmental, neurological, or behavioral problems; a diagnosis of mental retardation, pervasive developmental disorder, or specific language impairment. TD children were screened and excluded for autism with the Social Communication Questionnaire (29) (scores > 11) (SCQ—Lifetime Edition). All participants were native English speakers, were ambulatory, and had no suspected vision or hearing problems. The exclusion criteria for all subjects consisted of the presence of Fragile X or other serious neurological (e.g., seizures), psychiatric (e.g., bipolar disorder), the presence of any known genetic polymorphism that causes ASD, or known medical conditions including autoimmune disease and inflammatory bowel diseases/celiac disease. All subjects were screened *via* parental interview for current and past physical illness. Children with known endocrine, cardiovascular, pulmonary, liver, or kidney disease were excluded from enrollment in the study. No participant presented with a cold, fever, or other common illness, if such a condition occurred, the blood draw were delayed until the child's health status was stable for 48 h.

Peripheral Blood Mononuclear Cell (PBMC) Collection

Peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences, San Jose, CA, USA) following behavioral assessments. Whole blood was centrifuged at 960 *g* for 10 min to separate cell fractions from plasma. The cell fraction was diluted in 25 ml of Hank's Balanced Salt Solution (HBSS) (Mediatech, Manassas, VA, USA) and layered at room temperature over lymphocyte separation medium (LSM) (Mediatech) and centrifuged at 300 *g* for 30 min to isolate PBMCs. PBMCs were harvested at the interface between LSM and HBSS fractions and washed twice with 50 ml HBSS. The number of viable PBMC was determined by 1:1 dilution of Trypan Blue (Mediatech) exclusion counting on a Hausser phase contrast hemacytometer. Cells were then diluted in a chilled (4°C) magnetic separation buffer (MACS buffer) (Miltenyi Biotec, Auburn, CA, USA) to a concentration of 2.5×10^8/ml.

T Cell Isolation

T cells were isolated with a Pan T cell isolation kit according to the protocol provided by the manufacturer (Miltenyi Biotec). Briefly, a biotinylated antibody cocktail containing antibodies reactive to CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (glycophorin A) was added at a concentration of 10 µl of antibody cocktail/10^7 cells and incubated for 10 min at 4°C. Following incubation, an additional 30 µl of MACS buffer per 10^7 cells was added followed by 10 µl of magnetic beads conjugated to antibiotin antibodies at a concentration of 20 µl/10^7 cells and incubated at 4°C for an additional 15 min. Cells were then washed in 20-fold incubation volume of MACS buffer and centrifuged at 300 *g* for 10 min. Supernatants were aspirated and discarded, and PBMC were resuspended in 500 µl. This suspension was transferred to a MACS LS column on a MACS magnetic platform. The column was washed with a total of 9 ml MACS buffer, and eluent was again collected. All of the T cell-enriched eluent that passed through the LS column from the column was collected and centrifuged at 300 *g* for 10 min. The T cell-enriched pellet was washed and centrifuged twice in 10 ml of Roswell Park Memorial Institute (RPMI) media (Life technologies) containing 10% low endotoxin, heat-inactivated fetal bovine serum (Life technologies, Carlsbad, CA, USA), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Sigma, St. Louis, MO, USA) (complete RPMI). The T cells were then resuspended in 1×10^6 cell/ml and plated 1 ml/well in a 12-well sterile tissue culture plate (Greiner Bio-One, Monroe, NC, USA). T cells were allowed to rest in complete RPMI at 37°C for 16 h prior to stimulation and lysis.

T Cell Stimulation, Lysis, and Protein Quantification

T cells were either left unstimulated or stimulated with 10 nM phorbol myristate acetate (PMA) for 15 or 45 min in complete RPMI at 37°C. Following stimulation, T cells were quickly washed in 10 ml of ice-cold PBS and centrifuged for 10 min at 500 *g* at 4°C. Supernatants were aspirated, and cell pellet was resuspended in ice-cold lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 µg/ml leupeptin supplemented with freshly added 1% AEBSF. Cells were mixed in lysis buffer with vigorous pipetting and sonicated for 30 s. Cells were then incubated in lysis buffer on ice for 10 min. Following incubation, cell suspensions were centrifuged at 18,000 *g* for 10 min. Supernatants were collected, and aliquots were stored at −80°C until date of protein quantification or phosphorylation analysis. Protein levels were quantified using Bradford assay. In brief, diluted lysate and BSA standards were combined 1:1 with room temperature Bradford reagent (Bio-Rad, Hercules, CA, USA) and incubated for 10 min. Following incubation, optical density was measured with a 595 λ filter on a Bio-Rad spectrophotometer. Protein levels of T cell lysate samples were derived from a linear seven-point BSA standard curve (Bio-Rad).

Phosphorylation Assay

Relative levels of phosphorylated and total IRS1, PTEN, ERK1/2, Akt, GSK3α, GSK3β, TSC2, mTOR, p70S6K, and RPS6 were measured with Milliplex™ multiplexing bead immunoassays (Millipore, Billerica, MA, USA). This assay contains antibodies that have been standardized and optimized for sensitivity and specificity to detect the phosphorylation and total protein levels of the molecules tested. Assay detection of the total and phosphorylated proteins detected was confirmed using Western blot analysis of individual phosphorylated or total proteins prior to testing, as well as the manufacturer's own internal research quality controls. A subset of samples was also put through a second assay provided by Bio-Rad that contains many of standardized antibodies used in Western blot analyses, with strong correlation between assays (data not shown). Luminex technology provides a unique platform for analysis of limited sample volume, as is the

case in this study, where precious blood samples from pediatric patients are used. GAPDH was used as an internal control for both phoshporylated and total protein assays, which were measured separately. The multiplex assay was performed according to the manufacturer's instructions. Briefly, lysates were incubated with antibody-conjugated beads at a total protein concentration of 500 µg/ml. After protein capture, beads were washed twice followed by incubation with biotinylated detection antibody. Beads were then incubated with streptavidin-conjugated phycoerythrin (PE), followed by several washes. Finally, the bead sets were analyzed using a flow-based Luminex™ 100 suspension array system to determine bead identity and corresponding PE mean fluorescent intensity (MFI) (Bio-Plex 200; Bio-Rad Laboratories). Relative levels of phosphorylated and total proteins were determined by dividing MFI signal of each protein by GAPDH signal for each sample. Relative phosphorylation was determined by further dividing the GAPDH relative levels of phosphorylated proteins to their respective GAPDH relative total protein levels. All phosphorylation values are expressed as ratios.

Statistical Analysis

The data were non-parametrically distributed, *p* values were determined with Mann–Whitney *U*-test. All analyses were two tailed, and values of $p < 0.05$ were considered statistically significant. For these novel and previously untested experiments, unadjusted *p* values are presented (30). All analyses were conducted using GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). Associations between Akt/mTOR/pathway measures and behaviors assessed by ADOS were calculated using a two-tailed, non-parametric Spearman's correlation test with 95% confidence intervals.

RESULTS

Given that Akt/mTOR genetic mutations are potentially associated with increased ASD risk, we hypothesized that aberrations in many parts of the Akt/mTOR pathway will contribute to an overall pattern of increased Akt/mTOR pathway activity. To test this theory, we examined several proteins in the Akt/mTOR pathway. We observed increased IRS1 and RSP6 total protein in children with ASD compared with TD controls under unstimulated conditions ($p < 0.03$; **Table 2**). Similarly, total IRS1 and RSP6 were increased, in T cells following 15 or 45 min of stimulation, in the ASD group compared to TD controls ($p < 0.04$). No other total protein levels were different between groups (**Table 2**). For phosphorylated proteins, in unstimulated T cells, GSK3α, GSK3β, PTEN, TSC2, and mTOR were increased in children with ASD compared to TD controls ($p < 0.006$; **Table 3**). After 15 min of stimulation, T cells from children with ASD had higher phosphorylation of proteins, p7056K, IRS1, GSK3α, GSK3β, AKT, PTEN, TSC2, mTOR, and ERK ($p < 0.04$; **Table 3**). After 45 min of T cell stimulation, levels of phosphorylated protein were still increased in children with ASD for p7056K, IRS1, GSK3α, GSK3β, AKT, PTEN, TSC2, mTOR, and ERK, as well as RPS6 ($p < 0.02$; **Table 3**).

By calculating the ratio of phosphorylated to total protein levels, we observed higher ratios for mTOR and GSK3β ($p < 0.02$) in unstimulated ASD T cells when compared to unstimulated TD control T cells (**Table 4**; and Figures S1 and S2 in Supplementary Material). Given the effects of phosphorylation on these proteins (**Table 5**), it would indicate increased activity of mTOR and decreased activity of GSK3β in ASD T cells. This may suggest

TABLE 2 | Akt/mTOR pathway total protein in T cells.

Protein	ASD		Control		*p* Value
	Median	Interquartile range (IQR)	Median	IQR	
Activating					
Unstimulated					
Akt	0.175	0.132	0.169	0.088	0.708
Mammalian target of rapamycin (mTOR)	0.016	0.012	0.015	0.005	0.741
p70S6 kinase (p70S6K)	0.250	0.092	0.221	0.094	0.406
Ribosomal protein S6 (RPS6)	0.005	0.002	0.004	0.001	**0.017**
Extracellular receptor kinase (ERK)	0.655	0.149	0.646	0.076	0.551
Stimulated (15 min)					
Akt	0.173	0.124	0.171	0.085	0.792
mTOR	0.014	0.008	0.016	0.007	0.377
p70S6K	0.263	0.110	0.265	0.109	0.474
RPS6	0.006	0.004	0.005	0.002	**0.001**
ERK	0.604	0.196	0.605	0.155	0.669
Stimulated (45 min)					
Akt	0.168	0.107	0.169	0.090	0.853
mTOR	0.018	0.008	0.016	0.009	0.071
p70S6K	0.270	0.089	0.273	0.141	0.966
RPS6	0.014	0.016	0.009	0.008	**0.003**
ERK	0.607	0.115	0.617	0.118	0.664
Inactivating					
Unstimulated					
Insulin receptor substrate-1 (IRS1)	0.027	0.026	0.018	0.022	**0.030**
PTEN	0.289	0.156	0.255	0.079	0.129
Glycogen synthase kinase 3 (GSK) 3α	0.282	0.275	0.254	0.203	0.729
GSK3β	0.269	0.238	0.260	0.130	0.544
TSC2	0.040	0.049	0.039	0.056	0.590
Stimulated (15 min)					
IRS1	0.030	0.023	0.021	0.020	**0.041**
PTEN	0.307	0.146	0.266	0.075	0.067
GSK3α	0.209	0.245	0.280	0.214	0.053
GSK3β	0.153	0.169	0.168	0.143	0.378
TSC2	0.043	0.050	0.036	0.056	0.538
Stimulated (45 min)					
IRS1	0.040	0.028	0.024	0.031	**0.004**
PTEN	0.284	0.114	0.245	0.074	0.078
GSK3α	0.210	0.186	0.276	0.131	0.065
GSK3β	0.115	0.109	0.159	0.128	0.147
TSC2	0.040	0.049	0.035	0.056	0.866

All p values were calculated with two-tailed Mann–Whitney U-tests.
Values in bold and gray shades signifies a p < 0.05.

TABLE 3 | Akt/mTOR pathway phosphorylated protein in T cells.

Protein	ASD		Control		*p* Value
	Median	Interquartile range (IQR)	Median	IQR	
Activating					
Unstimulated					
Akt	0.006	0.004	0.005	0.003	0.110
Mammalian target of rapamycin (mTOR)	0.024	0.017	0.015	0.012	**0.006**
p70S6 kinase (p70S6K)	0.011	0.007	0.008	0.004	0.064
Ribosomal protein S6 (RPS6)	0.009	0.006	0.009	0.005	0.473
Extracellular receptor kinase (ERK)	0.006	0.004	0.006	0.002	0.211
Stimulated (15 min)					
Akt	0.008	0.005	0.005	0.006	**0.013**
mTOR	0.099	0.088	0.057	0.088	**0.011**
p70S6K	0.093	0.142	0.027	0.125	**0.018**
RPS6	0.060	0.071	0.013	0.070	0.085
ERK	0.153	0.150	0.080	0.195	**0.029**
Stimulated (45 min)					
Akt	0.008	0.005	0.005	0.005	**0.006**
mTOR	0.127	0.094	0.071	0.107	**0.002**
p70S6K	0.142	0.140	0.069	0.145	**0.003**
RPS6	0.137	0.221	0.035	0.200	**0.023**
ERK	0.104	0.094	0.043	0.103	**0.005**
Inactivating					
Unstimulated					
Insulin receptor substrate-1 (IRS1)	0.005	0.004	0.004	0.003	0.064
PTEN	0.219	0.097	0.170	0.074	**0.000**
Glycogen synthase kinase 3 (GSK3)α	0.015	0.012	0.011	0.005	**0.002**
GSK3β	0.023	0.021	0.012	0.014	**0.000**
TSC2	0.016	0.012	0.013	0.006	**0.001**
Stimulated (15 min)					
IRS1	0.009	0.007	0.004	0.008	**0.009**
PTEN	0.208	0.119	0.175	0.111	**0.045**
GSK3α	0.317	0.326	0.161	0.361	**0.009**
GSK3β	0.116	0.099	0.064	0.118	**0.023**
TSC2	0.052	0.039	0.034	0.054	**0.027**
Stimulated (45 min)					
IRS1	0.010	0.016	0.005	0.006	**0.003**
PTEN	0.233	0.176	0.164	0.050	**0.001**
GSK3α	0.307	0.303	0.107	0.263	**0.001**
GSK3β	0.113	0.077	0.067	0.089	**0.002**
TSC2	0.054	0.046	0.028	0.039	**0.000**

Median and IQR of phosphorylated Akt/mTOR pathway proteins. All p values were calculated with two-tailed Mann-Whitney U-tests.
Values in bold and gray shades signifies a $p < 0.05$.

TABLE 4 | Akt/mTOR pathway phosphorylated/total protein ratios in T cells.

Protein	ASD		Control		*p* Value
	Median	Interquartile range (IQR)	Median	IQR	
Activating					
Unstimulated					
Akt	0.039	0.050	0.028	0.021	0.544
Mammalian target of rapamycin (mTOR)	1.262	1.529	0.941	0.866	**0.024**
p70S6 kinase (p70S6K)	0.041	0.038	0.035	0.019	0.254
Ribosomal protein S6 (RPS6)	1.685	1.532	1.995	1.395	0.590
Extracellular receptor kinase (ERK)	0.009	0.009	0.009	0.004	0.597
Stimulated (15 min)					
Akt	0.053	0.046	0.039	0.038	0.129
mTOR	5.954	6.491	2.770	5.730	**0.016**
p70S6K	0.342	0.653	0.104	0.357	**0.002**
RPS6	5.787	7.306	3.298	10.334	0.274
ERK	0.227	0.288	0.127	0.367	**0.027**
Stimulated (45 min)					
Akt	0.062	0.058	0.036	0.042	0.078
mTOR	6.769	6.872	3.929	5.815	**0.008**
p70S6K	0.503	0.588	0.225	0.417	**0.004**
RPS6	9.008	8.853	4.325	17.095	0.087
ERK	0.178	0.168	0.078	0.186	**0.050**
Inactivating					
Unstimulated					
Insulin receptor substrate-1 (IRS1)	0.235	0.294	0.250	0.224	0.346
PTEN	0.681	0.461	0.647	0.278	0.115
Glycogen synthase kinase 3 (GSK3)α	0.072	0.283	0.043	0.090	0.057
GSK3β	0.073	0.111	0.050	0.042	**0.010**
TSC2	0.428	2.717	0.360	1.653	0.153
Stimulated (15 min)					
IRS1	0.305	0.695	0.275	0.347	0.834
PTEN	0.680	0.452	0.673	0.276	0.636
GSK3α	1.409	6.295	0.505	1.964	**0.004**
GSK3β	0.808	0.921	0.484	0.969	0.055
TSC2	1.797	7.840	0.867	2.747	**0.039**
Stimulated (45 min)					
IRS1	0.232	0.374	0.254	0.352	0.874
PTEN	0.860	0.445	0.663	0.207	**0.025**
GSK3α	1.199	4.969	0.364	1.290	**0.004**
GSK3β	0.977	0.783	0.523	0.899	**0.017**
TSC2	1.548	5.008	0.786	1.909	**0.029**

Median and IQR of phosphorylated/total Akt/mTOR pathway proteins. All p values were calculated with two-tailed Mann–Whitney U-tests.
Values in bold and gray shades signifies a $p < 0.05$.

higher activity of Akt/mTOR signaling in ASD T cells (**Figure 1**). ASD T cells also trended toward increased phosphorylation of GSK3α under unstimulated conditions (**Table 5**), which would indicate lower activity of GSK3α consistent with higher Akt/mTOR pathway activity (**Table 1**). Together these data indicate Akt/mTOR signaling is higher in resting T cells of children with ASD when compared with T cells from TD controls.

After stimulation with PMA for 15 min, T cells from children with ASD exhibited higher phosphorylation of ERK, mTOR,

TABLE 5 | Effect of phosphorylation on target sites of Akt/mTOR pathway proteins.

Activating		Inactivating	
Akt	Ser473	IRS1	Ser312
mTOR	Ser2448	PTEN	Ser380
p70S6K	Thr412	GSK3α	Ser21
RPS6	Ser235/Ser236	GSK3β	Ser9
ERK	Thr185/Tyr187	TSC2	Ser939

Proteins are organized according to whether the effect of phosphorylation on a specific phosphorylation site is activating or inactivating. The sites listed in the table are those measured in this article.

p70S6K, GSK3α, and TSC2 compared with T cells from children with TD ($p < 0.04$; **Table 4**), indicating increased activity of ERK, mTOR, and p70S6K but a decreased activity of inhibitory signals by TSC2 and GSK3α, suggesting that Akt/mTOR pathway activity may be increased in stimulated ASD T cells (**Table 1**). ASD T cells also trended toward increased phosphorylation of GSK3β ($p < 0.054$), which would indicate lower activity of inhibitory GSK3β consistent with higher Akt/mTOR pathway activity. After 45 min of PMA stimulation, increased phosphorylation of ERK, mTOR, p70S6K, GSK3α, GSK3β, PTEN, and TSC2 were detected in ASD T cells (**Table 4**). There was also a trend for increased AKT but which did not quite reach statistical significance ($p = 0.077$). Together these data suggest overall increased AKT/mTOR pathway activity in ASD T cells following stimulation.

Associations were observed for total p7056k and autism severity at 15 min poststimulation ($r = 0.327$, $p = 0.04$). Restrictive and repetitive behaviors were associated with the PTEN ratio after 15 min stimulation also ($r = -0.3316$, $p = 0.03$). For social affect, several measures were associated including total p7056k and the IRIS ratio in unstimulated and 45 min after stimulation ($p < 0.05$).

DISCUSSION

In this study, we report differential activity of several Akt/mTOR signaling molecules in young children with ASD. To observe dynamic phosphorylation activity, freshly isolated T lymphocyte cells were chosen as a cellular representative that could be acquired efficiently, safely, and easily from relatively non-invasive blood samples. From our experiments, we determined that ASD T cells generally exhibit phosphorylation to total protein ratios that would indicate higher activity of mTOR, ERK, and p70S6K as well as lower activity of GSK3α, GSK3β, TSC2, and PTEN than TD control T cells. This indicates a shift toward higher Akt/mTOR pathway activity in the ASD group (**Table 5**; **Figure 1**). An increased Akt/mTOR activity is consistent with deficiencies of FMR1, TSC1/2, or PTEN found in Fragile X, TSC, and Cowden syndrome, respectively (31–33). Moreover, suppression of this increased Akt/mTOR activity has been demonstrated to improve ASD-associated symptoms in mice deficient for PTEN and TSC1 (34, 35). Together these data suggest that increased Akt/mTOR activity may have a role in the pathophysiology of the general ASD population and not limited to known ASD-associated Akt/mTOR genetic mutations.

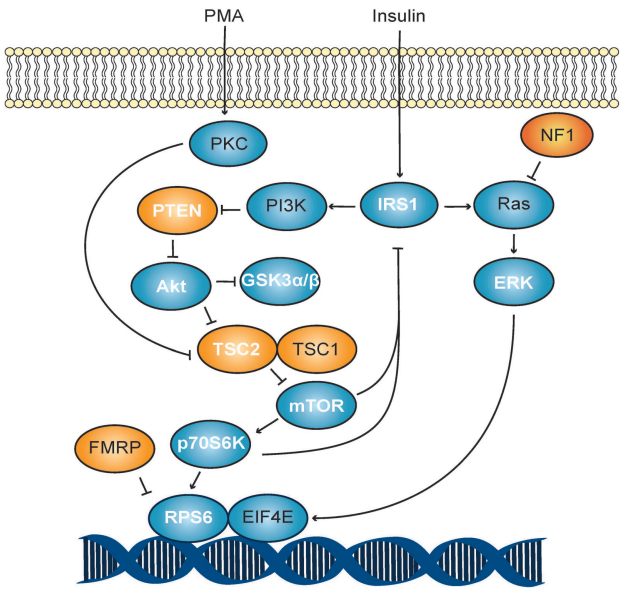

FIGURE 1 | Akt/mTOR signaling schematic. The PI3K pathway in response to stimulation with phorbol myristate acetate (PMA). Autism spectrum disorder-associated mutations are shown in orange, while all others are shown in blue. Molecules measured in this study are shown with white lettering.

The Akt/mTOR pathway is involved in a large number of physiological functions, in both the central nervous and immune systems (36–39). Atypical Akt/mTOR signaling may be related to many previous observations of abnormal T cell function (40–44) in children with ASD. The aberrancies in Akt/mTOR signaling observed in this study are likely not limited to T cells but will have relevance to signaling also in other immune cells and as such these data have relevance to other immune abnormalities previously observed in ASD involving multiple leukocyte subsets (25, 45–50). Aberrant Akt/mTOR signaling has the potential to impact cellular growth, proliferation, and cytokine production in the immune system (38), which can in turn affect behavior (26).

Our data show that immune dysfunction of children with ASD previously demonstrated may stem from aberrant T cells signaling *via* the Akt/mTOR pathway. To probe directly for dysregulation in the Akt/mTOR pathway, we sought to examine the phosphorylation activity of several proteins in the Akt/mTOR pathway in children with ASD and TD controls. As ASD manifests in early childhood, it is difficult to find suitable research tools and accessible tissues for experimentation. For example, postmortem human tissue can never provide the substrate for dynamic functional studies, and finding suitable control material is problematic. Immune cells, in contrast, provide a readily accessible model system that has many advantages including easy acquisition, high availability, and fine matching with controls. The advantages of using lymphocytes as an easily accessible "neural probe" in the investigation of psychiatric disorders in living subjects has been previously reviewed (24). We therefore utilized T lymphocytes as a neuronal surrogate in our experiments to examine dynamic signaling activity. T lymphocyte cells were chosen as a cellular

model in which to test Akt/mTOR pathway activity for several characteristics including their long life span and high numbers within the blood. Moreover, the Luminex technology was chosen as it provides a platform for analyses of a number of analytes simultaneously from small volumes of tissue. As this study utilized pediatric blood samples, T cell numbers were limited, and thus, performing Western blot analyses on all phosphorylated or total proteins would be prohibitive. Importantly, the antibodies used had been previously standardized and optimized, and we also checked for detection of the same proteins in Western blot analysis and intracellular flow cytometry techniques, using Jurkat cells and primary T cells from adults, prior to running the Luminex assays. In addition, on a subset of samples, a number of phosphorylated or total proteins were compared between two Luminex assays from different manufacturer's, with similar results. Our results suggest that the Luminex platform provides a quick and efficient means of identifying possible changes in the Akt/mTOR pathway, in pediatric samples that are limited in volume. Although our data showed increased Akt/mTOR signaling in ASD, whether this reflects what happens *in vivo* or within other tissues such as the gastrointestinal tract or brain is not known and would need further investigation.

Further work needs to be performed to determine context-dependent effects on Akt/mTOR pathway in T cells and how they relate to the brain; however, many gene expression studies have taken the approach to look at primary or immortalized blood cells as a surrogate for inaccessible tissue such as the brain. The advantages of using lymphocytes as neural surrogates for *in vitro* examination has been previously established, but there is also evidence that the increased Akt/mTOR activity observed in Fragile X central nervous system (CNS) tissue is mirrored in lymphocytes (51), suggesting that Akt/mTOR signaling in T cells is applicable to cells of the CNS, including neurons and glial cells. In neurons, the Akt/mTOR pathway is essential in the regulation of dendritic arborization and spine formation (52), which are important features of synapse formation. Increased activity of this pathway in neuronal knockouts of *TSC1* or *PTEN* results in lower sociability and seizures in mouse models (53–55), suggesting that both sociability and seizures are Akt/mTOR pathway activity dependent. Increased activity of this pathway in glial cells can also have negative effects on neurobiology, such as aberrant neuronal organization and seizures in astrocyte-specific *TSC1* conditional knockout mice (56). Lack of social interactions is a central symptom of ASD (1), and seizures are a common comorbidity in the disorder (57). Together these data suggest that these ASD symptoms could be potentially related to the high Akt/mTOR signaling as described in this study in ASD cells (53, 56, 58).

Akt activation leads to a number of effects, some of which are mTOR independent. These include regulating cell survival and growth, such as phosphorylation of Forkhead box O family of transcription factors and of GSK3α and GSK3β (59–61). GSK3 exists in two genetically distinct isoforms but with near identical function. GSK3α and GSK3β share 85% amino acid identity and 98% amino acid sequence homology within their kinase domains (62). The GSK3 proteins are involved in regulating metabolic function and are phosphorylated and inhibited by Akt among other kinases such as p70S6K. Once phosphorylated, the kinase activity of GSK3 is inhibited, and their substrates such as glycogen synthase, Ap-1, β-catenin, c-myc, and p53, thereby initiating signaling mechanisms promoting cell survival and growth. GSK3s are expressed in virtually all cell types, but their expression is highly enriched in the CNS (63) and appears to be involved in regulating synaptic plasticity (64). These proteins have recently gained attention in the area of Alzheimer's disease (AD) research, and there have been several observations that both the activity and total levels of GSK3 is upregulated in AD patients (65). Interestingly, we observed lower GSK3 activity in children with ASD compared with TD controls. Current evidence suggests that low GSK3 activity impairs LTD (66), which could affect synaptic plasticity and in children with ASD.

Collectively, these data described in this study suggest a general dysregulation of the Akt/mTOR pathway in an idiopathic ASD population. This may suggest a convergent pathology in ASD that would affect multiple physiological symptoms. It is unclear whether Akt/mTOR aberrancies described in the study are due to as yet unknown genetic mutations, epigenetic changes, or environmental factors, and it is possible that it may be due to a combination of genetic and environmental influences. In fact, growth factors that imbue effects by signaling through the Akt/mTOR pathway such as HGF and MIF have also been reported as dysregulated in ASD (67, 68), suggesting that circulatory homeostatic factors can be an additional source of Akt/mTOR pathway activity. Similarly, a mutation in cMET, the receptor for HGF, has also been reported in ASD (69), suggesting that Akt/mTOR-associated receptors may also be a source for aberrant signaling activity. Together, these data present a novel finding Akt/mTOR pathway dysregulation in young children with ASD that could provide a focus for targeted therapeutics for at least a subset of individuals with ASD.

AUTHOR CONTRIBUTIONS

All authors designed the experiments, helped with data analysis and interpretation, and played a major role in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fped.2017.00043/full#supplementary-material.

FIGURE S1 | Akt/mammalian target of rapamycin (mTOR) pathway activating phosphorylated/total protein ratios in T cells. Box and whisker plots show activating AKT/mTOR pathway (phosphorylated/total) protein levels after 0, 15, and 45 min of stimulation between autism spectrum disorder (ASD) (black bars) and controls (gray bars). Data are depicted as box showing the lower (25%) and upper (75%) quartiles and the middle line representing the median, whiskers showing the minimum and maximum. *p Value less than 0.05, calculated with two-tailed Mann–Whitney *U*-tests.

FIGURE S2 | Akt/mammalian target of rapamycin (mTOR) pathway inactivating phosphorylated/total protein ratios in T cells. Box and whisker plots show inactivating AKT/mTOR pathway (phosphorylated/total) protein levels after 0, 15, and 45 min of stimulation between autism spectrum disorder (ASD) (black bars) and controls (gray bars). Data are depicted as box showing the lower (25%) and upper (75%) quartiles and the middle line representing the median, whiskers showing the minimum and maximum. *p Value less than 0.05, calculated with two-tailed Mann–Whitney *U*-tests.

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Can Mouse Imaging Studies Bring Order to Autism Connectivity Chaos?

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Functional Magnetic Resonance Imaging (fMRI) has consistently highlighted impaired or aberrant functional connectivity across brain regions of autism spectrum disorder (ASD) patients. However, the manifestation and neural substrates of these alterations are highly heterogeneous and often conflicting. Moreover, their neurobiological underpinnings and etiopathological significance remain largely unknown. A deeper understanding of the complex pathophysiological cascade leading to aberrant connectivity in ASD can greatly benefit from the use of model organisms where individual pathophysiological or phenotypic components of ASD can be recreated and investigated via approaches that are either off limits or confounded by clinical heterogeneity. Despite some obvious limitations in reliably modeling the full phenotypic spectrum of a complex developmental disorder like ASD, mouse models have played a central role in advancing our basic mechanistic and molecular understanding of this syndrome. Recent progress in mouse brain connectivity mapping via resting-state fMRI (rsfMRI) offers the opportunity to generate and test mechanistic hypotheses about the elusive origin and significance of connectional aberrations observed in autism. Here we discuss recent progress toward this goal, and illustrate initial examples of how the approach can be employed to establish causal links between ASD-related mutations, developmental processes, and brain connectional architecture. As the spectrum of genetic and pathophysiological components of ASD modeled in the mouse is rapidly expanding, the use of rsfMRI can advance our mechanistic understanding of the origin and significance of the connectional alterations associated with autism, and their heterogeneous expression across patient cohorts.

Keywords: fMRI, functional connectivity, autism, mouse, resting-state, DMN, CNTNAP2, pathoconnectomics

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THE CONNECTIVITY THEORY OF AUTISM: OPEN QUESTIONS AND CONTROVERSIES

Autism is a heterogeneous syndrome characterized by core behavioral features including deficits in social communication and interaction, as well as restricted, repetitive patterns of behavior, and interests (Association AP, 2013). Although a primary and unitary etiology for autism spectrum disorder (ASD) has not been identified, its high heritability has been consistently documented, revealing a contribution of complex and highly heterogeneous genetic mutations (Geschwind, 2009; Geschwind and State, 2015; Sanders et al., 2015). Remarkably, although previously identified mutations, genetic syndromes, and *de novo* copy number variations (CNVs) account for about 10–20% of ASD cases, none of these single known genetic causes accounts for more than

1–2% of cases (reviewed in Abrahams and Geschwind, 2008). The phenotypic expression (i.e., "penetrance") of these genetic components is also highly variable, ranging from fully penetrant point mutations to polygenic forms with multiple gene–gene and gene–environment interactions. Remarkable variability exists also in the extent of cognitive and behavioral abnormalities presented by affected individuals (Georgiades et al., 2013; Lai et al., 2014; Chang et al., 2015), making heterogeneity a dominant theme for this group of disorders.

The advent of non-invasive brain imaging raised hopes that such clinical heterogeneity could be narrowed down to a small number of identifiable "imaging endophenotypes" that could help ASD diagnosis, patient stratification, and possibly provide clues as to the elusive etiology of this group of disorders. Unfortunately, the results of imaging studies have proven overall as variable as the clinical manifestations of ASD (Stanfield et al., 2008; Ecker et al., 2015). A notable exception to this scenario was the initial observation of reduced connectivity between brain regions in ASD patients, a finding first reported by Horwitz et al. (1988) using PET, and later corroborated by task-based (Just et al., 2004) and resting-state fMRI (rsfMRI) studies (Cherkassky et al., 2006; Kennedy and Courchesne, 2008; Assaf et al., 2010), which revealed impaired long-range synchronization in spontaneous brain activity. Together with evidence of reduced white matter connectivity detected with MRI (reviewed in Anagnostou and Taylor, 2011), these observations form the basis of the so called "under-connectivity theory of autism" (Anagnostou and Taylor, 2011; Just et al., 2012), according to which deficient long-range communication between brain regions may underlie ASD symptoms and pathophysiology. However, recent imaging studies have strongly challenged this view, highlighting a much more heterogeneous picture (see Vasa et al., 2016 for a recent review). For example, rsfMRI mapping in a large cohort of patients has revealed the presence of concomitant hypo- and hyper-connectivity (Di Martino et al., 2014), although a clear prevalence of hypo-connected regions was apparent. Similarly, widespread hyper-connectivity during childhood has also been recently described (Keown et al., 2013; Supekar et al., 2013; Uddin et al., 2013a), suggesting a possible neurodevelopmental origin for these alterations. More recently, the hypothesis that such conflicting findings could reflect greater inter-subject variability in ASD patients than in neurotypical controls (i.e., idiosyncratic connectivity) has been proposed (Hahamy et al., 2015). A putative confounding contribution of ASD-related motion and its effect on functional connectivity readouts is also the subject of an open controversy in the imaging community (Deen and Pelphrey, 2012; Power et al., 2012, 2015; Pardoe et al., 2016).

Collectively, the extensive literature published to date points at the presence of major functional connectivity alterations in ASD populations, although the identified regional patterns vary considerably across studies and patient cohorts (Kana et al., 2011; Müller, 2014; Ecker and Murphy, 2014; Ameis and Catani, 2015; Ecker et al., 2015; Bernhardt et al., 2016; Vasa et al., 2016). Despite this rapidly accumulating evidence, many fundamental questions as to the origin and significance of connectional alterations in ASD remain unanswered. For one, the neurophysiological underpinnings of these connectional aberrancies are largely unknown, and a causal etiopathological contribution of specific genetic variants to impaired connectivity in ASD remains to be firmly established. More broadly, it is unclear whether these abnormalities are a causative or epiphenomenal consequence of the disease, and whether their heterogeneous expression reflects cohort effects, different genetic etiologies, or neurodevelopmental trajectories. The exact relationship between connectivity alterations and the severity of ASD manifestation remains also obscure, with the vast majority of the human neuroimaging literature being focused on high-functioning ASD cohorts (Vissers et al., 2012).

A deeper understanding of the origin and significance of these phenomena is greatly complicated by our very limited understanding of the neurobiological foundations of macro-scale neuroimaging readouts commonly employed in ASD research, such as white matter microstructural parameters (e.g., fractional anisotropy, Owen et al., 2014) or the elusive functional couplings underlying rsfMRI-based functional connectivity. This has left us with a major explanatory gap between mechanistic models of brain function at the cellular and microcircuit level, and the emergence of macroscale functional activity in health and pathological states such as those that are observed in autism. As a result, we are currently unable to properly interpret and back-translate clinical evidence of aberrant connectivity into interpretable neurophysiological events/models that can help understand, diagnose or treat these disorders. It is also becoming apparent that a full disambiguation of the multifactorial and complex determinants of aberrant functional connectivity in ASD can only be obtained through the combined use of refined clinical imaging methods and multimodal-multiscale investigational approaches that currently can only be applied in experimental animal models.

BRIDGING THE GAP: FUNCTIONAL CONNECTIVITY MAPPING IN MOUSE AUTISM MODELS

The identification of several high-confidence ASD-risk genes involved in syndromic forms of autism (Sanders et al., 2015) has been paralleled by the generation of mouse lines recapitulating human mutations. Despite predictable limitations in reliably modeling the full phenotypic spectrum of a complex (and possibly only human) developmental disorder like ASD, mouse models can be harnessed to understand how genetic alterations translate into relevant changes in cells and circuits, and ultimately to identify points of convergence for molecular pathways, cells, circuits, and systems that may result in a deeper understanding of the pathophysiology of ASD and related behavioral deficits (Arguello and Gogos, 2012; Nelson and Valakh, 2015; Vasa et al., 2016). For example, molecular investigations in ASD mouse models have been instrumental in the identification of a limited set of molecular pathways to which ASD-involved genes seem to converge, including, among others, synaptogenesis, synaptic function, and neuronal translational regulation (reviewed in de la Torre-Ubieta et al., 2016). This

effort has been accompanied by the development of ASD-relevant behavioral phenotyping assays, primarily targeted at social, communication, and repetitive behaviors (Silverman et al., 2010a; Wöhr and Scattoni, 2013; Kas et al., 2014; Homberg et al., 2016). Interestingly, many—but not all—models showed autism-like traits, with manifestations ranging from repetitive behaviors to reduced social communication (ultrasonic vocalizations) and social interest (reviewed in Ellegood and Crawley, 2015). However, despite the widespread application and high face validity of ASD behavioral phenotyping, the significance and translational relevance of mouse behavioral alterations to human ASD remain debated (Wöhr and Scattoni, 2013) and should be extrapolated with caution.

Recent advances in mouse rsfMRI mapping (reviewed in Gozzi and Schwarz, 2016) offer the opportunity of extending mouse modeling of ASD to the investigation of the neurobiological underpinnings and etiopathological significance of ASD-related connectivity aberrations. Specifically, improvements in MRI imaging hardware, together with tighter control of physiological and motion artifacts (Weber et al., 2006; Ferrari et al., 2012) have led to robust and reproducible identification of homotopic rsfMRI networks covering known cortical and subcortical systems in the mouse by several research groups (Mechling et al., 2014; Nasrallah et al., 2014; Sforazzini et al., 2014; Zerbi et al., 2015; Shah et al., 2016). Interestingly, distributed networks encompassing heteromodal prefrontal and posterior cortical regions have also been identified (Sforazzini et al., 2014; Zerbi et al., 2015; Shah et al., 2016), leading to the suggestive hypothesis of the presence of evolutionary precursors of the human salience network and default mode network (DMN) in this species (reviewed in Gozzi and Schwarz, 2016). This notion is empirically corroborated by the recent observation that cytoarchitecturally homologous regions such as anterior cingulate and retrosplenial cortices (Vogt and Paxinos, 2014) similarly serve as connectivity hubs in humans and mice (Cole et al., 2010; Tomasi and Volkow, 2011; Liska et al., 2015). Moreover, the application of rsfMRI to the mouse brain comes with several important advantages, including the possibility to use quantitative imaging modalities for an objective endo-phenotypic characterization of ASD-related pathology complementary to behavioral assays, and to validate its readouts with invasive techniques that are off limits for human research, including local field potentials (LFPs) coherence mappings (Zhan et al., 2014), local injection of neuronal tracers (Sforazzini et al., 2016), as well as an ever-increasing array of histopathological, stereological, or immunohistochemical post-mortem analyses.

Collectively, these correspondences strongly support the use of rsfMRI as a means to bridge research of functional connectivity aberrancies in autism across species (human vs. mouse) and levels of inquiry (from cellular- and microscale to meso- and macroscale, **Figure 1**), along two main investigational routes. First, rsfMRI can be used to establish *causal* (rather than *associative*) etiopathological contributions between specific ASD-associated genetic variants and macroscale connectivity, thus complementing analogous clinical research efforts using imaging genetics (Scott-Van Zeeland et al., 2010; Rudie et al., 2012). One notable experimental advantage of mouse imaging with respect to current human imaging genetic approaches is the possibility of mapping and comparing the effect of multiple mutations (via the use of different autism mouse models) under rigorously controlled experimental conditions, thus reducing the confounding contribution of experimental variables that can be only minimally controlled in human research, such as genetic and environmental variability, age (Uddin et al., 2013b), ASD-related motion, and group differences in cognitive states (Vasa et al., 2016). The main goal of this line of investigation is to assess whether seemingly unrelated ASD-risk mutations do converge on a limited number of distinct functional connectivity endophenotypes. An elegant demonstration of this approach has been recently described using morpho-anatomical MRI. Brain-volumetric phenotypes of 26 ASD mouse models as defined by structural MRI methods exhibited clustering into three main groups, each with a distinct set of concomitant changes in size across different brain regions (Ellegood et al., 2015). Such reduction of morpho-anatomical heterogeneity is not surprising, given the wide (and sometimes opposing) stream of pathophysiological alterations observed in syndromic forms of autism, which range from basic molecular or synaptic mechanism such as protein synthesis (Geschwind and Levitt, 2007; Auerbach et al., 2011) up to homeostatic regulations of excitatory and inhibitory neurotransmission (Nelson and Valakh, 2015). Analogous analyses with regards to functional connectivity phenotypes should be possible in the future to associate basic pathophysiological traits with macroscale connectional aberrancies.

A second main line of investigation is the combined use of mouse rsfMRI and multiscale neurobiological techniques to obtain a mechanistic description of ASD-related phenotypes and pathophysiological pathways leading to aberrant functional connectivity. This research can include, but is not limited to, a deeper investigation of syndromic ASD mutations associated with specific pathological traits [e.g., Tuberous Sclerosis 2 as a key mediator of impaired autophagy and increased synaptic density (Tang et al., 2014)], and can possibly be extended to investigate risk factors that have been also more loosely implicated in autism. This research effort may generate crucial mechanistic information that can be used to back-translate clinical evidence of aberrant connectivity into interpretable neurophysiological events/models that can help understand, diagnose, or treat these disorders. A brief description of initial steps toward these two main goals is reported in the next two sections.

FUNCTIONAL CONNECTIVITY MAPPING IN GENETIC MODELS OF AUTISM

An outstanding question in ASD connectivity studies is whether genetic mutations associated with syndromic forms of autism are sufficient to produce aberrant macroscale functional connectivity. Initial mouse rsfMRI studies seem to corroborate this hypothesis. Specifically, Haberl and colleagues have recently investigated functional and structural connectivity in the

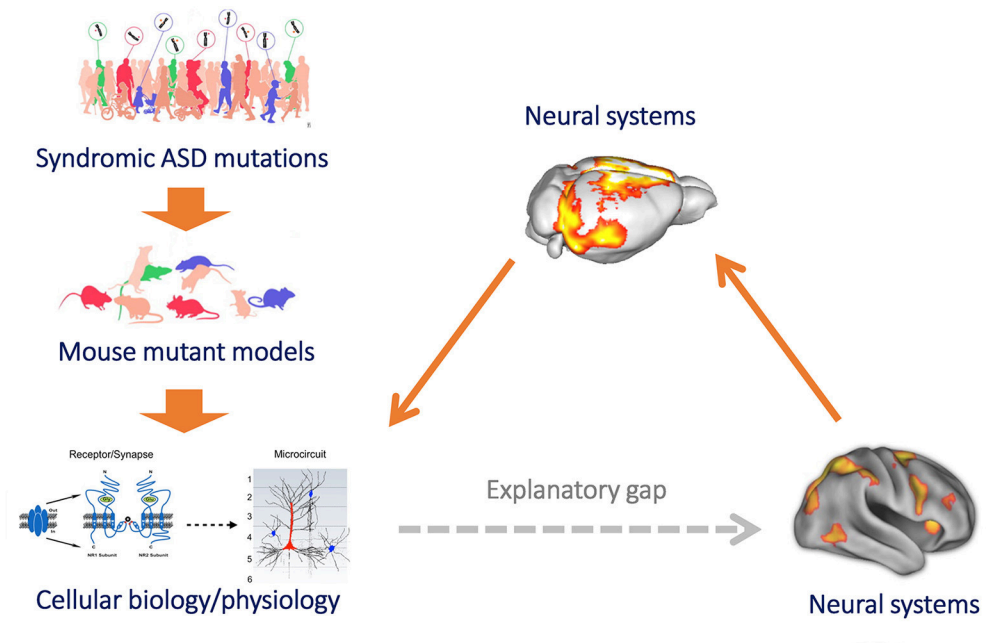

FIGURE 1 | Mouse imaging can bridge the gap between microscale models of brain function and clinical research of macroscale functional connectivity. Mouse models provide a powerful reductive platform that can be employed to link etiological determinants of ASD, such as syndromic mutation or neurodevelopmental traits, to basic molecular and cellular signatures of pathology (left, top to down). However, until recently we have been unable to use this approach to study the neurobiological underpinnings of macroscale functional connectivity, owing to difficulty in translating models of brain function across levels of inquiry. This results in a major explanatory gap between clinical research (heavily relying on macroscale neuroimaging measures of brain function, such as rsfMRI) and preclinical neurobiological investigation in rodent models (bottom, right). The implementation of functional connectivity mapping via rsfMRI in the mouse (right) can bridge this gap, by permitting to causally relate connectional changes with basic molecular or cellular processes, and by permitting a direct translation of these findings from and to humans owing to the shared biophysical principle underlying these measurements (figure adapted from Arguello and Gogos, 2012; Anticevic et al., 2013 with permission).

Fmr1$^{-/y}$ model of fragile X syndrome (FXS; Budimirovic and Kaufmann, 2011) and described connectional aberrations in sensory networks (Haberl et al., 2015). These included reduced structural integrity of the corpus callosum and an increase in local connectivity of the primary visual cortex, as probed by viral tracers, an effect accompanied by reduced rsfMRI coupling between visual and other neighboring sensory cortical regions. The authors suggested that the observed decoupling could explain sensory processing defects that are often observed in FXS patients (Boyd et al., 2010).

In another recent study, homozygous mice lacking the ASD-risk gene CNTNAP2 (Peñagarikano et al., 2011) exhibited reduced long-range and local functional connectivity in cingulate and prefrontal regions (Liska et al., 2016), two key heteromodal areas of the mouse brain previously characterized as functional connectivity hubs, owing to their rich connectivity with other brain areas (Liska et al., 2015). Interestingly, impaired antero-posterior prefrontal connectivity between components of the mouse DMN was associated with reduced social investigation, a behavioral measure regarded as a core "autism trait" in mice (Wöhr and Scattoni, 2013). This finding recapitulates analogous imaging results obtained in human carriers of CNTNAP2 gene polymorphisms (Scott-Van Zeeland et al., 2010), hence providing a first example of the translational value of this approach. This finding is consistent with the presence of impaired GABAergic neurotransmission in these animals (Peñagarikano et al., 2011), a trait that could result in aberrant oscillatory rhythms. It is interesting to note that analogous prefrontal hypo-connectivity has been observed using rsfMRI in BTBR mice, an idiopathic model of autism characterized by agenesis of the corpus callosum and by analogous excitatory/inhibitory imbalances (Sforazzini et al., 2016).

rsfMRI mapping has also been recently carried out in a mouse model of human 15q13.3 microdeletion, a CNV associated with schizophrenia, intellectual disability, and ASD (Shinawi et al., 2009). Compared to wild-type mice, 15q13.3 mice showed widespread patterns of hyper-connectivity along the hippocampal-prefrontal axis, a network commonly affected in schizophrenic patients (Gass et al., 2016). Notably, Gass and colleagues also showed that aberrant functional connectivity could be acutely rescued by pharmacological stimulation of nicotinic acetylcholine alpha 7 receptors, in keeping with a contribution of this mechanism to the development of schizophrenia-related phenotypes in these mice (Gass et al., 2016). Although the phenotypic traits of this mouse line appear to be more closely related to schizophrenia rather than to ASD (Fejgin et al., 2014), the results of this study are important as they show that CNVs and genetic alterations with partial penetrance

to ASD could produce divergent connectional phenotypes (e.g., hyper- and hypo-connectivity), suggesting a plausible contribution of genetic heterogeneity to some of the discrepant imaging findings in humans. Importantly, these initial mouse studies argue against an artifactual (e.g., motion-driven) origin of connectivity aberrations reported in human ASD research, because the use of light sedation in mice along with artificial ventilation allows for the acquisition of virtually motion-free images.

NEUROBIOLOGICAL PATHWAYS LEADING TO ABERRANT FUNCTIONAL CONNECTIVITY

A few recent studies have provided important mechanistic investigations of ASD-relevant phenotypes associated with aberrant functional connectivity. In the first of such studies, Zhan et al. (2014) investigated whether deficits in synaptic pruning, a putative pathophysiological determinant of autism (Hutsler and Zhang, 2010), result in connectivity alterations. To probe this hypothesis, the authors measured rsfMRI connectivity in $Cx3cr1^{KO}$ mice, a mouse line characterized by microglia-dependent synaptic pruning deficits as a result of deficient neuronal-microglia signaling (Paolicelli et al., 2011). Synaptic pruning deficits in $Cx3cr1^{KO}$ were found to be associated with long-range functional connectivity impairments, a finding corroborated by LFPs coherence recordings in freely-behaving animals. Interestingly, the authors also showed that impaired pruning was associated with core mouse "autism traits," and that long-range fronto-hippocampal connectivity was a good predictor of social behavior. This study is of special importance, as it was the first to suggest a role for dysfunctional synaptic maturation in shaping long-range functional synchronization and to postulate a contribution of immune system mediators to this cascade. Empirical evidence in support of this hypothesis comes from another recent study (Kim et al., 2016), where analogous phenotypes where observed in mice characterized by defective autophagy in microglia, including increased synaptic density, impaired social activity, and a trend for impaired connectivity between posterior-sensory and prefrontal regions. Similarly, Filiano et al. (2016) recently showed that deficiency in interferon-γ, a key immune signaling protein, is associated with social deficits and frontal rsfMRI hyper-connectivity in SCID mice, thus corroborating a putative mechanistic link between immune dysfunction, impaired social behavior, and functional connectivity. Although promising and mechanistically relevant, these initial results should be extrapolated to autism research with great caution, as a pathophysiological contribution of immune and microglia deficits to ASD has yet to be unambiguously demonstrated (Estes and McAllister, 2015). They, however, powerfully illustrate how the combined use of rsfMRI, mouse genetics and state-of-the-art neuro-biological approaches can elucidate pathways leading to aberrant functional connectivity, an approach that can be extended to investigate the role of multiple ASD-relevant pathophysiological factors, including syndromic genetic mutations.

LIMITATIONS AND FUTURE PERSPECTIVES

Like any other experimental approach, mouse rsfMRI is accompanied by limitations that should be taken into account when the approach is used to investigate the basis of connectivity alterations in ASD. First and foremost, as mouse rsfMRI experiments normally employ sedation to minimize stress and motion of animals during scans, the contribution of possible genotype-dependent differences in sensitivity to anesthesia (Petrinovic et al., 2016) should be controlled. The fact that to date only a minority of studies (Zhan et al., 2014; Liska et al., 2016; Sforazzini et al., 2016) have reported genotype-dependent measures of anesthesia sensitivity is a factor for concern, as differences in anesthesia depth/sensitivity can affect connectivity strength and distribution of the imaged networks (Nasrallah et al., 2014). The impact of anesthesia *per-se* as a putative modifier of intrinsic connectional architecture appears to be less of an issue, as a large body of human and rodent research shows that, under light controlled sedation, the regional patterns of functional correlation seem to be largely preserved (reviewed in Gozzi and Schwarz, 2016). As pointed out in previous work, a rigorous control of motion and physiological state is also of paramount importance to obtain reliable network mapping (Jonckers et al., 2015; Gozzi and Schwarz, 2016). It should also be mentioned that, although the field is still lacking in standardized protocols and methods that would facilitate comparison of experimental results across studies and sites, this issue is receiving increased attention and collaborative efforts are underway to address it.

The initial studies described here represent only the first step toward a greater understanding of the origin and underpinnings of connectional alterations in ASD. Future investigations are required to describe commonalities and differences between brain functional networks in the mouse and human from multiple points of view, including topology (Sporns and Betzel, 2016; van den Heuvel et al., 2016a), biological underpinnings (Richiardi et al., 2015; Wang et al., 2015; van den Heuvel et al., 2016b), and functional equivalence (Li et al., 2015). Similarly, studies of additional genetic etiologies associated with ASDs, covering heterogeneous pathophysiological pathways, are crucial to achieve a deeper understanding of whether the connectional signatures are mutation specific or can be regarded as a generalizable phenomenon. When coupled to analogous clinical efforts aimed at identification of connectional aberrancies in genetically homogeneous populations [e.g., 16p11.2 deletion (Simons Vip Consortium, 2012; Owen et al., 2014)], the method can also be used to investigate the cellular and physiological basis of clinically relevant neuroimaging readouts and, via a comparison between human and mouse imaging findings, to obtain an assessment of the translational and construct validity of mouse models of ASD. The developmental trajectory of these alterations could in principle also be investigated in mouse models, although critical limitations in the accuracy of physiological control in young mice and pups exist.

Much of mouse ASD modeling has been so far primarily addressed at monogenic ASD syndromes, which represent ∼10%

of ASDs (Silverman et al., 2010a; Nelson and Valakh, 2015). The recapitulation, in mice, of high-confidence genetic etiologies associated with ASD offers the opportunity to probe specific hypotheses about circuit dysfunction and ASD pathology that can be directly extrapolated to homologous clinical populations [e.g., 16p11.2 microdeletion (Simons Vip Consortium, 2012; Owen et al., 2014)]. An important limitation of current ASD translational research is its inability to reliably model "idiopathic" autism, which is the most frequent diagnostic label for ASD-related behavioral manifestations. Attempts to use forward genetic approaches in inbred mouse lines exhibiting ASD-like behaviors without a specific genetic determinant have been proposed, with the inbred BTBR mouse line probably being the most notable example in the field (Silverman et al., 2010b; Gogolla et al., 2014; Squillace et al., 2014). Translational relevance of neuro-behavioral findings obtained by comparing genetically homogeneous inbred lines like asocial BTBR and "normosocial" B6 mice is, however, debated (Dodero et al., 2013; Squillace et al., 2014). Nevertheless, novel neuromolecular approaches and the use of induced pluripotent stem cells (iPSCs) from patients have begun to reveal common downstream neurobiological pathways in idiopathic forms of autism characterized by shared neuroanatomical features [e.g., macrocephaly (Nicolini et al., 2015; Marchetto et al., 2016)]. Controlled manipulation of such signaling and molecular pathways in animal models is a foreseeable strategy that can be employed to expand our translational framework to the investigation of macroscale brain network aberrancies in idiopathic forms of ASD.

Finally, studies in which connectivity alterations are pharmacologically or genetically rescued may help clarify the relevance of functional connectional alterations to ASD pathology and its behavioral manifestations. Specifically, if connectivity alterations are an underlying cause of observed behavioral deficits, then behavioral phenotypic "rescue" should be accompanied by normalized patterns of brain functional connectivity. This research could indicate whether connectivity alterations are *necessary* for the expression of ASD-related behaviors in mice, or are instead an epiphenomenal manifestation of underlying pathophysiology, thus providing an empirical assessment of the pathophysiological relevance of connectivity aberrancies in ASD. "Rescue" studies may also help identify putative endo-phenotypes (complementary to behavior) that could serve as measurable readouts for early clinical translation and evaluation of novel ASD treatments in genetically defined autism syndromes (Smucny et al., 2014).

In conclusion, functional imaging of the mouse has now reached a turning point such that accurate modeling and investigation of ASD-connectivity aberrations is currently possible, via the use of readouts amenable to direct translation to human research (e.g., rsfMRI). Despite caveats, in the next few years the approach is poised to offer breakthroughs in our understanding of the pathogenesis of ASD-related connectivity aberrancies, possibly bringing some order to the intricate and often contradictory body of research detailing connectional alterations in patient populations.

AUTHOR CONTRIBUTIONS

AG conceived and wrote the manuscript with input from AL.

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Language Impairments in ASD Resulting from a Failed Domestication of the Human Brain

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Autism spectrum disorders (ASD) are pervasive neurodevelopmental disorders entailing social and cognitive deficits, including marked problems with language. Numerous genes have been associated with ASD, but it is unclear how language deficits arise from gene mutation or dysregulation. It is also unclear why ASD shows such high prevalence within human populations. Interestingly, the emergence of a modern faculty of language has been hypothesized to be linked to changes in the human brain/skull, but also to the process of self-domestication of the human species. It is our intention to show that people with ASD exhibit less marked domesticated traits at the morphological, physiological, and behavioral levels. We also discuss many ASD candidates represented among the genes known to be involved in the "domestication syndrome" (the constellation of traits exhibited by domesticated mammals, which seemingly results from the hypofunction of the neural crest) and among the set of genes involved in language function closely connected to them. Moreover, many of these genes show altered expression profiles in the brain of autists. In addition, some candidates for domestication and language-readiness show the same expression profile in people with ASD and chimps in different brain areas involved in language processing. Similarities regarding the brain oscillatory behavior of these areas can be expected too. We conclude that ASD may represent an abnormal ontogenetic itinerary for the human faculty of language resulting in part from changes in genes important for the "domestication syndrome" and, ultimately, from the normal functioning of the neural crest.

Keywords: autism, domestication, language evolution, neural oscillations, language deficits

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INTRODUCTION

Autism spectrum disorders (ASD) are pervasive neurodevelopmental conditions characterized by several and severe cognitive and social deficits, including language and communication problems, repetitive and stereotypical behavior, and problems with social interaction (Bailey et al., 1996). In DSM-V, language deficits are no longer explicitly postulated as a central feature of ASD because they are subsumed in its distinctive communication problems. Nevertheless, it is clear that ASD entails a typical language profile and language developmental path (reviewed in Benítez-Burraco and Murphy, 2016; see also Tager-Flusberg et al., 2005; Tager-Flusberg, 2006; Eigsti et al., 2007; Bourguignon et al., 2012). Because of the masking effect of a variable IQ, and the variable degree of

functionality exhibited by ASD patients, it is difficult to hypothesize a core language deficit in this condition. The impairment of the oromotor function has been claimed to account for expressive language problems in some autistic subjects (Belmonte et al., 2013). Comprehension problems seemingly result from other underlying deficit(s), including a reduced effect of semantic priming (Preissler, 2008), problems with phonological processing (Lindgren et al., 2009), or impairment of procedural memory (Walenski et al., 2006).

At the neural level, ASD entails atypical development, wiring and interconnection of areas involved in language processing (Stefanatos and Baron, 2011; Bourguignon et al., 2012). Not surprisingly, functional differences in language processing tasks of ASD compared with unaffected subjects have been attested as well (Courchesne and Pierce, 2005; Scott-Van Zeeland et al., 2010a,b). For instance, microstructural anomalies and reduced lateralization patterns have been observed in the arcuate fasciculus of ASD patients (Fletcher et al., 2010), suggesting that a constraint on the integrative processes during development may contribute to language impairment in this condition (Schipul et al., 2011). We also wish to highlight both increased and decreased intra- and inter-hemispheric connectivity (Hahamy et al., 2015), and abnormal responses to linguistic stimuli (reviewed in Stefanatos and Baron, 2011, pp 259–262). Intriguingly, the ASD phenotype is characterized by increased intrinsic functional connectivity during the first years of life (the time window where language is acquired) and reduced connectivity in adolescent and adult states (Uddin et al., 2013).

In spite of this growing body of neurobiological data, a comprehensive view of language processing in the ASD brain is still lacking. Specifically, ASD studies need to move beyond simplistic models of language processing and focus instead on how collections of brain areas jointly engaged in specific, impaired cognitive operations (see Fedorenko and Thompson-Schill, 2014, for a general discussion). This is a real challenge, provided that abnormal brain profiles are not expected to easily map on to anomalous categories or computations of linguistic theories (see Poeppel, 2012; Murphy, 2016a, for discussion). We have recently proposed a translational theory of language deficits in ASD as amounting to abnormal patterns of brain rhythms (Benítez-Burraco and Murphy, 2016); although a clarification and empirical validation of this hypothesis is still pending.

Finally, we wish to emphasize that ASD has been associated with sequence variants, copy number variation (CNVs), and/or changes in the expression patterns of an extensive number of genes (Geschwind and State, 2015). Despite the remarkable genetic heterogeneity, it is noteworthy that all these genes tend to converge on specific pathways and neural mechanisms, functionally relevant in this condition and expected to account for its associated deficits (Willsey and State, 2015). Specifically, several candidates for language impairment in ASD have been proposed, including *MET*, *CTTNBP2*, *EN2*, *NBEA*, *HRAS*, and *PTEN* (Comings et al., 1996; Naqvi et al., 2000; Cheung et al., 2001; Castermans et al., 2003; Benayed et al., 2005; Campbell et al., 2006). Nonetheless, the gap between genes and language deficits in ASD still remains open (see Jeste and Geschwind, 2014, for a general discussion, and Benítez-Burraco and Murphy, 2016, for a specific discussion on candidates for language dysfunction in ASD).

The aim of this paper is to contribute to the bridging of the gap between the genetic backdrop and language deficits observed in ASD. To this end, we will primarily focus on language evolution. There exists a strong, deep link between evolution and (abnormal) development. Recently-evolved neural networks seem to be more sensitive to damage because of their lower levels of resilience (Toro et al., 2010). As a consequence, aspects of brain development and function that are preferably impaired in modern populations are expected to be involved in recently evolved, human-specific cognitive abilities. Some comprehensive accounts of the human condition set against the cognitive profiles of other primates have been recently put forth (Seed and Tomasello, 2010; Platt et al., 2016). Comparative genomics also provides valuable information about the sources of the observed differences and similarities in the human genome (Rogers and Gibbs, 2014; Franchini and Pollard, 2015). Likewise, we are beginning to achieve an advanced understanding of the genetic changes that occurred after our split from extinct hominins (Pääbo, 2014; Zhou et al., 2015). We expect that the same factors that prompted the transition from an ape-like cognition to our specific mode of cognition are involved in the etiology of cognitive disorders involving language deficits and, particularly, of ASD (see Benítez-Burraco, 2016a, for a general discussion).

In what follows, the focus is placed on one aspect of this evolutionary process: the self-domestication of the human species. At present, we have a decent understanding of how our language-readiness (that is, our species-specific ability to learn and use language) may have evolved. Accordingly, among the changes brought about by human evolution, one very relevant aspect is the ability to transcend (better than other species) the signature limits of core knowledge systems and thus go beyond modular boundaries (Mithen, 1996; Spelke, 2003; Carruthers, 2006; Hauser, 2009; Boeckx, 2011; Wynn and Coolidge, 2011). As hypothesized in Boeckx and Benítez-Burraco (2014a), our language-readiness boils down to this enhanced cognitive ability, but also to its embedding inside cognitive systems responsible for interpretation (*thought*) and externalization (*speech*). This language-readiness was seemingly brought about by specific changes in the skull/brain developmental path (resulting in a more globular brain), which entailed new patterns of long-distance connections among distributed neurons and, ultimately, new patterns of brain rhythmicity, including an adequate degree and pattern of cortical inhibition. Interestingly, brain rhythms are heritable components of brain function (Linkenkaer-Hansen et al., 2007; Hall et al., 2011) and have been linked to computational primitives of language (Murphy, 2015a,b, 2016a), allowing for a good explanation (and not just a description) of linguistic computation (and of language deficits) at the brain level, and specifically, for a satisfactory mapping of language deficits to neural dysfunction and its genetic basis in ASD (Benítez-Burraco and Murphy, 2016). We have found many candidates for ASD among the genes known to be involved in the emergence of language-readiness (Benítez-Burraco and Boeckx, 2015).

At the same time, the emergence of modern-like languages (and perhaps of core aspects of language too) was seemingly favored by changes in the cultural niche of our ancestors. The archeological record shows that cognitive modernity (encompassing language-readiness) did not automatically entail behavioral modernity (seemingly resulting from using fully-fledged languages), which only appeared long after the emergence of anatomically-modern humans (AMHs) together with changes in human cultural dynamics. Current linguistic research has shown that aspects of linguistic complexity (including core aspects of grammatical knowledge) correlate with aspects of social complexity (Wray and Grace, 2007; Lupyan and Dale, 2010). Moreover, core properties of human languages (like duality of patterning) can develop in response to environmental pressure, as research into emergent sign languages has nicely illustrated, implying that they cannot be regarded as part of the biological endowment (see Benítez-Burraco, 2016b, for discussion). Importantly, language acquisition by the child demands a prolonged socialization window that enables her to receive the proper amount of triggering stimuli and to interact with other conspecifics. All this means that the intrinsic cognitive machinery may be not enough for granting the acquisition of a successful tool for linguistic cognition and that the environment has to be of the right kind too (see Sterelny, 2011 on behavioral modernity set against cognitive modernity).

It has been hypothesized that the social conditions (or the *cultural niche*) that facilitated the enhancement of linguistic structure through a cultural mechanism were brought about by a process of human self-domestication (see Thomas, 2014, for details, and Hare and Tomasello, 2005; Deacon, 2009, on relaxed selective pressures resulting from self-domestication as explanations of the emergence of key aspects of behavioral modernity). Different factors may have contributed to human self-domestication, from adaptation to the human-made environment to selection against aggression to sexual selection. We have hypothesized (Benítez-Burraco et al., in press) that the very changes that brought about our globular skull/brain and our language-readiness may have also fuelled the emergence of a (self-domesticated) phenotype in the human species. Accordingly, we have found numerous links between the candidates for globularization and language-readiness, and genes important for the development and function of neural crest cells (NCC). Indeed, the hypofunction of the neural crest (NC) has been claimed to account for the constellation of distinctive traits observed in domestic mammals (the "domestication syndrome") (Wilkins et al., 2014).

Because of the deep link between evolution and development, we expect that examining the signatures of the domesticated phenotype in people with ASD contributes to a better understanding of etiology of ASD, and specifically, of language deficits in this condition. In a recent paper Reser (2014) found similarities between autism and species of solitary mammals. Although the focus was put on behavior, the author suggests that future research will benefit from investigating the neurobiological, genetic and epigenetic causes of these similarities. Here we try to push research in this direction. The paper is structured as follows. First, we provide a general account of the domesticated traits that are absent or attenuated in ASD. Then we move to the genes and focus on candidates for ASD that are found among the set of genes involved in the domestication syndrome and the evolution of language-readiness, as characterized in Benítez-Burraco et al. (in press), showing that they exhibit a distinctive expression profile in the brain of autists. Finally, we compare the ASD phenotype with wild primates, focusing on the expression profile of these genes, but also on oscillatory signatures of areas important for language processing, considering that language impairment in ASD can be interpreted as an "oscillopathic" condition (see Benítez-Burraco and Murphy, 2016). We will conclude that ASD (and language deficits in ASD) can be viewed as an abnormal ontogenetic itinerary for the human faculty of language, resulting in part from changes in genes important for the domestication syndrome and seemingly from changes in the normal functioning of the NC.

DOMESTIC TRAITS IN THE ASD PHENOTYPE

Wilkins et al. (2014) provide a comprehensive summary of traits known to be modified in domesticated mammals, many of them concerning the cranial region. These include changes in ear size and shape, changes in the orofacial area (including shorter snouts and smaller jaws), changes in dentition (particularly, smaller teeth), and a reduced brain capacity (specifically, of components of the forebrain such as the amygdala or parts of the limbic system). Other distinctive traits commonly found in domesticated strains are depigmentation, neoteny, shorter reproductive cycles, and increased docility, which is thought to result from adrenal size reduction and adrenal hypofunction as well as from reduced levels of stress hormones (including adrenocorticoids, adrenocorticotropic hormone, cortisol, and corticosterone). This delayed adrenal maturation also involves a hypofunction of the sympathetic nervous system and an increase of the duration of the immaturity of the hypothalamic-pituitary-adrenal system (the HPA axis), which provides the animal with a longer socialization window. According to Wilkins et al. (2014), the multiple phenotypic traits that characterize the domestication syndrome emerge as unselected by-products from a developmental reduction in NCC inputs, resulting from selection for tameness. Interestingly, compared to extinct hominins, AMHs exhibit a number of domesticated traits, including reduced brains (at least during the last 50,000 years), changes in dentition, reduction of aggressiveness, and retention of juvenile characteristics (see Thomas, 2014, for details). Intriguingly, most of these features are generally attenuated in ASD (**Figure 1**).

To begin with, ASD subjects show significant differences with healthy controls regarding minor physical anomalies, particularly in the craniofacial region (assumed to result from deviations during fetal development and suggested to constitute external markers of atypical brain growth) (Tripi et al., 2008; Manouilenko et al., 2014). Specifically, in adults the abnormal shape of the ears is robustly associated with autistic traits, with

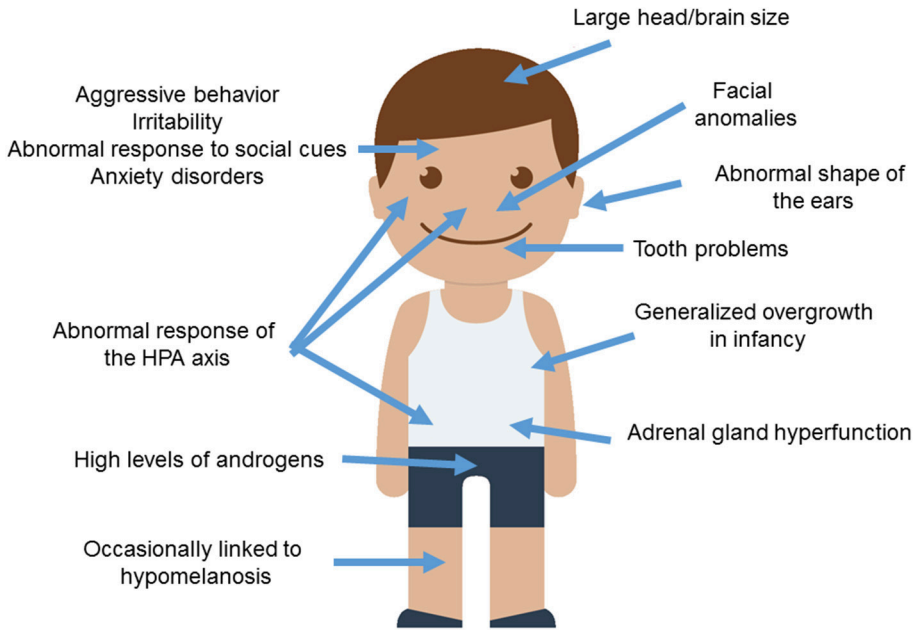


FIGURE 1 | Anomalous presentation of domesticated traits in people with ASD. Main clinical features observed in ASD patients and concerning the domestication syndrome are shown. The child diagram was gathered from Iconfinder output (available at http://www.iconfinder.com/icons/525448/boy_child_kid_male_man_person_white_icon).

higher scores correlating with poorer functioning (Manouilenko et al., 2014). Regarding the changes in the orofacial region, prepubertal boys with ASD show significant differences in facial morphology compared to typically developing (TD) boys (Aldridge et al., 2011). This distinctive facial phenotype is more pronounced in subjects with severe symptoms, significant cognitive impairment, and language regression (Obafemi-Ajayi et al., 2015). Concerning tooth peculiarities, children with ASD show greater abnormalities in dentition, including missing teeth, diastemas, or reverse overjets (Luppanapornlarp et al., 2010). With respect to brain size, head circumference is significantly larger in people with ASD, with nearly 15% suffering from macrocephaly. Higher brain volumes correlate with lower functioning abilities; indeed nearly 9% ASD individuals exhibit brain overgrowth (Sacco et al., 2015). It is worth noticing that higher head circumference and brain size values are observed only during early childhood (Fukumoto et al., 2008; Courchesne et al., 2011, although see Raznahan et al., 2013), particularly when ASD is presented with regression (Nordahl et al., 2011). Typically, early brain overgrowth is followed by a decrease in structural volumes (Courchesne et al., 2011). Although brain overgrowth may result from a dysregulation of the overall systemic growth (see below), it is thought to impact on cognition. This is believed to occur as a result of the reduced networking efficiency among widespread regions of the cortex, due to the increased long-distance connections (Lewis et al., 2013). Specifically, people with ASD show increased volumes of the amygdala (Mosconi et al., 2009; Murphy et al., 2012), which correlate with the severity of their social and communication impairments (Schumann et al., 2009). In the TD population, higher amygdala volumes are associated with poorer language abilities in infancy (Ortiz-Mantilla et al., 2010).

Regarding the behavioral traits associated with the domestication syndrome, we wish to highlight that aggressive behaviors are frequent in children with ASD (with about 25% of them having scores in the clinical range), and correlate with lower cognitive outcomes (Hill et al., 2014). Children with ASD display more reactive than proactive aggression attitudes (Farmer et al., 2015). Likewise, irritability is also commonly observed in affected individuals (Mikita et al., 2015). Additionally, ASD is commonly found to be comorbid with generalized anxiety disorder (Hollocks et al., 2014; Bitsika et al., 2015). Several studies have been carried out to learn more about the physiological basis of this anomalous response to the social environment. Interestingly, higher serum cortisol responses are usually found in children with ASD, particularly after stressor stimulation, when prolonged duration and recovery of cortisol elevation is also observed (Spratt et al., 2012). Moreover, children with ASD show a distinctive diurnal rhythm of cortisol compared to their TD peers; this involves elevated cortisol levels at the end of the day and dampened linear decline across the day in some children (Tomarken et al., 2015). Dysregulation of the diurnal rhythm as a whole has been found in low functioning ASD (Taylor and Corbett, 2014). Also, anxiety symptoms correlate with high cortisol levels in ASD pediatric patients (Bitsika et al., 2015). Plasma levels of adrenocorticotropic hormone are also significantly higher in children with ASD, and correlate positively with the severity of the symptoms (Hamza et al., 2010). The HPA axis in ASD responds in a more sluggish way to physiological or physical manipulation. Accordingly, Taylor

and Corbett (2014) found hyper-responsiveness of the HPA axis when unpleasant stimuli or relatively benign social situations are involved, whereas they observed hypo-responsiveness in conditions involving social evaluative threat. On the whole, the HPA axis may be more reactive to stress in social anxiety disorder and ASD (Spratt et al., 2012; Jacobson, 2014). Because children with autism and anxiety disorders show a blunted cortisol response to psychosocial stress, and given that reduced cortisol responsiveness is significantly related to increased anxiety symptoms, Hollocks et al. (2014) suggested that a non-adaptive physiological response to psychosocial stress may exist in ASD.

Finally, it is worth considering some other traits commonly observed in domesticated mammals: neoteny, alterations of reproductive cycles, and pigmentation changes. Regarding neotenic features, it is noteworthy that children with ASD exhibit an early generalized overgrowth (van Daalen et al., 2007; Fukumoto et al., 2008; Chawarska et al., 2011). Typically, boys with ASD show increased body size at birth and during infancy, with postnatal overgrowth correlating with lower adaptive functioning, greater severity of social deficits, and poorer verbal skills (Chawarska et al., 2011; Campbell et al., 2014). Interestingly, higher levels of androgens are found in children and adolescents with ASD. This correlates with the severity of autistic traits and might account for the precocious puberty also reported in this condition (El-Baz et al., 2014). These findings emphasize the role of elevated pre- and postnatal testosterone levels in the liability for ASD (see Hauth et al., 2014). Testosterone significantly affects brain development, particularly targeting the hypothalamus, the amygdala and the hippocampus, impacting on aspects of memory consolidation (Filová et al., 2013). High perinatal testosterone concentration negatively correlates with early vocabulary development in TD boys (Hollier et al., 2013). Interestingly, children with elevated androgen levels due to congenital adrenal hyperplasia show atypical patterns of brain asymmetry in the perisylvian areas, and language/learning disabilities (Plante et al., 1996). Less data on reproductive functions in females is available, due to the lower prevalence of ASD among women. Nevertheless, women with ASD reported significantly more irregular menstrual cycles and dysmenorrhea (Ingudomnukul et al., 2007; Hamilton et al., 2011). Likewise, an increase in premenstrual syndrome has been observed in women with ASD (Obaydi and Puri, 2008; Hamilton et al., 2011), who are more likely to exhibit behavioral issues related to the onset of periods (Burke et al., 2009). In addition, delayed age of menarche seems to correlate with the severity of autistic traits (Hergüner and Hergüner, 2016). These findings lend support to the androgen theory of ASD, according to which elevated levels of testosterone during fetal development may contribute to the development of ASD. Finally, concerning changes in pigmentation, it is of interest that hypomelanotic diseases usually entail autistic symptoms, as is commonly observed in hypomelanosis of Ito (OMIM#300337; Akefeldt and Gillberg, 1991; von Aster et al., 1997; Gómez-Lado et al., 2004). It has been hypothesized that the comorbidity between hypomelanosis and ASD may result from a deficiency in vitamin D (Eyles, 2010; Bakare et al., 2011). In fact, core symptoms of ASD improve after vitamin D supplementation (Jia et al., 2015). Interestingly, core candidates for the globularization of the AMH skull/brain and the evolution of language-readiness are involved in vitamin D homeostasis and function (see Benítez-Burraco and Boeckx, 2015, for details).

As noted above, regardless of the different selectionist scenarios that may account for the traits commonly found in domesticated mammals, a role for NC hypofunction during embryonic development has been proposed (see Wilkins et al., 2014, for details). No comprehensive view of the role (if any) of the NC in the aetiopathogenesis of ASD has been provided to date. Still, it is important to note that neurocristopathies (that is, conditions resulting from NC defects) commonly involve autistic features. For instance, in CHARGE syndrome (OMIM#214800) autistic traits coexist with developmental abnormalities affecting endocrine, reproductive, urinary and digestive systems, along with skeletal and craniofacial features (Fernell et al., 1999). Given this background, we will now examine whether candidates for ASD are overrepresented among the genes believed to play a central role in NC development and function, with a special emphasis on those that interact with genes important for the globularization of the AMH skull/brain.

ASD AND THE GENETICS OF THE DOMESTICATION SYNDROME

In order to improve our characterization of the domesticated traits in ASD, it is of interest to assess whether candidate genes for this condition (with a particular emphasis on language disabilities) are overrepresented among, or are functionally related to, candidates for domestication. We have relied on an extended list of candidates, which includes the core set of genes proposed by Wilkins et al. (2014), plus a subset of the genes involved in the globularization of the AMH skull/brain and the emergence of language-readiness that are functionally related to them, through direct interaction, and/or that play a role in the development and function of the NC (see Benítez-Burraco et al., in press, for details). Our list also comprises NC-related genes known to play a key role in craniofacial development and/or disorders. As noted above, most of the domesticated traits result from the modification of the cranial region and many of the ASD distinctive features concern the skull, face and brain. Moreover, as reasoned in Boeckx and Benítez-Burraco (2014a,b), we expect that our language-readiness resulted from changes in the development of the skull/brain, but also from the refinement of the externalization devices, specifically, the orofacial region: As also noted above, the impairment of oromotor function has been hypothesized to account for some language deficits in ASD. **Table 1** provides a full list and a schematic characterization of these genes.

When we tried to identify ASD-candidates among this extended list of genes via PubMed (http://www.ncbi.nlm.nih.gov/pubmed), we found out that nearly 25% of them have been suggested to play a role in the aetiopathogenesis of ASD. If we also consider genes that we found differentially expressed in postmortem brain tissues isolated from patients (as discussed

TABLE 1 | List of putative candidate genes for domestication and ASD.

Gene symbol	Gene name	Domestication[a]	Language-readiness[b]	NCC[c]	Craniofacial[d]	Brain rhythmicity[e]	ASD	
							Candidate[f]	Differentially expressed[g]
ALX1	Aristaless-like homeobox protein 1			+	+			
ALX3	Aristaless-like homeobox protein 3			+	+			
ALX4	Aristaless-like homeobox protein 4			+	+			
AXIN2	Axin 2			+	+			+
BAZ1B	Bromodomain adjacent to zinc finger domain 1B	+		+				
BMP2	Bone morphogenetic protein 2	+	+	+	+			
BMP7	Bone morphogenetic protein 7	+	+	+				+
CDC42	Cell division cycle 42		+	+				+
CHD7	Chromodomain helicase DNA binding protein 7	+		+			+	+
CITED2	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2		+	+				+
CTNNB1	Catenin Beta 1		+	+			+	+
DLX1	Distal-less homeobox 1		+	+				+
DLX2	Distal-less homeobox 2	+	+	+				
DLX5	Distal-less homeobox 5	+	+	+	+	+	+	
DLX6	Distal-less homeobox 6		+	+	+	+	+	
EDN1	Endothelin 1			+	+			+
EDN3	Endothelin 3	+		+				+
EDNRA	Endothelin receptor type A			+	+			+
EDNRB	Endothelin receptor type B	+		+				+
ERF	ETS2 repressor factor			+	+			+
FGF7	Fibroblast growth factor 7		+	+				
FGF8	Fibroblast growth factor 8	+	+	+				+
FGFR1	Fibroblast growth factor receptor 1	+	+	+	+			
FGFR2	Fibroblast growth factor receptor 2			+	+		+	
FOXD3	Forkhead box D3	+		+			+	
FOXP2	Forkhead box P2	+	+					
FREM1	FRAS1 related extracellular matrix 1			+	+			
GDNF	Glial-derived neurotrophic factor	+		+				
GLI3	GLI family zinc finger 3	+	+	+	+			
GRHL3	Grainyhead like transcription factor 3			+	+			
GSC	Goosecoid homeobox			+	+			
HES1	Hes family bHLH transcription factor 1	+	+	+				+
HOXA2	Homeobox A2			+	+	+		
HSH2D	Hematopoietic SH2 domain containing			+	+			
KIT	KIT proto-oncogene receptor tyrosine kinase	+		+			+	+
MAGOH	Mago homolog, exon junction complex core component	+		+				+
MITF	Microphthalmia-associated transcription factor	+		+				+
MSX1	Msh homeobox 1			+	+		+	+
MSX2	Msh homeobox 2			+	+			+
NCAM1	Neural cell adhesion molecule 1	+	+				+	+
NODAL	Nodal growth differentiation factor	+	+					+
NOG	Noggin			+	+			
NTN1	Netrin 1			+	+			+

(Continued)

TABLE 1 | Continued

Gene symbol	Gene name	Domestication[a]	Language-readiness[b]	NCC[c]	Craniofacial[d]	Brain rhythmicity[e]	ASD	
							Candidate[f]	Differentially expressed[g]
PAX3	Paired box 3	+	+	+				
PAX6	Paired box 6		+	+		+	+	+
PAX7	Paired box 7				+			+
POLR1A	Polymerase (RNA) I subunit A				+		+	
POU3F2	POU class 3 homeobox 2	+	+				+	+
PQBP1	Polyglutamine binding protein 1	+	+					+
PTCH1	Patched 1			+	+		+	+
RET	Ret proto-oncogene	+		+				+
ROBO1	Roundabout guidance receptor 1		+	+				+
ROBO2	Roundabout guidance receptor 2		+	+			+	
RUNX2	Runt related transcription factor 2	+	+	+	+			
SATB2	Special AT-rich sequence binding-homeobox 2		+	+				+
SHH	Sonic hedgehog	+	+	+	+			
SIX2	Sine oculis-related homeobox 2			+	+			
SLIT1	Slit guidance ligand 1		+	+				
SLIT2	Slit guidance ligand 2		+	+				+
SOX2	Sex determining region Y-box 2	+	+	+				+
SOX9	Sex determining region Y-box 9	+	+	+	+		+	+
SOX10	Sex determining region Y-box 10	+	+	+			+	
SPECC1L	Sperm antigen with calponin homology and coiled-coil domains 1-like			+	+			+
TCF12	Trascription factor 12			+	+			+
TCOF1	Treacle ribosome biogenesis factor 1	+		+				
VCAN	Versican		+	+				+
ZIC1	Zinc finger protein family member 1			+	+			+

[a] *Core candidates for the "domestication syndrome" according to Wilkins et al. (2014) (bold italicized tags) plus language-readiness genes functionally interacting with them according to Benítez-Burraco et al. (in press) (regular tags).*

[b] *Genes highlighted as candidates for globularization of the AMH skull/brain and the emergence of language-readiness according to Boeckx and Benítez-Burraco (2014a,b) and Benítez-Burraco and Boeckx (2015).*

[c] *Involved in neural crest (NC) development and function.*

[d] *Involved in craniofacial development and/or found mutated in craniofacial syndromes.*

[e] *Involved in brain oscillation and rhythmicity.*

[f] *Candidate for ASD as resulting from genomic studies (pathogenic SNPs, association studies, CNVs, functional studies, etc.).*

[g] *Differentially expressed in postmortem brain tissues of ASD-vs.-control individuals (see text for details).*

in the subsequent section), the percentage rises above 50%. Interestingly, some of these genes are thought to be involved in brain rhythmicity (see **Table 1**), plausibly contributing to the oscillopathic signature of the ASD brain during language processing.

We expect that the genes we highlight here are functionally interconnected and map on to specific pathways, signaling cascades, or aspects of brain development and function, of interest for language processing and the aetiopathology of ASD. *In silico* analyses offer promising insights. Accordingly, String 10 (http://www.string-db.org) predicts quite robust links between most of these genes (**Figure 2**). Likewise, ontology analyses by Panther (http://www.pantherdb.org) suggest that they might play biological functions important for ASD and be part of signaling pathways known to be impaired in this condition (**Table 2**).

Candidate Genes: A Functional Characterization

Some of Wilkins et al.'s (2014) original candidates for the domestication syndrome are candidates for ASD. *KIT* mutations have been found in patients featuring ASD symptoms (Kilsby et al., 2013). KIT is a tyrosine kinase receptor (Kasamatsu et al., 2008), which acts as a key developmental regulator in the NC-derived processes of hematopoiesis, melanogenesis, and gametogenesis (Rothschild et al., 2003). In rats mutations of *Kit* impair hippocampal synaptic potentiation and spatial learning and memory (Katafuchi et al., 2000). Likewise, whole-genome sequencing analyses have identified deleterious variants of *CHD7* in ASD probands (Jiang et al., 2013). *CHD7* is known to be the main candidate for CHARGE syndrome (Vissers et al., 2004; Lalani et al., 2006), mentioned above. Interestingly, CHARGE

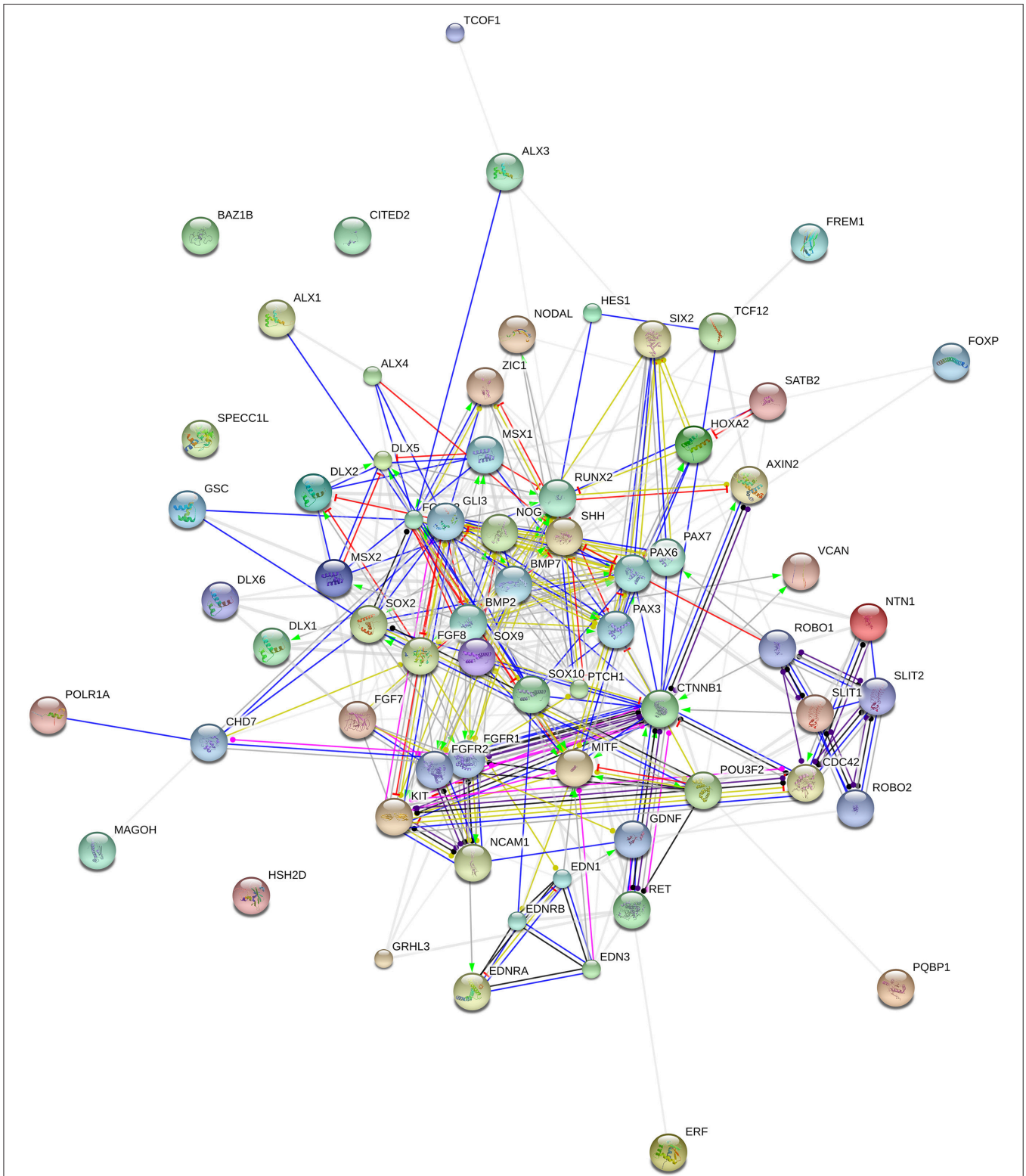


FIGURE 2 | Functional links among candidates for domestication and ASD. The chart (drawn using String 10.0 license-free software, http://string-db.org/) shows the network of known and predicted interactions among all genes listed in **Table 1**. The colored nodes symbolize proteins: small nodes are proteins with unknown 3D structure, while large nodes are those with known 3D structures. The color of the edges represent different kind of known protein-protein associations. Green: activation, red: inhibition, dark blue: binding, light blue: phenotype, dark purple: catalysis, light purple: posttranslational modification, black: reaction, yellow:

(Continued)

FIGURE 2 | Continued
transcriptional regulation. Edges ending in an arrow symbolize positive effects, edges ending in a bar symbolize negative effects, whereas edges ending in a circle symbolize unspecified effects. Gray edges symbolize predicted links based on literature search ((co-mention in PubMed abstracts). Stronger associations between proteins are represented by thicker lines. The medium confidence value was 0.0400 (a 40% probability that a predicted link exists between two enzymes in the same metabolic map in the KEGG database: http://www.genome.jp/kegg/pathway.html). String 10 predicts associations between proteins that derive from a limited set of databases: genomic context, high-throughput experiments, conserved coexpression, and the knowledge previously gained from text mining (Szklarczyk et al., 2015). This is why the figure does not represent a fully connected graph (evidence for additional links are provided in the main text). Importantly, the diagram only represents the potential connectivity between the involved proteins, which has to be mapped onto particular biochemical networks, signaling pathways, cellular properties, aspects of neuronal function, or cell-types of interest that can be confidently related to aspects of language development and function.

TABLE 2 | GO classifications of candidates for domestication and ASD.

Biological process	%[a]	Pathway	
Metabolic process (GO:0008152)	21.60%	Axon guidance mediated by Slit/Robo (P00008)	12.20%
Biological regulation (GO:0065007)	18.80%	TGF-beta signaling pathway (P00052)	10.20%
Developmental process (GO:0032502)	17.00%	Endothelin signaling pathway (P00019)	8.20%
Cellular process (GO:0009987)	13.80%	Gonadotropin releasing hormone receptor pathway (P06664)	8.20%
Multicellular organismal process (GO:0032501)	11.50%	Wnt signaling pathway (P00057)	8.20%
Immune system process (GO:0002376)	4.10%	FGF signaling pathway (P00021)	8.20%
Apoptotic process (GO:0006915)	3.20%	Angiogenesis (P00005)	6.10%
Response to stimulus (GO:0050896)	3.20%	Hedgehog signaling pathway (P00025)	6.10%
Biological adhesion (GO:0022610)	3.20%	Axon guidance mediated by netrin (P00009)	4.10%
Localization (GO:0051179)	1.80%	CCKR signaling map (P06959)	4.10%

[a]*Numbers refer to percent of gene hit against total of process or pathway hits. Only the top 10 functions, filted after Bonferroni post-hoc correction, have been included.*

syndrome also involves microcephaly, face asymmetry, cleft lip/palate, along with variable degrees of intellectual disability (Pisano et al., 2014; Hale et al., 2016, for review). Changes in the expression pattern of *CDH7* can also result in behavioral anomalies resembling the autistic phenotype. Accordingly, *in utero* exposure to heavy metals in mice increases autism-like behavioral phenotypes in adult animals through inducing the hypomethylation of *Chd7* (Hill et al., 2015). *FOXD3,* encoding a transcription factor, is downregulated by DISC1 (Drerup et al., 2009), a robust candidate for schizophrenia that has been also associated to ASD (Williams et al., 2009; Zheng et al., 2011; Kanduri et al., 2016). *FOXD3* maps within one of the present-day human-specific differentially-methylated genomic regions (DMRs) (Gokhman et al., 2014). Interestingly, loss of *Disc1* results in abnormal NCC migration and differentiation (Drerup et al., 2009). Also, DISC1 downregulates *SOX10*, another NC gene, involved in the maintenance of precursor NCC pools, in the timing of NCC migration onset, and in the induction of their differentiation; it is also implicated in oligodendrocyte differentiation (Hattori et al., 2014). In turn, SOX10 interacts with PAX3, another core candidate proposed by Wilkins et al. (2014), and with POU3F2 (Smit et al., 2000). Sequence and CNVs affecting *POU3F2* have been found in subjects with ASD, and in individuals with different developmental and language delays (Huang et al., 2005; Lin et al., 2011). POU3F2 is a known interactor of *FOXP2*, the renowned "language gene" (Maricic et al., 2013). AMHs bear a derived allele of the binding site which is less efficient in activating transcription than the Neanderthal/Denisovan counterpart (Maricic et al., 2013). Likewise, *POU3F2* has been associated with human accelerated conserved non-coding sequences (haCNSs) (Miller et al., 2014). Also, it interacts with PQBP1, which has been linked to intellectual disability (Wang et al., 2013) and developmental delay and microcephaly (Li et al., 2013). Also *SOX9*, considered a master regulator of craniofacial development and related to several congenital skeletal malformations (Mansour et al., 2002; Gordon et al., 2009; Lee and Saint-Jeannet, 2011), is found among the candidates for ASD.

Accordingly, gene and miRNA expression profiling using cell-line derived total RNA has revealed *SOX9* as one of the genes dysregulated in ASD (Ghahramani Seno et al., 2011). As discussed in detail by Benítez-Burraco et al. (in press) *SOX9* interacts with *BMP2*, *BMP7*, *DLX2*, and *HES1*. All of them are core components of the network believed important for globularization and language-readiness (reviewed in Boeckx and Benítez-Burraco, 2014a). In addition, all of them are involved in NCC development and migration, and in the patterning of NC-derived tissues (Mallo, 2001; Gajavelli et al., 2004; Correia et al., 2007; Glejzer et al., 2011; Ishii et al., 2012). *BMP2* is a key osteogenic regulator, which has been associated to craniosynostosis (Justice et al., 2012; Lattanzi et al., 2013). *BMP2*, *BMP7*, and *DLX2* act upstream *SOX9* (Sperber et al., 2008; Li et al., 2013). In turn, *SOX9* mediates the retinoic acid-induced expression of *HES1*, known also to be involved in language function, craniofacial development, and neuron growth and interconnection (reviewed in Boeckx and Benítez-Burraco, 2014b). Importantly, retinoic acid also regulates the expression of other genes that are relevant for language, like *FOXP2* (Devanna et al., 2014), or for globularization, like *ASCL1* (see Benítez-Burraco and Boeckx, 2015, for details). Retinoic acid has proven to be important for brain plasticity (Luo et al., 2009), and memory and learning processes (Etchamendy et al., 2003; Jiang et al.,

2012). Recent whole-exome sequencing analyses have linked retinoic acid regulation pathways to ASD (Moreno-Ramos et al., 2015). In neuronal cells reduced levels of RORA downregulate multiple transcriptional targets that are significantly enriched in biological functions negatively impacted in ASD and which include known ASD-associated genes, like *A2BP1*, *CYP19A1*, *ITPR1*, *NLGN1*, and *NTRK2* (Sarachana and Hu, 2013a). *RORA* itself is downregulated in postmortem prefrontal cortex and cerebellum of subjects with ASD (Nguyen et al., 2010). *RORA* is differentially regulated in them by masculine and feminine hormones: Whereas it is under negative feedback regulation by androgens, it is under positive regulation by estrogens (Sarachana et al., 2011; Sarachana and Hu, 2013b). In certain regions of the brain this sexually dimorphic expression is also found in several of RORA's targets and this correlation is much higher in the cortex of males (Hu et al., 2015). Perhaps not surprisingly, synthetic RORα/γ agonist improve autistic symptoms in animal models of the disease, particularly, repetitive behavior (Wang et al., 2016).

We wish to highlight two other genes thought to be involved in the changes that brought about modern language that are also candidates for ASD and interact with core candidates for the domestication syndrome as posited by Wilkins et al. The first one is *DLX5*, involved in crucial aspects of NC development (McLarren et al., 2003; Ruest et al., 2003), but also of skull and brain development (Kraus and Lufkin, 2006; Wang et al., 2010). Accordingly, it plays a role in thalamic development (Jones and Rubenstein, 2004) and contributes to regulate the migration and differentiation of precursors of GABA-expressing neurons in the forebrain (Cobos et al., 2006). *DLX5* is a candidate for ASD (Nakashima et al., 2010), due to an ultraconserved cis-regulatory element (Poitras et al., 2010), which is bound by GTF2I, encoded by one of the genes commonly deleted in Williams-Beuren syndrome (OMIM#194050) and a candidate for ASD too (Malenfant et al., 2012). Additionally, *DLX5* is regulated by MECP2 (Miyano et al., 2008), encoded by the main candidate for Rett syndrome (OMIM#312750), a condition entailing problems for motor coordination, autistic behavior, and language regression (Uchino et al., 2001; Veenstra-VanderWeele and Cook, 2004). Interestingly, Dlx5/6(±) mice exhibit abnormal pattern of γ rhythms resulting from alterations in GABAergic interneurons, particularly in fast-spiking interneurons (Cho et al., 2015). In addition, DLX5 interacts with key candidates for language evolution, in particular, with *RUNX2* and *FOXP2* (see Boeckx and Benítez-Burraco, 2014a, for details). The second one is *NCAM1*, which is also a target of both RUNX2 (Kuhlwilm et al., 2013) and FOXP2 (Konopka et al., 2009). In mice mutations in the gene affect working/episodic-like memory (Bisaz et al., 2013), whereas overexpression of the Ncam1 extracellular proteolytic cleavage fragment impacts on GABAergic innervation, affecting long- and short-term potentiation in the prefrontal cortex (Brennaman et al., 2011). *NCAM1* encodes a cell adhesion protein involved in axonal and dendritic growth and synaptic plasticity (Rønn et al., 2000; Hansen et al., 2008). It interacts with VCAM1 which is involved in cell adhesion and the control of neurogenesis (Kokovay et al., 2012), and which bears a fixed (D414G) change in AMHs compared to Neanderthals/Denisovans (Pääbo, 2014). *VCAM1* is upregulated by CLOCK, which plays a key role in the modulation of circadian rhythm (Gao et al., 2014). Together with other circadian-relevant genes *CLOCK* seems to be involved in the psychopathology of ASD cases entailing sleep disturbances (Yang et al., 2016). The circadian modulation of synaptic function has been hypothesized to contribute decisively to ASD (Bourgeron, 2007). In turn, *CLOCK* interacts with *RUNX2* and with several other candidates for language-readiness, like *DUSP1*, involved in vocal learning (Doi et al., 2007), and *USF1*, which regulates synaptic plasticity, neuronal survival and differentiation (Tabuchi et al., 2002; Steiger et al., 2004). USF1 binds the promoter of *FMR1* (Kumari and Usdin, 2001), a strong candidate for Fragile-X syndrome (OMIM#300624), which presents with language problems and ASD features (Kaufmann et al., 2004; Smith et al., 2012). The regulatory region of *USF1* shows many fixed or high frequency changes compared to Denisovans (Meyer et al., 2012).

As shown in **Table 1**, several of the genes important for globularization and language-readiness are involved in NC development and function and some of them are also candidates for ASD. Accordingly, we expect them to contribute to the abnormal domesticated features observed in patients with ASD, and also to their distinctive language profile. Among them we wish mention *CTNNB1*, *DLX1*, *DLX6*, *PAX6*, and *ROBO2*. CTNNB1 is a component of the Wnt/β-catenin signaling pathway, known to be impaired in ASD (Cao et al., 2012; Zhang et al., 2012; Martin et al., 2013). CTNNB1 controls aspects of NC development, from NC induction, lineage decisions, to differentiation (Hari et al., 2012). As noted in Boeckx and Benítez-Burraco (2014b), CTNNB1 is expected to interact with many of the genes highlighted as important for the evolution of language-readiness, specifically with RUNX2 and SLIT2/ROBO1 signals. Regarding DLX1, it is a robust NC marker (Ishii et al., 2012), involved in patterning and morphogenetic processes in NC-derived tissues (Mallo, 2001). It also regulates the development of the skull and the brain (Andrews et al., 2003; Jones and Rubenstein, 2004). In mice *Dlx1* downregulation results in reduced glutamatergic input to the hippocampus (Jones et al., 2011), as well as in changes in interneuron subtypes and migration patterns in the cortex (Ghanem et al., 2008). *DLX1* is found to be downregulated in ASD (Voineagu et al., 2011; McKinsey et al., 2013). ROBO2 is one of the DLX1 interactors. Slit/Robo signaling regulates early NCC migration (Jia et al., 2005) *ROBO2* is also involved in thalamocortical axons (TCA) development, known to be important for the modulation of cognitive functions (López-Bendito et al., 2007; Marcos-Mondéjar et al., 2012). *ROBO2* is a candidate for ASD (Suda et al., 2011), but also for different types of language disorders, like dyslexia (Fisher et al., 2002) and speech-sound disorder and reading (Stein et al., 2004). It has been related as well to expressive vocabulary growth in the normal population (St Pourcain et al., 2014). Finally, *PAX6* controls the migration of NCCs from the anterior midbrain (Matsuo et al., 1993). *PAX6* is involved as well in the development of the brain (Valverde et al., 2000; Tyas et al., 2003; Caballero et al., 2014). Mutations on *PAX6* have been reported in some forms of ASD (Maekawa et al., 2009), although they also impact in working memory (Bamiou et al., 2007). Alterations of *PAX6* expression in the brain of people with ASD may account for the observed imbalance in excitatory/inhibitory

neuronal activity (Kim et al., 2014). And like many of the genes reviewed above, *PAX6* is functionally related to both *FOXP2* and *RUNX2*, and it also targets *POU3F2* (see Benítez-Burraco and Boeckx, 2015, for details).

Most of the NCC-genes mentioned here are known to play a key role in the development and patterning of the craniofacial complex, and to be associated to congenital craniofacial defects (**Table 1**) (see Twigg and Wilkie, 2015 for review). Many of these genes are known candidates for ASD, including *DLX5* and *DLX6* (reviewed above), *FGFR2*, *MSX1*, *POLR1A*, and *PTCH1*. Both *DLX5* and *DLX6* are indeed required for NC-derived facial morphogenesis (Gitton et al., 2011) FGFRs are among the main craniosynostosis-associated genes. In particular, gain-of-function mutations in *FGFR2* are typically associated to Apert (OMIM#101200) and Crouzon (OMIM#123500) syndromes, while both *FGFR1* and *FGFR2* are found mutated in Pfeiffer syndrome (OMIM#101600) (Lattanzi et al., 2012). All these syndromic craniosynostoses occasionally present with variable degree of ASD-like mental retardation (Morey-Canellas et al., 2003). *MSX1* encodes a transcriptional repressor involved in craniofacial development and shaping (particularly in odontogenesis) (Alappat et al., 2003; Lattanzi, forthcoming). It is expressed in the NC (Khadka et al., 2006), where it acts as a master regulator of gene expression (Attanasio et al., 2013). Although it has not been associated to ASD, *MSX1* is a direct downstream target of DLX5 during early inner ear formation (Sajan et al., 2011). The gene is also a critical intrinsic dopaminergic neuron determinant (Andersson et al., 2006) and is found mutated in some patients with Wolf-Hirschhorn syndrome (OMIM#194190), a clinical condition entailing profound mental retardation and craniofacial dysmorphism (Campbell et al., 1989). *POLR1A*, found mutated in acrofacial dysostosis (Cincinnati type, OMIM#616462) involving microcephaly, plays a role in the regulation of NC-derived skeletal precursor cells (Weaver et al., 2015). In some ASD subjects CNVs result in fusion transcripts involving *POLR1A*, although no fusion transcripts have been detected to date (Holt et al., 2012).

Finally, it is worth mentioning that genes encoding primary cilium signaling molecules, such as *SHH*, *GLI3,* and *PTCH1*, are all primarily involved in congenital malformations affecting the midline craniofacial compartment (Brugmann et al., 2010; Rice et al., 2010). Specifically, *PTCH1* is required in the NC-dependent orofacial development and gives rise to orofacial clefting, when mutated (Metzis et al., 2013). Heterozygous mutations of either *SHH* or *PTCH1* are typically found in holoprosencephaly (OMIM#610828, and #236100), a genetically heterogeneous, highly prevalent congenital forebrain anomaly in humans, associated with mental retardation and craniofacial malformations (Ming et al., 2002; Mercier et al., 2011). In addition, a 22-bp deletion in this gene has been found in a girl with ASD and Gorlin syndrome, a complex condition involving macrocrania and hypertelorism (Delbroek et al., 2011).

Candidate Genes: Expression Profiles in the ASD Brain

If our hypothesis is on the right track, we expect that the genes we highlight here are dysregulated in the brain of people with ASD, particularly in areas important for language processing. Accordingly, we surveyed the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/gds) searching for their expression profiles in the cerebellum and the temporal cortex (but also in the frontal and occipital cortices) in patients with ASD. This should help identify new candidates for ASD in the context of domestication and language-readiness (**Table 1**). Overall, we could find significant expression values for some of our candidates and learnt that they are up- or downregulated in the brain of autists (**Figure 3**).

Among the genes that are significantly downregulated in the cerebellum we found *AXIN2*, *EDNRB*, *SOX2*, *SPECC1L*, *TCF12,* and *VCAN*, whereas *CDC42*, and *PQBP1* are found upregulated in this region (**Figure 3**). Although none of them has been associated to ASD, they stroke us as promising candidates for the atypical presentation of the domestication syndrome in ASD. *AXIN2* is expressed in the cranial NC and is needed for NC-derived frontal bone osteogenesis (Yu et al., 2005; Li et al., 2015a). This gene is also expressed as a specific marker for suture stem cells (Maruyama et al., 2016), but also acts as a negative regulator of canonical Wnt pathway, contributing to the stability of CTNNB1 (Li et al., 2015a). Speech alterations are also observed in people with *AXIN2* mutations causing non-syndromic oligodontia (Liu et al., 2015). *EDNRB* encodes a receptor for endothelins, known to be potent vasoactive peptides. Mutations in this gene are associated to increased susceptibility to Hirschsprung disease (OMIM#600155), a neurocristopathy characterized by congenital absence of intrinsic ganglion cells in the enteric nervous plexa (Amiel et al., 2008). Waardenburg syndrome (OMIM#277580), a genetically heterogeneous condition which may involve developmental delay subsidiary to sensorineural hearing loss, has also been associated with mutations in *EDNRB* (Read and Newton, 1997). *SOX2*, one of core candidates for domestication (Wilkins et al., 2014), encodes an interactors of the GLI factors as part of the SHH-GLI signaling pathway involved in NCC fate (Oosterveen et al., 2012, 2013; Peterson et al., 2012), but also in the globularization of the AMH skull/brain (see Boeckx et al., submitted for details). SOX2 interacts as well with the BMP signaling (Li et al., 2015b). Interestingly, SOX2 regulates *PQBP1*, highlighted above as one of POU3F2 interactors. *SPECC1L* is found mutated in Opitz G/BBB syndrome (OMIM# #145410) and in facial clefting (Kruszka et al., 2015). This gene functions in NC development (Wilson et al., 2016) and is specifically involved in facial morphogenesis (Saadi et al., 2011). *TCF12* is highly expressed in embryonic precursors of skull/brain structures, including NC-derived head mesenchyme (Uittenbogaard and Chiaramello, 2002). *TCF12* directly interacts with *TWIST1*, mutated in Saethre-Chotzen syndrome (OMIM#601622), which features complex craniosynostosys with variable degrees of intellectual disability, including ASD traits (Maliepaard et al., 2014). Indeed, loss-of-function mutations of *TCF12* have been identified in patients with coronal synostosis, which sometimes involves intellectual disability (Sharma et al., 2013; di Rocco et al., 2014; Paumard-Hernández et al., 2015; Piard et al., 2015). *VCAN* encodes versican-1, a protein that guides migratory NCCs (Dutt et al., 2006) and which shows a fixed N3042D change in AMHs (Pääbo, 2014). Finally, *CDC42* controls NC stem

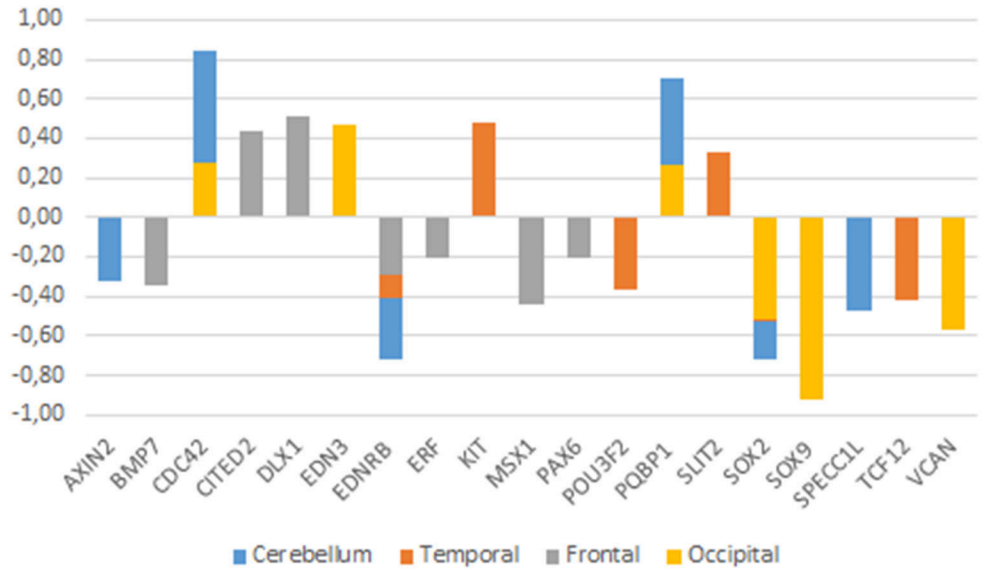


FIGURE 3 | Expression profiles of candidate genes in the ASD brain. Data were gathered from the following microarray expression datasets available on the Gene Expression Omnibus database (GEO datasets, http://www.ncbi.nlm.nih.gov/gds): GSE28521 (Voineagu et al., 2011) for the temporal and frontal cortices, GSE38322 (Ginsberg et al., 2012) for the cerebellum and the occipital cortex. Data are shown as log transformation of fold changes (logFC) between patients and corresponding controls. Only genes showing statistically significant ($p < 0.05$) differential expression were considered. Additional details may be found in the Supplemental information file.

cell proliferation (Fuchs et al., 2009). Inactivation of Cdc42 in NCCs causes craniofacial and cardiovascular morphogenesis defects (Liu et al., 2013). As discussed in detail in Boeckx and Benítez-Burraco (2014b) *CDC42* is an important gene regarding language evolution, because of its functional connections with core candidates for globularization and the externalization of language (including *FOXP2*, *RUNX2*, *SLIT2*, and *ROBO1*), with genes related to language disorders (like *CMIP*), and with genes known to have changed after our split from Neanderthals and Denisovans (like *ITGB4*, *ARHGAP32*, *ANAPC10,* and *CDC42EP4*).

With regards to the temporal cortex, we found that *EDNRB*, *POU3F2*, *SOX2*, *VCAN*, and *TCF12* are downregulated in subjects with ASD, whereas *KIT*, *PQBP1*, and *SLIT2* are upregulated in them (**Figure 3**). As noted above, *POU3F2*, *SOX2*, *KIT* are known candidates for ASD. We have already reviewed all these genes. Concerning the frontal cortex we found that *BMP7*, *EDNRB*, *PAX6*, *ERF*, and *MSX1* are significantly downregulated, whereas *CITED2* and *DLX1* are significantly upregulated (**Figure 3**). As noted in **Table 1**, *DLX1*, *PAX6*, and *MSX1* have been previously associated to ASD. Most of these genes have been already reviewed. *BMP7* is a NCC gene involved in regulation of osteogenesis (Cheng et al., 2003; Anderson et al., 2006) and in skull and brain development (Yuge et al., 2011; Segklia et al., 2012). *BMP7* is also closely related to some of the core candidates for globularization and language- readiness, like *BMP2*, *DLX1*, *DLX2*, and *RUNX2*. BMP7 is predicted (according to String 10) to interact with SOX2 via NOG, involved in dopamine neuron production and an inhibitor of BMP signaling (Chiba et al., 2008). Developmental delay and learning disabilities are commonly observed in people with mutations in *BMP7* (Wyatt et al., 2010). *ERF* encodes a member of the ETS family of transcription factors, expressed in migratory cells, including NCCs (Paratore et al., 2002). *ERF* haploinsufficiency gives rise to either coronal or multisuture synostosis, midface hypoplasia, often associated with behavioral and learning difficulties (Twigg et al., 2013). Concerning *CITED2*, this is a functional partner of both FOXP2 and RUNX2 (Luo et al., 2005; Vernes et al., 2011; Nelson et al., 2013), two important genes for the emergence of modern language. *CITED2* is also involved in craniofacial development (Bhattacherjee et al., 2009) and in the establishment of left-right axis through interactions with the BMP signaling and Nodal (Preis et al., 2006; Lopes Floro et al., 2011). Interestingly, 99% of AMHs bear a highly disruptive intergenic change near *CITED2* compared to Altai Neanderthals and Denisovans (Prüfer et al., 2014).

Finally, regarding the occipital cortex, we found that *EDN3*, *CDC42*, and *PQBP1* are significantly upregulated in ASD, whereas *SOX2*, *SOX9*, and *VCAN* are downregulated. We have already considered all these genes, with the exception of *EDN3*. This gene encodes an endothelium-derived vasoactive peptide which binds the product of *EDNRB*, playing a key role in the development of neural crest-derived cell lineages, such as melanocytes and enteric neurons. Although *EDN3* is a candidate for Waardenburg syndrome and Hirschsprung disease, the gene has been found significantly dysregulated in children with ASD (Glatt et al., 2011).

As shown in **Figure 3**, genes that exhibit significant changes in their expression levels in the ASD brain are consistently down- or upregulated across all the regions under analysis. Considering

their functions and the phenotypes resulting from their mutation, the most promising of these genes are *CDC42* and *PQBP1* (which are upregulated) and *EDNRB* and *SOX2* (which are found downregulated). As noted above, to date none of them have been associated to ASD, but they emerge as reasonably involved in the pathogenesis of this condition.

Noteworthy age-related differences in the intrinsic functional connectivity of the brain are observed in ASD: adult patients show reduced connectivity while children tend to exhibit an increased connectivity (Uddin et al., 2013). Therefore, we have further analyzed ASD brain expression data in an age-matched fashion. Due to the available sample characteristics, only expression data obtained from the cerebellum could be analyzed (see Supplemental file for further details). The reduction of the age-related bias in the patients-vs.-controls comparison, enabled finding a higher number of statistically significant dysregulated genes in the ASD brain. Accordingly, we found that in the cerebellum of ASD children (below 11 years old), *CDC42*, *MSX1*, *MSX2*, *NODAL*, *PQBP1*, and *SLIT2* were downregulated, whereas, *AXIN2*, *CHD7*, *CITED2*, *EDNRB*, *FGF8*, *NCAM1*, *PAX7*, *PTCH1*, *RET*, *ROBO1*, *SOX2*, *SPECC1L*, *TCF12*, *VCAN*, and *ZIC1* were upregulated. In turn, in the cerebellum of adult patients (aged 22–60 years) we found that *CDC42*, *CTNNB1*, *DLX1*, *EDNRA*, *EDNRB*, *HES1*, *KIT*, *MAGOH*, *MITF*, *NCAM1*, *NTN1*, *POU3F2*, *PQBP1*, *PTCH1*, *RET*, *ROBO1*, *SATB2*, *SOX2*, *SPECC1L*, *TCF12*, *VCAN*, and *ZIC1* are downregulated, whereas only *AXIN2*, *CTNNB1*, *DLX1*, *EDN1*, and *MSX1* are upregulated. Overall, we concluded that nearly one third of the candidates for domestication are dysregulated in the cerebellum of people with ASD, and that more than a half are specifically dysregulated in the cerebellum of either children and/or adults with this condition. Genes that are dysregulated in both children and adults with ASD can be regarded as significant contributors to the atypical presentation of the domestication syndrome in this condition. This list encompasses 14 genes: *AXIN2*, *CDC42*, *EDNRB*, *MSX1*, *NCAM1*, *PQBP1*, *PTCH1*, *RET*, *ROBO1*, *SOX2*, *SPECC1L*, *TCF12*, *VCAN*, and *ZIC1*. Interestingly, they exhibit opposite expression profiles in children and adults with ASD (**Figure 4**). Most of these genes have been already discussed here. In addition, *RET,* encoding a cadherin that plays a crucial role in NC development, is a candidate for Hirschsprung disease (OMIM# 142623; Edery et al., 1994) and is found to be differentially expressed after RUNX2 transfection in neuroblastoma cells (Kuhlwilm et al., 2013). *RET* is downstream *ASCL1* (another candidate for Hirschsprung disease) in noradrenergic brain stem neurons important for respiratory rhythm modulation (Dauger et al., 2001). Likewise, *ZIC1* is needed for NC development (along with *PAX3*) and plays a key role in craniofacial development (Milet et al., 2013; Plouhinec et al., 2014). Mutations in *ZIC1* result in severe coronal synostosis associated with learning difficulties (Twigg et al., 2015).

In the last section of the paper we attempt refining our characterization of ASD as an atypically-domesticated phenotype. In doing so, we will focus on brain function, with a special emphasis on brain oscillations. Accordingly, we will compare the oscillopathic profile of people with ASD during language processing with the oscillatory signature of the TD population and that of non-domesticated primates. Additionally, we will examine the expression profile in the primate brain of the candidate genes we highlight here.

ASD AND WILD PRIMATES: FROM BRAIN OSCILLATION TO GENE EXPRESSION PATTERNS

ASD and Primate Oscillomes

As we have discussed in detail in Benítez-Burraco and Murphy (2016) language impairment in ASD (as ASD itself) can be satisfactorily characterized as an oscillopathic condition. With cognitive disorders exhibiting disorder-specific abnormal oscillatory profiles, it is also noteworthy that species-specific oscillatory patterns seemingly emerge as slight variations within the network constellation that constitutes a universal brain syntax (Buzsáki and Watson, 2012; Buzsáki et al., 2013).

The differences in brain capacity between domesticated and non-domesticated animals (Wilkins et al., 2014) would be predicted to give rise to a corresponding alteration in oscillatory properties (i.e., features of neural oscillations which form part of an individual's "oscillome," as it is termed in Murphy and Benítez-Burraco, in press; Murphy, 2016a,b). Although we feel that current knowledge is too scarce to permit any reasonable linking hypotheses between the primate and ASD oscillomes, we would like to briefly sketch out a possible route to increasing our understanding of the neural signature of domestication (and failed domestication itineraries).

Call vocalizations have been found not to be impaired when the homolog of Broca's region in the monkey brain is lesioned, which suggests that other area (like the limbic system and brainstem) are involved (Sage et al., 2006). However, macaques appear to share similar call comprehension substrates with human language comprehension in the left posterior temporal gyrus (Heffner and Heffner, 1986). It would be of interest, for instance, to compare the rhythmic properties of this region of the TD brain with those of the primate brain to see if any particular activity (e.g., coupling and synchronization) is marked in humans. This would also yield insights into how the primate call comprehension system "interfaces" with other cognitive systems (given the appropriate experimental environment), and would also permit the exploration of similar interface properties of human language comprehension, which requires the transfer of information to two interfaces (**Figure 5**).

Discounting work on evoked potentials (which is itself fairly modest), there are currently only a handful of empirical studies of the monkey oscillome. Brincat and Miller (2015), for instance, discovered functional differences and frequency-specific interactions between the Rhesus hippocampus (HPC) and prefrontal cortex (PFC) during object pair association learning. θ synchrony was found to be greater after errors and decreased after learning; correct associations increased β-α synchrony, which was also greater in the HPC-PFC direction. Esghaei et al. (2015) also suggested that the macaque visual cortex employs phase-amplitude coupling to regulate inter-neuronal correlations, and so the potential for generic oscillomic processes to yield insights into cognitive (dys)function seems apparent. During the internally monitored continuation phase of a synchronization-continuation task, β also appears to increase in

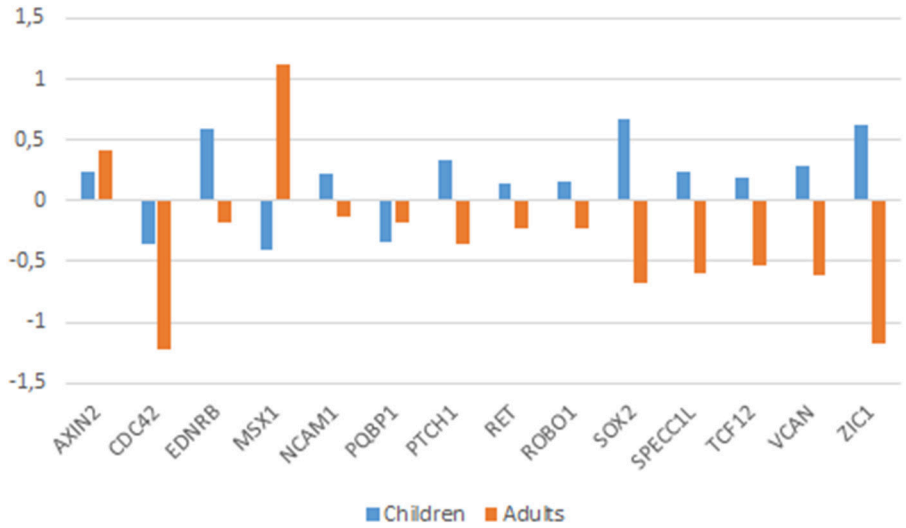
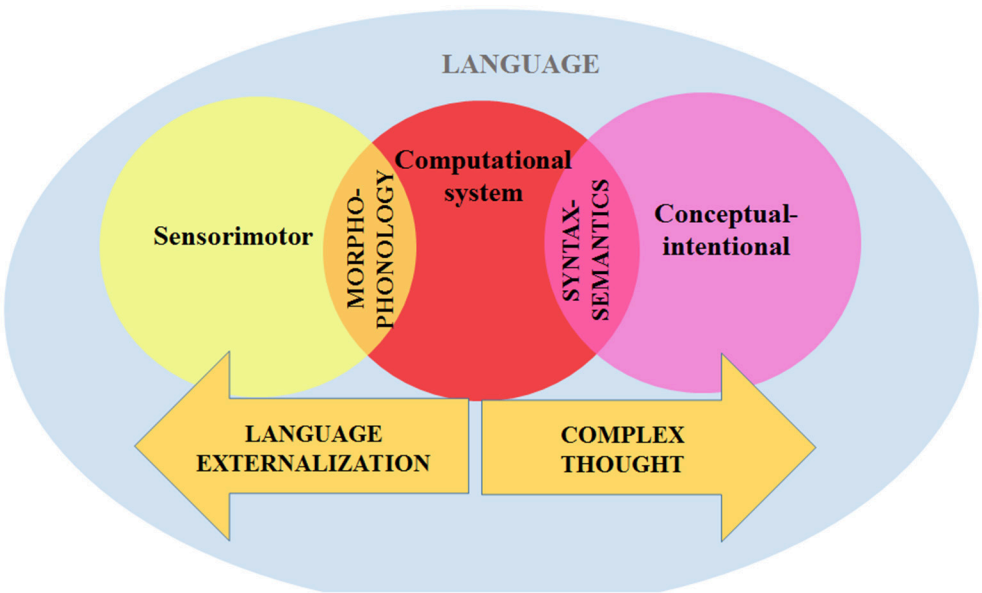


FIGURE 4 | Expression profiles of candidates genes in the cerebellum of children and adults with ASD. Expression data were obtained from the microarray expression dataset GSE38322 (Ginsberg et al., 2012) available on the Gene Expression Omnibus database (GEO datasets, http://www.ncbi.nlm.nih.gov/gds). Data are shown as log transformation of fold changes (logFC) between patients and corresponding controls. Only genes showing statistically significant ($p < 0.05$) differential expression were considered. Additional details may be found in the Supplemental information file.



Cognome	*Dynome*	*Connectome*
Set-formation	Frequency coupling	Assembly formation
Lexicalisation	Long-term potentiation	Ephaptic coupling
Cyclic transfer	Cycle skipping	
Labeling	γ-band synchronisation	
	Phase locking	

FIGURE 5 | A schematic view of language representing the systems and interfaces of interest and the levels of analysis. "Cognome" refers to the operations available to the human nervous system (Poeppel, 2012) and "dynome" refers to brain dynamics (Kopell et al., 2014; Murphy and Benítez-Burraco, in press). See text for details.

Rhesus monkeys (Bartolo and Merchant, 2015), suggesting that—as in humans (Murphy, 2015a)—β is responsible for maintaining the existing cognitive set in memory. β is also involved in "the attentive state and external cues as opposed to detailed muscle activities" in Japanese monkeys (*Macaca fuscata*) (Watanabe et al., 2015). Finally, pulvinar γ is involved in feedforward processing for snake images, and also in cortico-pulvinar-cortical integration for face images (Le et al., 2016), while Ramirez-Villegas et al.'s (2015) study of the macaque hippocampal CA3-CA1 network pointed to the role of γ in memory reactivation, transfer and consolidation.

Currently, there are no studies comparing the oscillome of domesticated and non-domesticated primates, but our prediction would be that non-domesticated primates display a degree of oscillomic difference with domesticated primates comparable to the difference between TD individuals and people with ASD. **Table 3** summarizes existing knowledge of the ASD and primate oscillome during a range of cognitive tasks, and it is hard to find any correlations or connections between the two. However, we feel that comparatively exploring these oscillomes will permit a greater understanding of the atavistic neural oscillations of the non-domesticated human and primate brains. Future research should also seek to compare the oscillomes of domesticated and non-domesticated primates in an effort to investigate neural signatures of domestication.

Candidate Genes: Expression Profiles in the Primate Brain

If our hypothesis turns to be on the right track, we further expect that the genes that we have found dysregulated in the brain of people with ASD show similar expression profiles in conditions where normal socialization failed to occur. Because feral children are scarce and not easily available we examined the expression profiles of these genes in wild primates (chimps). In particular, we selected available gene expression profiles obtained from chimp brain areas that are known to be involved in language processing in humans (the cerebellum, the temporal cortex, and the frontal cortex), as we did for people with ASD. We learnt that most of the genes that we had previously found differentially expressed in the ASD brain data exhibit the same expression pattern in the chimp brain, including *EDNRB* (in the cerebellum), *BMP7*, *DLX1*, *EDNRB*, *MSX1*, and *PAX6* (in the frontal cortex), and *VCAN* (in the temporal cortex) (**Figure 6**).

CONCLUSIONS

Socialization is a crucial step needed for the achievement of many cognitive abilities that are a signature of the human condition. Language is one of the most prominent of such abilities. Several high prevalent pathological conditions impact on human-specific cognitive abilities, including schizophrenia and ASD. In ASD, social abilities are seriously compromised, but other core cognitive skills, including language, also exhibit differences with the non-affected population. ASD is a multifactorial condition, with wide clinical and genetic heterogeneity. It is still not clear how ASD features emerge from genomic and/or environmental cues during development. In this paper we have focused on language deficits in ASD, although we expect that the lessons we draw here contribute to shedding light on the whole profile of this cognitive condition. In doing so we have adopted and evolutionary perspective, because of the robust link that exists between (abnormal) development and evolution. As we have shown, domesticated traits are absent or are attenuated in people with ASD, and genes that we believe important for the (self)domestication of our species and the evolution of our distinctive cognitive abilities (including language) show abnormal expression patterns in the brains of people with autism. What is more: abnormalities can be traced to the time window when crucial brain rewiring occurs during language acquisition and when changes in the normal configuration of the brain occurs in children with ASD. Additionally, some features of the ASD phenotype can be found (or expected to be found) in wild primates. On the whole, we think that our approach can help illuminate the etiology of ASD primarily because provides robust links between the genome and the environment, and between development and evolution, in line with the current evo-devo approaches to cognitive diseases (see Benítez-Burraco, 2016a, for review). In this sense, the putative involvement of the neural crest

TABLE 3 | Summary of the patterns of rhythmicity observed in wild primates and the observed oscillomic differences in ASD compared to TD subjects.

Frequency band	Oscillomic monkey profile	Oscillopathic profile of autism spectrum disorder
Delta (∼0.5–4 Hz)	Decreased phase-amplitude coupling with γ yields increased visual attention, suggesting that cross-frequency coupling suppression modulates attention.	Increased in eyes-closed resting state exam; predicted to be disrupted in processing phrases involving raising and passives.
Theta (∼4–10 Hz)	Decreased phase-amplitude coupling with γ yields increased visual attention; greater HPC-PFC synchrony after object pair association errors.	Reduced cross-frequency coupling with γ; does not synergistically engage with γ during speech; predicted to be disrupted in certain memory retrieval processes.
Alpha (∼8–12 Hz)	Increased synchrony with β during correct object pair associations.	Reduced cross-cortically; reduced resting-state α-γ phase amplitude coupling; increased in resting state; predicted to be disrupted during certain lexicalizations.
Beta (∼10–30 Hz)	Increased synchrony with α during object pair associations; increases during continuation phase of a synchronization-continuation task.	Reduced in picture-naming tasks; predicted to be disrupted in the maintenance of syntactic objects in raising, passives and *wh*-questions.
Gamma (∼30–100 Hz)	Involved in processing snake and face images increases during action sequence updating and memory consolidation, reactivation, and transfer.	Over-connectivity gives rise to increased γ; reduced in rSTG and lIFG during picture naming; predicted to be disrupted quite generally in linguistic cognition.

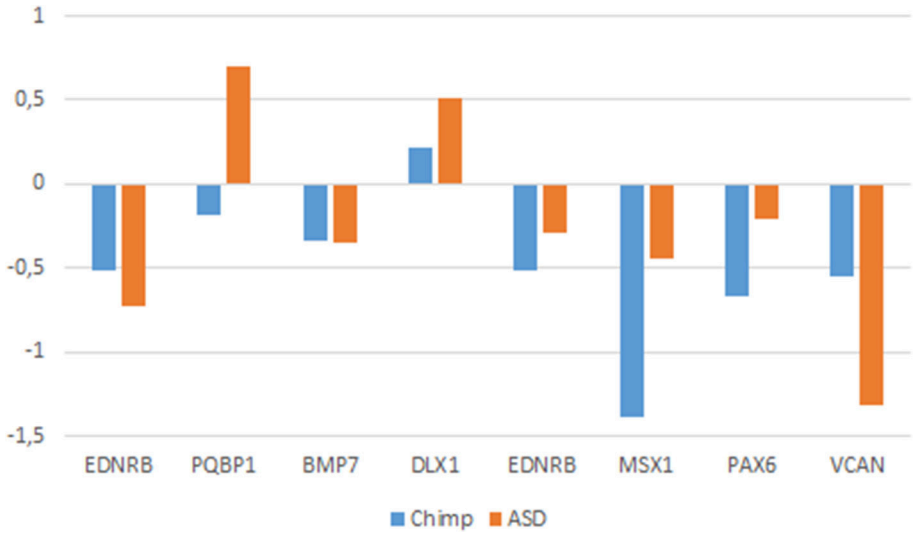


FIGURE 6 | Comparative expression profiles in chimpanzees and subjects with ASD of candidate genes. Data were obtained from microarray expression datasets available on the Gene Expression Omnibus database (GEO datasets, http://www.ncbi.nlm.nih.gov/gds): GSE28521 (Voineagu et al., 2011) for the temporal and frontal cortices, and GSE38322 (Ginsberg et al., 2012) for the cerebellum of subjects with ASD; GSE22569 (Somel et al., 2011; Liu et al., 2012) for the cerebellum, GSE18142 (Konopka et al., 2009) for the frontal cortex, and GSE7540 (Cáceres et al., 2003) for the temporal cortex of chimps. Data are shown as log transformation of fold changes (logFC) between patients and corresponding controls. Only genes showing statistically significant ($p < 0.05$) differential expression were considered. Additional details may be found in the Supplemental information file. Note that the plot is intended to display the overall trend of gene expression, given that the relative expression values (i.e., logFC) were obtained from comparative analyses performed on different datasets (based on different designs, samples, and batches).

in the aetiopathogenesis of ASD emerges as a promising avenue for future research. At the same time, we expect that our approach with help illuminate the evolutionary history of our language-readiness: Our results support the view that language evolution benefitted from a favorable social context that may have resulted from our (self)-domestication.

AUTHOR CONTRIBUTIONS

AB contributed to all Sections and drafted the manuscript, WL contributed to Sections "Domestic Traits in the ASD Phenotype" and "ASD and the Genetics of the Domestication Syndrome" and revised the manuscript, EM contributed to Sections "Introduction" and "ASD and Wild Primates: from Brain Oscillation to Gene Expression Patterns" and revised the manuscript.

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Comparative Gene Expression Analysis of Two Mouse Models of Autism: Transcriptome Profiling of the BTBR and *En2*$^{-/-}$ Hippocampus

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Autism spectrum disorders (ASD) are characterized by a high degree of genetic heterogeneity. Genomic studies identified common pathological processes underlying the heterogeneous clinical manifestations of ASD, and transcriptome analyses revealed that gene networks involved in synapse development, neuronal activity, and immune function are deregulated in ASD. Mouse models provide unique tools to investigate the neurobiological basis of ASD; however, a comprehensive approach to identify transcriptional abnormalities in different ASD models has never been performed. Here we used two well-recognized ASD mouse models, BTBR T^{+} *Itpr3*^{tf}/J (BTBR) and Engrailed-2 knockout (*En2*$^{-/-}$), to identify conserved ASD-related molecular signatures. *En2*$^{-/-}$ mice bear a mutation within the EN2 transcription factor homeobox, while BTBR is an inbred strain with unknown genetic defects. Hippocampal RNA samples from BTBR, *En2*$^{-/-}$ and respective control (C57Bl/6J and *En2*$^{+/+}$) adult mice were assessed for differential gene expression using microarrays. A total of 153 genes were similarly deregulated in the BTBR and *En2*$^{-/-}$ hippocampus. Mouse phenotype and gene ontology enrichment analyses were performed on BTBR and *En2*$^{-/-}$ hippocampal differentially expressed genes (DEGs). Pathways represented in both BTBR and *En2*$^{-/-}$ hippocampal DEGs included abnormal behavioral response and chemokine/MAP kinase signaling. Genes involved in abnormal function of the immune system and abnormal synaptic transmission/seizures were significantly represented among BTBR and *En2*$^{-/-}$ DEGs, respectively. Interestingly, both BTBR and *En2*$^{-/-}$ hippocampal DEGs showed a significant enrichment of ASD and schizophrenia (SCZ)-associated genes. Specific gene sets were enriched in the two models: microglial genes were significantly enriched among BTBR DEGs, whereas GABAergic/glutamatergic postsynaptic genes, FMRP-interacting genes and epilepsy-related genes were significantly enriched among *En2*$^{-/-}$ DEGs. Weighted correlation network analysis (WGCNA) performed on BTBR and *En2*$^{-/-}$ hippocampal transcriptomes together identified six modules significantly enriched in ASD-related genes. Each of these modules showed a specific enrichment profile in neuronal and glial genes, as well as in genes associated to ASD comorbidities

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such as epilepsy and SCZ. Our data reveal significant transcriptional similarities and differences between the BTBR and *En2*$^{-/-}$ hippocampus, indicating that transcriptome analysis of ASD mouse models may contribute to identify novel molecular targets for pharmacological studies.

Keywords: autism, hippocampus, gene expression, microarray, WGCNA, BTBR, Engrailed, mouse

INTRODUCTION

Autism spectrum disorders (ASD) are a family of neurodevelopmental disorders characterized by a high degree of genetic heterogeneity. Recent advances in genetics and genomics allowed to attribute the heterogeneous clinical manifestations of ASD to shared pathophysiological processes (de la Torre-Ubieta et al., 2016). Integrative analysis of large-scale genetic data revealed distinct gene networks affected in ASD, mainly related to the formation and function of brain synapses. Network-based analysis of large-scale transcriptome data also highlighted that co-expression modules related to synapse development, neuronal activity and immune function are deregulated in ASD (Voineagu et al., 2011; Gupta et al., 2014; de la Torre-Ubieta et al., 2016). Thus, pathophysiological processes in ASD seem to converge on specific molecular pathways and networks, with a clear interplay between immune and synaptic functions (Estes and McAllister, 2015).

Gene transcriptional profiling in ASD is mainly performed on post-mortem brain samples, but the restricted availability of human ASD brain tissues represents a significant challenge. For this reason, ASD mouse models provide a unique tool to identify conserved pathological mechanisms at the gene expression level. BTBR T^{+} *Itpr3*^{tf}/J (BTBR) is an inbred strain of mice that incorporates behavioral phenotypes relevant to all diagnostic symptoms of ASD, including reduced social interactions in juveniles and adults, repetitive self-grooming and an unusual pattern of ultrasonic vocalizations resembling the atypical vocalizations seen in some autistic children (Scattoni et al., 2008, 2013). Moreover, BTBR mice show a severely reduced hippocampal commissure and absent corpus callosum (Wahlsten et al., 2003). Noteworthy, corpus callosum abnormalities have been reported in autistic individuals (Egaas et al., 1995; Alexander et al., 2007). However, it is important to emphasize that genetic abnormalities causing behavioral deficits in BTBR mice are still under investigation (Jones-Davis et al., 2013).

Engrailed-2 (EN2) is a homeodomain transcription factor involved in regionalization and patterning of the midbrain and hindbrain regions (Joyner et al., 1991). Genome-wide association studies revealed that EN2 is a candidate gene for ASD (Benayed et al., 2009), and an abnormal expression and methylation profile of the EN2 gene has been reported in the cerebellum of ASD patients (James et al., 2013, 2014; Choi et al., 2014). Mice lacking the homeobox domain of *En2* (*En2*$^{hd/hd}$ mice; Joyner et al., 1991; here referred to as *En2*$^{-/-}$) display neuropathological changes related to ASD. These defects include cerebellar hypoplasia and reduced number of Purkinje neurons (Joyner et al., 1991; Kuemerle et al., 1997), defective GABAergic innervation in the forebrain (Sgadò et al., 2013a; Allegra et al., 2014; Provenzano et al., 2014), reduced monoaminergic innervation to the forebrain (Brielmaier et al., 2014; Genestine et al., 2015; Viaggi et al., 2015) and ASD-like behavioral traits such as decreased sociability, spatial learning deficits, and increased seizure susceptibility (Cheh et al., 2006; Tripathi et al., 2009; Brielmaier et al., 2012; Provenzano et al., 2014).

So far, a comprehensive approach to identify common and distinct abnormalities in ASD models has been conducted by brain imaging-based neuroanatomical phenotyping (Ellegood et al., 2015; Ellegood and Crawley, 2015). However, few efforts have been made to identify conserved genes signatures at the transcriptome level in brain tissues from ASD mice. The hippocampal and cortical transcriptome has been examined in BTBR mice (Daimon et al., 2015; Kratsman et al., 2016), while we evaluated the gene expression signature of the *En2*$^{-/-}$ hippocampus and cerebellum (Sgadò et al., 2013b). Similar studies have been performed on brains from *Fmr1* (Prilutsky et al., 2015) and *Pten* (Tilot et al., 2016) mutant mice. However, a comprehensive approach to identify transcriptional abnormalities in ASD mouse models has never been performed so far. Here we compared the transcriptome profile of the BTBR and *En2*$^{-/-}$ hippocampus, and describe common and distinct transcriptional signatures for these two ASD mouse models.

MATERIALS AND METHODS

Animals

Experiments were conducted in conformity with the European Community Directive 2010/63/EU and were approved by the Animal Welfare Committee of University of Trento, Istituto Superiore di Sanità and 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. *En2* mutants were originally generated on a mixed 129Sv x C57BL/6 genetic background (Joyner et al., 1991) and then backcrossed at least five times into a C57BL/6 background. *En2*$^{+/+}$ and *En2*$^{-/-}$ mice used in this study were obtained by heterozygous mating (*En2*$^{+/-}$ x *En2*$^{+/-}$) and genotyped by PCR as previously described (Sgadò et al., 2013a). BTBR T^{+} *Itpr3*^{tf}/J (BTBR) and C57Bl/6J (B6) inbred mice were purchased from the Jackson Laboratory (Bar Harbour, ME, USA) and bred in the mouse vivarium of the Istituto Superiore di Sanità (Rome, Italy). A total of 32 adult (3–5 months old) mice were used: 4 BTBR and 4 B6 mice for microarray experiments, and 6 mice per strain/genotype (BTBR, B6, *En2*$^{+/+}$ and *En2*$^{-/-}$) for quantitative RT-PCR.

Microarrays and Single-Gene Differential Expression Analysis

In this study, we aimed to identify common and distinct molecular signatures across the hippocampi of BTBR and $En2^{-/-}$ mice. To obtain a comparable and reliable hippocampal transcriptomic profile of BTBR mice and reduce any source of variability during microarray analysis, which might arise from pre-scanning and/or post-scanning steps (Kadanga et al., 2008), we applied the same experimental procedures previously described for the transcriptome analysis of $En2^{-/-}$ hippocampus (Sgadò et al., 2013b). Briefly, hippocampal RNAs from BTBR and B6 mice ($n = 4$ per experimental group) were purified using standard column purification according to the manufacturer's protocol (RNAeasy Mini Kit, QIAGEN). RNA quality was analyzed by microfluidic gel electrophoresis on RNA 6000 NanoChips using the Agilent 2100 Bioanalyzer. Only RNA with a high (>9) RNA integrity number was selected and used for subsequent retrotranscription, labeling, and array hybridization according to Agilent protocols. Mouse gene expression arrays (Agilent 4X44K slides) were hybridized and scanned with the Agilent microarray station. The images obtained from the microarray scanner were analyzed with Agilent Feature Extraction version 10.7.3.1. Hippocampal gene expression dataset of BTBR and B6 mice have been deposited in the NCBI's Gene Expression Omnibus (GEO) database (accession number GSE81501). Intensity values were processed with Agi4x44PreProcess using default parameters to remove low-quality probes. Signals were then normalized by means of the quantile normalization method. Multiple replicas of the same probes were summarized using the median. To evaluate differential expression, the Rank Product (RP) non-parametric method was used (Sgadò et al., 2013b). The RP is equivalent to calculating the geometric mean rank with a statistical method (average rank) that is slightly more sensitive to outlier data and puts a higher premium on consistency between the ranks in various lists.

Quantitative RT-PCR (qRT-PCR)

Total RNAs were extracted by Trizol reagent (Invitrogen) from explanted hippocampi. DNase-treated RNAs were purified by RNA extraction RNAeasy Kit (QIAGEN). cDNA was synthesized from pooled RNAs by SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer' instructions. qRT-PCR was performed in a C1000 Thermal Cycler (Bio-Rad) with real-time detection of fluorescence, using the KAPA SYBR FAST Master Mix reagent (KAPA Biosystems). Mouse mitochondrial ribosomal protein L41 (mRPL41) was used as a standard for quantification. Primer sequences (Eurofins Genomics) are reported in **Supplementary Table 1**. Ratios of comparative concentrations of each mRNA with respect to L41 mRNA were then calculated and plotted as the average of three independent reactions (technical replicates) obtained from each RNA. Expression analyses were performed using the CFX3 Manager (Bio-Rad) software (Sgadò et al., 2013b). Statistical analysis of qRT-PCR was performed with Prism 6 (GraphPad) software. Values were expressed as mean ± s.e.m and quantitative gene expression differences between each autistic mouse (BTBR and $En2^{-/-}$) strain and their respective controls (B6 and $En2^{+/+}$) were assessed by Student's *t*-test, with the level of statistical significance set at $p < 0.05$.

Phenotype/Pathway Ontology and Enrichment Analysis on DEGs

DEGs in BTBR and $En2^{-/-}$ hippocampi were analyzed for "phenotype ontology" using Enrichr (http://amp.pharm.mssm.edu/Enrichr/). Enrichr is an enrichment analysis web-based tool providing various types of gene-set libraries, including the knockout mouse phenotypes ontology developed by the Jackson Lab from their Mouse Genome Informatics-Mammalian Phenotype (MGI-MP) browser (Blake et al., 2009). Mammalian phenotypes predefined by MGI are sorted by *z*-score considering only terms with an adjusted *p*-value less than 0.05. Phenotype/pathway ontology of $En2^{-/-}$ hippocampal DEGs was performed on our previous dataset from $En2^{+/+}$ and $En2^{-/-}$ adult mice, already deposited in NCBI's GEO database (accession number GSE51612; Sgadò et al., 2013b). In order to visualize enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we used DAVID v6.7 (http://david.abcc.ncifcrf.gov/). To focus the functional analysis on hippocampal expressed genes we used as background a list of tissue-specific expressed genes for both autistic (BTBR and $En2^{-/-}$) strains. These background lists were obtained by filtering the genes by the normalized expression values and excluding the ones with the lowest expression levels (<10th percentile).

We also performed the direct enrichment analysis of different gene set categories present in the DEGs from BTBR and $En2^{-/-}$ mice, using the hypergeometric test present in R (*P*-value cut-off 0.05). The background used to compute the enrichments was the same used for KEGG pathway analysis (see below for the gene lists used for enrichment analyses).

Weighted Correlation Network Analysis (WGCNA) and Enrichment on Co-expression Modules

Microarray data from the BTBR and $En2^{-/-}$ hippocampi were analyzed with the WGCNA R package in order to find highly correlated modules of co-expressed genes (Langfelder and Horvath, 2008). Two different batches of samples, autistic (BTBR and $En2^{-/-}$) vs. control (B6 and $En2^{+/+}$), were used to obtain an appropriate number for the WGCNA analysis. All samples were normalized and filtered using the same method described above. The R program ComBat (Johnson et al., 2007) included in the SVA package (Leek et al., 2012) was used to remove the batch effect present on different chips, in which samples were run. In order to keep the most informative probes, only the top 20% (in terms of per-probe variance) were used as input for the WGCNA analysis. The WGCNA method is based on soft threshold approach in order to obtain an adjacency matrix that describes the relations among the genes (Zhang and Horvath, 2005). The scale-free criterion is used to select the power to apply to the correlations in order to obtain the adjacency matrix, a power of 11 was chosen in this study. The Topological Overlap

Measure (TOM) is then calculated starting from the previously obtained adjacency matrix. TOM is a highly robust measure of network interconnectedness (proximity) and expresses the strength characterizing the connection between each pair of genes. Genes with high TOM are clustered into co-expression modules. kME (representing the connection strength of each gene in each module) is then calculated as the correlation of each gene to the module eigengene (ME, defined as the first principal component of the expressions of the genes within the module). The ME defines measures of module membership (MM), which quantify how close a gene is to a given module eigengene. For each expression profile, Gene Significance (GS) was calculated as the absolute value of the correlation between expression profile and trait (autistic phenotype). The statistical significance of MM and GS (denoted as p.MM and p.GS) is carried out from the correlation test *p*-value of the WGCNA package. All values are tabulated in **Supplementary Table 3**. Given autism is a complex and very heterogeneous group of disorders, simple correlation of the modules with the trait produced meaningless results. A different approach was hence used: the modules were ranked based on their enrichment in term of genes present in the SFARI list. Then, modules significantly enriched in ASD-associated genes were functionally characterized for enrichment in markers of specific synapse, cell and disease types, using the same method described above. Moreover, to evaluate the biological and functional relevance of ASD-associated modules we used the Enrichr web tool. We analyzed the overrepresentation of the biological processes GO category for each gene list of modules.

Gene Lists Used for Enrichment Analyses

SFARI database (https://gene.sfari.org/autdb/Welcome.do) was used to calculate enrichment with ASD-associated genes on BTBR and $En2^{-/-}$ hippocampal DEGs. For disease annotations, we used the following gene sets from Autworks database (http://autworks.hms.harvard.edu): epilepsy, parkinsonian disorders and schizophrenia. For searching the recurrence and overlaps with FMRP-associated genes, we used a set of 842 genes (herein termed "FMRP Interacting Genes"), from a crosslinking and immunoprecipitation (CLIP) experiment (Darnell et al., 2011). Other gene lists used in this study (neuronal markers, astrocyte markers, type 1 microglial markers, type 2 microglial markers, oligodendrocyte markers, postsynaptic density, asdM12, asdM16) are available at http://www.arkinglab.org/resources. GABAergic synapse, glutamatergic synapse, dopaminergic synapse lists were taken from http://www.genome.jp/kegg/. In addition, the following gene lists were compiled from MsigDB (http://www.broadinstitute.org/gsea/msigdb/collections.jsp#C1): GABAergic presynaptic markers (GABA synthesis, release, reuptake and degradation); GABAergic postsynaptic markers (GABA A receptor activation, GABA B receptor activation, GABA receptor activation); glutamaergic presynaptic markers (glutamate neurotransmitter release cycle); glutamaergic postsynaptic markers (glutamate receptor activity; glutamate signaling pathway); dopaminergic markers (dopamine neurotransmitter release cycle).

RESULTS

Recently, we showed by transcriptome analysis that several genes related to ASD, abnormal synaptic transmission and GABA signaling are markedly deregulated in the hippocampus of $En2^{-/-}$ mice (Sgadò et al., 2013b). Here we extended this analysis to the hippocampus of BTBR mice, to test the hypothesis that common and distinct downstream mechanisms may be altered in these two ASD mouse models. Hippocampi from BTBR and B6 control adult mice were assessed for differential gene expression by microarray and bioinformatic analysis, as previously reported for $En2^{-/-}$ mice (Sgadò et al., 2013b). We found 1016 differentially expressed genes in the hippocampus of BTBR mice compared to their B6 controls (**Figure 1A**). Among these, 436 and 580 were up- and down-regulated, respectively. **Supplementary Table 2** shows the entire list of genes differentially expressed in the BTBR hippocampus, with fold change, percentage of false prediction (pfp) and *P*-values calculated by RankProd. We next validated microarray findings by qRT-PCR analysis, selecting seven representative genes from the DEGs list. Except for Gabra5, for which decreased protein and mRNA levels were found in the brains of ASD patients (Fatemi et al., 2010), many of the selected genes belong to immune/inflammatory categories. All genes showed statistically significant differential expression in the BTBR hippocampus, as compared to B6 controls [chemokine (C-C motif) ligand 21A (Ccl21a) $p = 0.0424$; gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5 (Gabra5) $p = 0.0069$; glial fibrillary acidic protein (Gfap) $p = 0.009$; polymerase (RNA) E (DNA directed) polypeptide E (Polr3e) $p = 0.025$; cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a) $p = 0.0052$; protein kinase C, delta polypeptide E (Polr3e) $p = 0.0029$; SLIT and NTRK-like family, member 6 (Slitrk6) $p = 0.0018$] (**Figure 1B**). In all tested genes the expression difference reported by qRT-PCR significantly correlated with microarray data (Pearson $r = 0.97$, $p < 0.0003$).

We next compared the BTBR hippocampal transcriptional profile to that of $En2^{-/-}$ mice. We first identified a total number of 153 commonly expressed genes between BTBR and $En2^{-/-}$ mice Interestingly, some of the common DEGs are related to inflammatory pathways (Aldh1a2, C2, Ccnd1, Cox7b2, Eif2ak2, Gsk3a, H2-Aa, H2-A1, H2-Bl, Map3k6, Pglyrp1, Slpi, Tia1; **Figure 2A**). qRT-PCR confirmed the differential expression of two of these genes [cyclin D1 (Ccdn1), $p = 0.0018$ and $p = 0.0051$ for BTBR and $En2^{-/-}$ mice, respectively; peptidoglycan recognition protein 1 (Pglyrp1), $p = 0.0052$ and $p = 0.0284$ for BTBR and $En2^{-/-}$ mice, respectively] (**Figure 2B**).

We next explored whether shared functional categories or common pathways were perturbed in both ASD mouse models. To increase accuracy of the functional analysis, we decided to compare the two lists of DEGs using updated databases and different bioinformatic tools, respect to those previously employed for the analysis of the hippocampal trascriptome of $En2^{-/-}$ mice (Sgadò et al., 2013b). We tested BTBR and $En2^{-/-}$ hippocampal DEGs for enrichment of mouse phenotype terms (Mouse Genome Informatics Mammalian Phenotype Level 4) using Enrichr (**Figure 3**). Two significantly

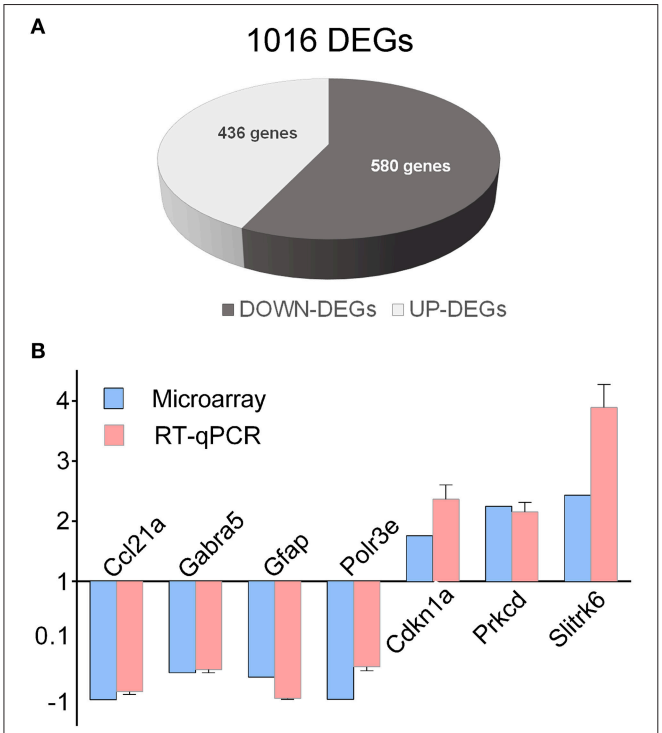
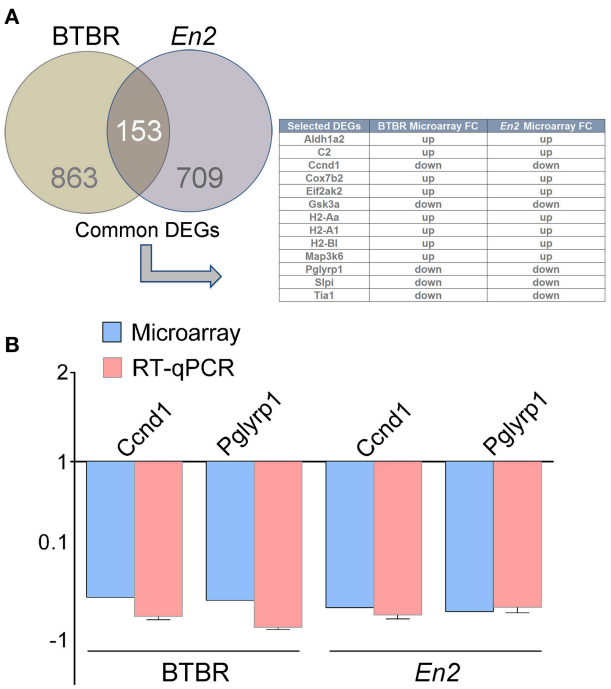


FIGURE 1 | Differentially expressed genes in the BTBR hippocampus. **(A)** Schematic representation of BTBR hippocampal DEGs, as compared to control B6 mice. A total of 1016 DEGs were identified, (580 down-regulated, 436 up-regulated). **(B)** qRT-PCR validation of differentially expressed genes. qRT-PCR results for all the evaluated genes were in agreement with microarray results. Values are expressed as each gene/L41 comparative quantitation ratios normalized on the expression of WT (mean ± s.e.m of three replicates from pools of six animals per genotype; $p < 0.05$, Student's *t*-test, BTBR vs. B6).



Selected DEGs	BTBR Microarray FC	*En2* Microarray FC
Aldh1a2	up	up
C2	up	up
Ccnd1	down	down
Cox7b2	up	up
Eif2ak2	up	up
Gsk3a	down	down
H2-Aa	up	up
H2-A1	up	up
H2-Bl	up	up
Map3k6	up	up
Pglyrp1	down	down
Slpi	down	down
Tia1	down	down

FIGURE 2 | DEGs commonly expressed in the BTBR and *En2*$^{-/-}$ hippocampus. **(A)** Venn diagram of differentially expressed genes in the BTBR and *En2*$^{-/-}$ adult hippocampus. A total of 1016 and 862 differentially expressed genes were identified in BTBR (brown) and *En2*$^{-/-}$ (gray) mice, respectively. Among these, 153 show differential expression in both strains. The table shows a subset of these common DEGs, all belonging to inflammatory pathways. **(B)** qRT-PCR validation of Ccnd1 and Pglyrp1, two genes differentially expressed in both BTBR and *En2*$^{-/-}$ hippocampi. qRT-PCR results for both genes were in agreement with microarray results. Values are expressed as each gene/L41 comparative quantitation ratios normalized on the expression of respective control (mean ± s.e.m of three replicates from pools of six animals per genotype; $p < 0.05$, Student's *t*-test, BTBR vs. B6 and *En2*$^{-/-}$ vs. *En2*$^{+/+}$).

enriched phenotypes were common to both mouse strains: abnormal behavioral responses and abnormal eating/drinking behavior. Conversely, specific phenotype ontologies enriched in BTBR were abnormal sensory capabilities, neurodegeneration, abnormal innate immunity and abnormal antigen presenting (**Figure 3A**). For *En2*$^{-/-}$ mice, we confirmed a significant enrichment for terms related to seizure and altered synaptic transmission (**Figure 3B**), as previously reported (Sgadò et al., 2013b). KEGG pathways analysis for BTBR and *En2*$^{-/-}$ hippocampal DEGs was performed with DAVID, using tissue-specific lists of expressed genes as background for each ASD mouse model (see Materials and Methods). The common pathways that were most significantly enriched across the differentially expressed genes of both ASD mouse models were related to chemokine signaling, MAPK signaling, systemic lupus erythematosus, Fc gamma receptor mediated phagocytosis and pathways in cancer (**Figure 4**).

To further reveal similarities and differences between the BTBR and *En2*$^{-/-}$ hippocampal transcriptome, we tested whether the two lists of DEGs were specifically enriched for cell-type specific markers and genes implicated in neurodevelopmental and neurodegenerative diseases (ASD, SCZ, epilepsy, Parkinson's). Remarkably, when compared to these repositories, BTBR and *En2*$^{-/-}$ hippocampal DEGs were enriched in SCZ- and ASD-related genes according to SFARI 2014 dataset (**Figure 5A**), consistent with reports showing overlap in candidate genes between ASD and SCZ (Crespi et al., 2010). Furthermore, when we separately analyzed up- and down-regulated genes, we found a significant over-representation of neuronal markers for both BTBR and *En2*$^{-/-}$ down-regulated DEGs; conversely, a significant over-representation of astrocyte markers was found in BTBR and *En2*$^{-/-}$ up-regulated DEGs (**Figure 5A**). Significant differences were also detected between the two strains. Among the genes down-regulated in the *En2*$^{-/-}$ hippocampus the following terms were significantly enriched: epilepsy, asdM12 (a neuronal module enriched for ASD-associated genes; Voineagu et al., 2011), FMR1 interacting genes, dopaminergic markers, GABAergic postsynaptic markers and glutamatergic presynaptic markers; conversely, type I microglia markers were significantly enriched among BTBR down-regulated genes (**Figures 5A,B**).

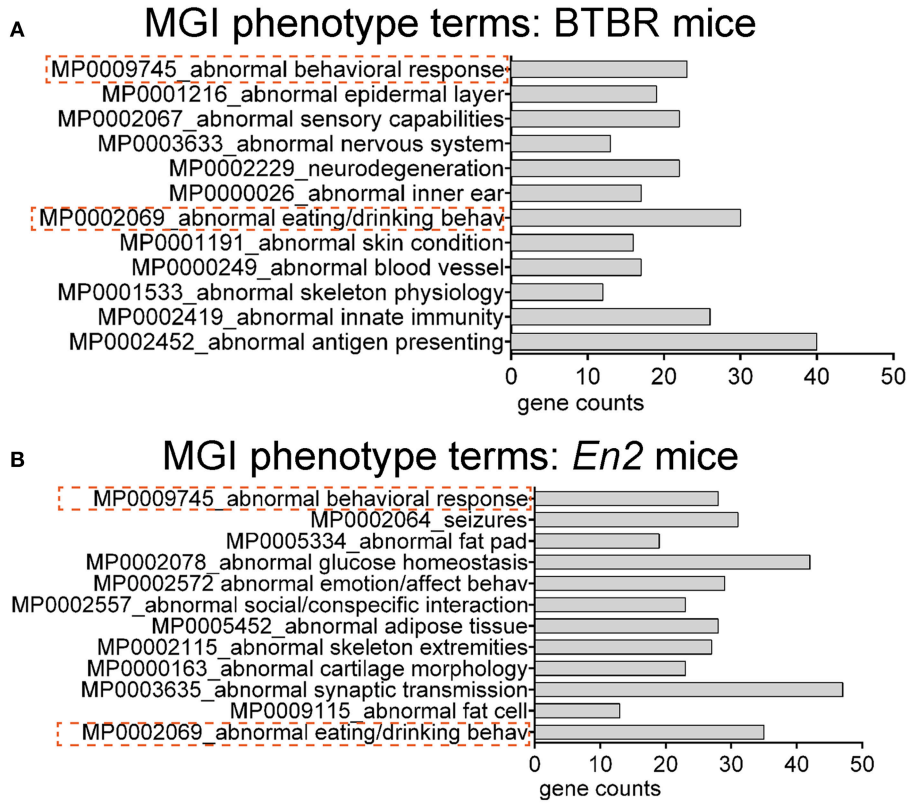


FIGURE 3 | Overrepresented mouse phenotype categories for differentially expressed genes in BTBR and *En2*$^{-/-}$ hippocampi. BTBR and *En2*$^{-/-}$ hippocampal DEGs were analyzed for enrichment in phenotype ontology categories using Enrichr, with an adjusted $p < 0.05$. For each category, the number of genes is indicated by the length of the horizontal bars (gene counts). Dashed red lines highlight phenotype categories common to BTBR **(A)** and *En2*$^{-/-}$ **(B)** DEGs.

To further investigate the presence of common molecular networks deregulated across autistic mice as compared to their respective controls, we analyzed the entire gene expression dataset (BTBR+*En2*$^{-/-}$ vs. B6+*En2*$^{+/+}$) using WGCNA (Zhang and Horvath, 2005). This analysis allows to identify discrete gene modules based on co-expression profiles (Voineagu et al., 2011). WGCNA identified 18 main modules for groups of genes with high topological overlap, ranging between 28 and 653 probes in size (**Supplementary Table 3**). The modules were correlated to the disease trait using a linear mixed regression framework and then ranked based on their enrichment in term of genes present in the SFARI list (retrieved on 2015). Six out of the eighteen modules were significantly enriched in ASD-related genes (Blue, $p = 9.072043e\text{-}09$; Brown, $p = 6.297529e\text{-}08$; Black, $p = 7.691951e\text{-}06$; Pink, $p = 0.00021$; Greenyellow, $p =$ value 0.00054; Red, $p = 0.0044$) (**Figure 6A**). We next tested the association of each of the six ASD-enriched modules for enrichment analysis. Except the Greenyellow module, all modules shared a significant enrichment for neuronal markers, as well as for genes associated to epilepsy and SCZ (**Figure 6A**). The Greenyellow module was enriched only for FMRP-interacting and glutamatergic synapse genes. Brown and Red modules were significantly enriched for oligodendrocyte markers and genes associated with M2-microglial cell states. For a better functional characterization of ASD-associated modules, we also used Enrichr to assess the GO biological process annotation of the six ASD gene-enriched modules. Blue, Black, Pink, and Red modules, which are enriched for neuronal markers (**Figure 6A**) also contain genes with the GO term "regulation of ion transmembrane transport" or "synaptic transmission" (**Figure 6B**). The Brown module, one of most enriched for categories (neuronal markers, type 2 microglial markers, oligodendrocyte markers, postsynaptic density, FMRP interacting genes, GABAergic/glutamatergic/dopaminergic synapse, epilepsy, schizophrenia; **Figure 6A**), showed an over-representation of genes involved in cognition, learning, and memory. Finally, the Greenyellow module did not show any significant over-representation of GO categories (**Figure 6B**).

DISCUSSION

Brief Summary of Results

In this study, we compared the hippocampal transcriptome of BTBR and *En2*$^{-/-}$ adult mice, two robust animal models of ASD. We identified both common and distinct gene pathways represented in the BTBR and *En2*$^{-/-}$ transcriptome. Common pathways included chemokine and MAP kinase signaling, whereas genes involved in immune dysfunction and abnormal synaptic transmission were specifically represented among BTBR

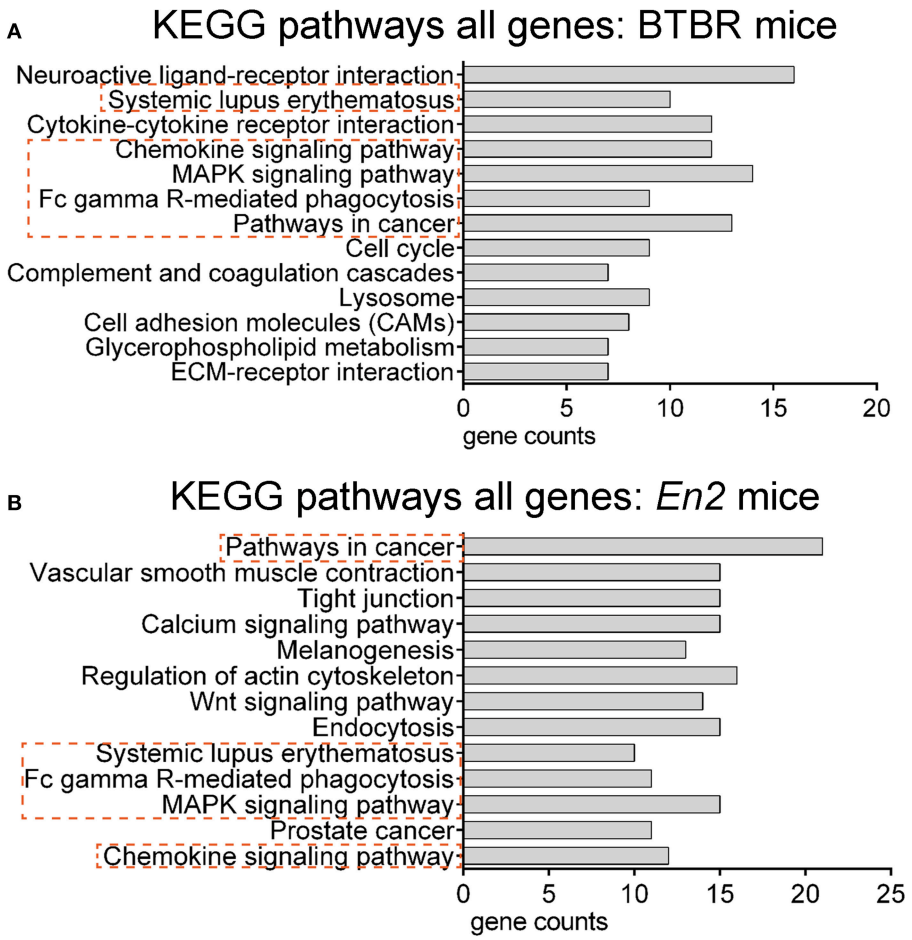


FIGURE 4 | Overrepresented pathways categories for differentially expressed genes in BTBR and *En2*$^{-/-}$ hippocampi. BTBR and *En2*$^{-/-}$ hippocampal DEGs were analyzed for enrichment in KEGG pathways categories using DAVID, with an adjusted $p < 0.05$. For each category, the number of genes is indicated by the length of horizontal bars (gene counts). Dashed red lines highlight pathways common to BTBR **(A)** and *En2*$^{-/-}$ **(B)** DEGs.

and *En2*$^{-/-}$ DEGs, respectively. Both BTBR and *En2*$^{-/-}$ hippocampal DEGs showed a significant enrichment of ASD and SCZ-associated genes, with specific gene sets enriched in the two models (glial genes in BTBR; GABAergic, glutamatergic FMRP-related and epilepsy-related genes in *En2*$^{-/-}$). Finally, network analysis (WGCNA) performed on BTBR and *En2*$^{-/-}$ hippocampal DEGs together identified six modules significantly enriched in ASD-associated genes, with specific enrichment profile in neuronal, glial, epilepsy-related, and SCZ-associated genes.

Common Pathways Deregulated in the BTBR and *En2*$^{-/-}$ Hippocampus

Our comparative microarray analysis revealed that 155 genes (out of a total of 44,000) are differentially expressed in both BTBR and *En2*$^{-/-}$ hippocampus (**Figure 1**), indicating that a very low of number of genes (0.35%) are commonly deregulated among these two ASD mouse models. Nevertheless, ontology analyses for phenotypes and cellular pathways revealed that some common gene categories are significantly over-represented among the genes differentially expressed in the BTBR and *En2*$^{-/-}$ hippocampus, as compared to their respective controls (B6, *En2*$^{+/+}$ mice). Mammalian Phenotype ontology revealed that genes involved in abnormal behavioral responses are significantly deregulated in both mouse models (**Figure 2**). Most importantly, KEGG pathways analysis showed that genes related to immune response and inflammation are significantly deregulated in both BTBR and *En2*$^{-/-}$ hippocampus (KEGG pathways: chemokine signaling, MAPK signaling, systemic lupus erythematosus, Fc gamma receptor mediated phagocytosis; **Figure 3**). This is in line with our current knowledge about the role of the immune system in ASD pathogenesis. Genetic studies indicate that several genes encoding components of the immune system are associated to ASD. In addition, data from both ASD individuals and animal models indicate a significant dysregulation of immune processes in ASD, such as up-regulation of pro-inflammatory cytokines/chemokines, increased expression of major histocompatibility complex, and activation

A

Gene lists	BTBR All-DEGs	*En2* All-DEGs	BTBR Up-DEGs	*En2* Up-DEGs	BTBR Down-DEGs	*En2* Down-DEGs
Neuronal Markers						
Astrocyte Markers						
Type 1 Microglial Markers						
Type 2 Microglial Markers						
Oligodendrocyte Markers						
Postsynaptic Density (PSD)						
asdM12						
asdM16						
SFARI_2014						
SFARI_2015						
FMR1 Interacting Genes						
Epilepsy						
Parkinsonian disorders						
Schizophrenia						

B

Gene lists	BTBR All-DEGs	*En2* All-DEGs	BTBR Up-DEGs	*En2* Up-DEGs	BTBR Down-DEGs	*En2* Down-DEGs
GABAergic presynaptic						
GABAergic postsynaptic						
Glutamatergic presynaptic						
Glutamatergic postsynaptic						
Dopaminergic markers						

	P < 0,05
	P > 0,05

FIGURE 5 | Enrichment analysis on BTBR and *En2*$^{-/-}$ hippocampal DEGs. Lists of BTBR and *En2*$^{-/-}$ hippocampal DEGs (all, up-regulated and down-regulated) were analyzed for enrichment in genes belonging to specific categories relevant to the autistic phenotype **(A)** and neuronal subtypes **(B)**. Gene lists were compiled from the literature (see Materials and Methods and references therein). Significance of enrichment is indicated (white, $p > 0.05$; gray, $p < 0.05$).

of microglia in the brain (reviewed in Estes and McAllister, 2015). Significantly increased levels of pro-inflammatory cytokines (IL-1β, IL-6) and microglia activation have been reported in BTBR brains (Heo et al., 2011; Onore et al., 2013; Zhang et al., 2013), similarly to what observed in post-mortem brain tissue from ASD patients (Vargas et al., 2005; Li et al., 2009; Suzuki et al., 2013).

It is important to point out that, although the B6 strain is commonly used to compare with BTBR in molecular and behavioral studies (reviewed in Silverman et al., 2010), it exists the possibility that it is not an ideal or sufficient control for BTBR in gene expression studies. Indeed, linkage disequilibrium studies showed that B6 and BTBR mice are on different branches of the mouse genetic tree (Petkov et al., 2005). Thus, further genetic characterization is needed to identify the ideal control strain for inbred BTBR mice. In the absence of such a control, we conformed to previous studies using B6 as a control for BTBR in transcriptional profiling analyses (Daimon et al., 2015).

Differently from what reported in this study, our previous transcriptome profiling revealed a significant deregulation of immune- and inflammation-related genes in the cerebellum but not hippocampus of *En2*$^{-/-}$ adult mice (Sgadò et al., 2013b). One possible explanation is that our previous gene ontology analysis of the *En2*$^{-/-}$ hippocampal transcriptome was performed using different databases than those used for the present study. Preliminary unpublished data from our laboratory however indicate that the expression of several immune mediators (IL-1β, TNF-α, toll-like receptor 2, CCL2, CCL5) is significantly deregulated in the hippocampus (as well as neocortex and cerebellum) of *En2*$^{-/-}$ adult mice.

Our enrichment analyses revealed that both sets of BTBR and *En2*$^{-/-}$ hippocampal DEGs are enriched in ASD-related genes according to the SFARI 2014 dataset. *En2*$^{-/-}$ hippocampal DEGs also showed a significant enrichment in genes contained in two additional datasets of autism-related genes (asdM12 and SFARI15). This confirms that both BTBR and *En2*$^{-/-}$ mice are valuable mouse models to investigate ASD-relevant gene signatures. Most importantly, these enrichment analyses showed that both BTBR and *En2*$^{-/-}$ DEGs dataset are significantly enriched in SCZ-associated genes (**Figure 5**). This is in agreement with recent results from large-scale genomic studies showing that ASD and SCZ are neurodevelopmental disorders characterized by overlapping genetics and phenotypes (Murdoch and State, 2013). Genetic lesions at the origin of these disorders are thought to affect brain circuit formation and synaptic function during embryonic and/or postnatal development, ultimately leading to a varying range of (partially overlapping) pathological behaviors in ASD and SCZ. Recent genomic studies show that control subjects carrying copy-number variants (CNVs) conferring risk of ASD or SCZ poorly perform in cognitive tests (Stefansson et al., 2014), suggesting that conserved genetic mechanisms might underlie shared co-morbidities in these two neurodevelopmental disorders.

Enrichment analyses also showed that both sets of BTBR and *En2*$^{-/-}$ hippocampal DEGs are enriched in neuronal and astrocyte markers. The importance of glial cells in ASD pathophysiology was initially suggested by RNA sequencing studies showing a significant enrichment of glial genes belonging to immune/inflammatory categories in ASD brain tissues (Voineagu et al., 2011). These gene expression results are supported by neuroanatomical data showing glial cell activation in ASD postmortem brains (reviewed in Petrelli et al., 2016).

A

Gene lists	Modules					
	Blue *9,44% of SFARI enrich. p value 9.072043e-09*	**Brown** *9,05 % of SFARI enrich. p value 6.297529e-08*	**Black** *10,9 % of SFARI enrich. p value 7.691951e-06*	**Pink** *10,9 % of SFARI enrich. p value 0.00021*	**Greenyellow** *11,1 % of SFARI enrich. p value 0.00054*	**Red** *6,4 % of SFARI enrich. p value 0.0044*
Neuronal Markers						
Astrocyte Markers						
Type 1 Microglial Markers						
Type 2 Microglial Markers						
Oligodendrocyte Markers						
Postsynaptic Density (PSD)						
FMRP Interacting Genes						
GABAergic synapse						
Glutamatergic synapse						
Dopaminergic synapse						
Epilepsy						
Schizophrenia						
Parkinsonian disorders						

P < 0,05
P > 0,05

B

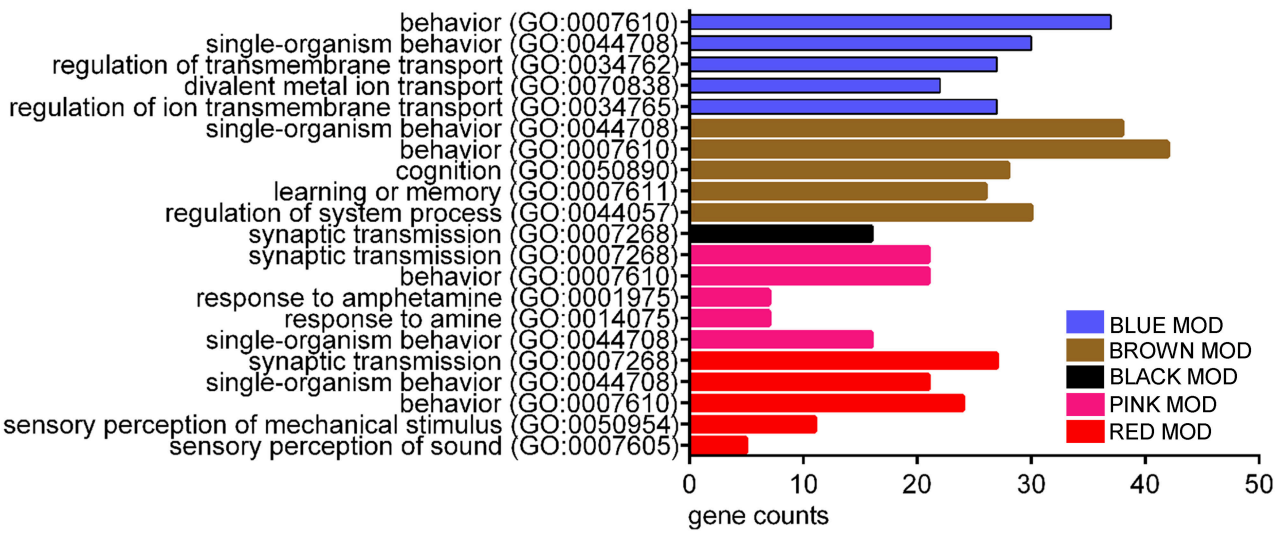


FIGURE 6 | Enrichment and gene ontology analyses on co-expression modules resulting from WGCNA on BTBR and *En2*$^{-/-}$ hippocampal DEGs. (A) Co-expression modules enriched in SFARI genes were analyzed for enrichment in genes belonging to specific categories relevant to the autistic phenotype, as indicated. Gene lists were as in **Figure 5**. Significance of enrichment is indicated (white, $p > 0.05$; gray, $p < 0.05$). **(B)** Co-expression modules were analyzed for enrichment in GO categories using Enrichr, with an adjusted $p < 0.05$. For each category, the number of genes is indicated by the length of horizontal bars (gene counts). Modules are indicated in their respective colors. The Greenyellow module is not indicated in the graph since it did not show any significant over-representation of GO terms.

Distinct Pathways Deregulated in the BTBR and *En2*$^{-/-}$ Hippocampus

Phenotype and gene ontology analyses, along with enrichment analyses, also confirmed that marked differences are present between the hippocampal gene expression profiles of BTBR and *En2*$^{-/-}$ adult mice. Mammalian Phenotype ontology analysis showed that the "abnormal innate immunity" and "seizure/abnormal synaptic transmission" pathways are significantly over-represented among hippocampal DEGs in BTBR and *En2*$^{-/-}$ adult mice, respectively (**Figure 2**). Abnormal immune response has been clearly demonstrated in BTBR mice (see Careaga et al., 2015, and references above). As an example, when pregnant dams of BTBR and C57BL/6J inbred strains were exposed to the viral mimic polyinosinic-polycytidylic acid (polyI:C), severe ASD-like behaviors and persistent dysregulation of adaptive immune system function were only observed in BTBR offspring (Schwartzer et al., 2013).

The gene expression signature of the BTBR hippocampus resulting from the present study is markedly different from that previously published by other authors. We detected a total of 1016 BTBR DEGs, whereas the number of BTBR DEGs detected by Daimon et al. (2015) was significantly lower (301). Only 69 BTBR DEGs were common to the two datasets. In both studies, the hippocampal BTBR transcriptome was compared to that of C57BL/6J inbred mice, and adult mice of comparable age were used (5 months in our study, 4 months in Daimon et al., 2015). However, Daimon et al. (2015) used a different microarray platform (Illumina), microarray analysis software (DIANE 6.0) and pathways analysis software (WebGestalt, http://bioinfo.vanderbilt.edu/), which can justify the difference in the obtained results.

As for the specific gene expression signature of the $En2^{-/-}$ hippocampus, KEGG pathway analysis performed in the present study confirmed our previous analysis (Sgadò et al., 2013b). Indeed, a marked excitation/inhibition unbalance is present in the $En2^{-/-}$ hippocampus and neocortex, as suggested by the increased seizure susceptibility (Tripathi et al., 2009) and loss of GABAergic interneurons (Sgadò et al., 2013a; Allegra et al., 2014; Provenzano et al., 2014) observed in $En2^{-/-}$ mice. These data are also supported by this study (**Figure 5**), which reveals a significant enrichment of GABAergic/glutamatergic postsynaptic markers among the genes down-regulated in the $En2^{-/-}$ hippocampus; genes interacting with fragile X mental retardation protein (FMRP) are also enriched, in keeping with the marked down-regulation of the FMRP pathway detected in the $En2^{-/-}$ hippocampus (Provenzano et al., 2015). Finally, dopaminergic markers are also enriched among the genes down-regulated in the $En2^{-/-}$ hippocampus, in keeping with the established role of Engrailed proteins in regulating the development and function of dopaminergic neurons (Gherbassi and Simon, 2006). Conversely, microglia markers are significantly enriched among genes down-regulated in the BTBR hippocampus, thus confirming the marked dysregulation of glial cell function in this ASD model. Accordingly, histopathological studies revealed glial cells but not GABAergic neurons alterations in the hippocampus of BTBR mice (Stephenson et al., 2011).

Weighted Correlation Network Analysis Reveals ASD-Relevant Gene Expression Modules

Based on the significant similarities detected between the BTBR and $En2^{-/-}$ hippocampal DEGs (**Figures 2**, **3**), we considered the gene expression signature of these two ASD mouse models together, and compared it to that of their respective control strains (C57Bl/6J and $En2^{+/+}$). We then performed weighted gene co-expression network analysis (WGCNA; Langfelder and Horvath, 2008) to identify correlation patterns among genes across the ASD models vs. control microarray samples, and find gene clusters (modules) whose expression is highly correlated. Genes differentially expressed in ASD mouse models were clustered in 18 modules, and our WGCNA showed that 6 out of these 18 modules were significantly enriched in ASD-related genes. Each of these 6 ASD genes-enriched modules showed a specific enrichment profile in neuronal and glial genes, as well as in genes associated to epilepsy and SCZ. This is in line with WGCNA performed on gene signatures from ASD post-mortem cortical brain tissue, which showed a significant deregulation of glial and neuronal activity-dependent genes in autism (Gupta et al., 2014). It is also important to note that ASD and epilepsy show a high degree of comorbidity (Buckley and Holmes, 2016), and conserved genetic mechanisms have been proposed to underlie ASD and SCZ (see above in this discussion; Stefansson et al., 2014). Thus, WGCNA performed on microarray samples from autistic vs. control mouse strains is able to identify conserved gene expression signatures across different ASD mouse models.

CONCLUSIONS

The present study reveals significant transcriptional similarities and differences between the BTBR and $En2^{-/-}$ hippocampus, confirming the idea that transcriptome profiling of specific brain areas from ASD mouse models may contribute to identify novel molecular targets for pharmacological studies.

AUTHOR CONTRIBUTIONS

GP designed and performed experiments, analyzed data and wrote the paper. ZC and LR performed experiments. KM and TF analyzed data. MS conceived the study and provided funding. YB conceived the study, analyzed data, provided funding, and wrote the paper. ZC and KM equally contributed to this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00396

Supplementary Table 1 | Primers used for qRT-PCR analyses.

Supplementary Table 2 | List of genes differentially expressed in the BTBR hippocampus, as compared to B6 controls.

Supplementary Table 3 | Raw data of WGCNA analysis on autistic (BTBR and $En2^{-/-}$) vs. control (B6 and $En2^{+/+}$) differentially expressed genes.

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Premutation in the Fragile X Mental Retardation 1 (*FMR1*) Gene Affects Maternal Zn-milk and Perinatal Brain Bioenergetics and Scaffolding

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Fragile X premutation alleles have 55–200 CGG repeats in the 5′ UTR of the *FMR1* gene. Altered zinc (Zn) homeostasis has been reported in fibroblasts from >60 years old premutation carriers, in which Zn supplementation significantly restored Zn-dependent mitochondrial protein import/processing and function. Given that mitochondria play a critical role in synaptic transmission, brain function, and cognition, we tested FMRP protein expression, brain bioenergetics, and expression of the Zn-dependent synaptic scaffolding protein SH3 and multiple ankyrin repeat domains 3 (Shank3) in a knock-in (KI) premutation mouse model with 180 CGG repeats. Mitochondrial outcomes correlated with FMRP protein expression (but not *FMR1* gene expression) in KI mice and human fibroblasts from carriers of the pre- and full-mutation. Significant deficits in brain bioenergetics, Zn levels, and Shank3 protein expression were observed in the Zn-rich regions KI hippocampus and cerebellum at PND21, with some of these effects lasting into adulthood (PND210). A strong genotype × age interaction was observed for most of the outcomes tested in hippocampus and cerebellum, whereas in cortex, age played a major role. Given that the most significant effects were observed at the end of the lactation period, we hypothesized that KI milk might have a role at compounding the deleterious effects on the *FMR1* genetic background. A higher gene expression of *ZnT4* and *ZnT6,* Zn transporters abundant in brain and lactating mammary glands, was observed in the latter tissue of KI dams. A cross-fostering experiment allowed improving cortex bioenergetics in KI pups nursing on WT milk. Conversely, WT pups nursing on KI milk showed deficits in hippocampus and cerebellum bioenergetics. A highly significant milk type × genotype interaction was observed for all three-brain regions, being cortex the most influenced. Finally, lower milk-Zn levels were recorded in milk from lactating women carrying the premutation as well as other Zn-related outcomes (Zn-dependent alkaline phosphatase activity and lactose biosynthesis—whose limiting step is the Zn-dependent β-1,4-galactosyltransferase). In premutation carriers, altered

Zn homeostasis, brain bioenergetics and Shank3 levels could be compounded by Zn-deficient milk, increasing the risk of developing emotional and neurological/cognitive problems and/or FXTAS later in life.

Keywords: bioenergetics, brain, *FMR1*, milk, mitochondria, premutation, Shank3, zinc

INTRODUCTION

A 55–200 expanded CGG nucleotide repeats in the 5′-UTR of the fragile X mental retardation one gene (*FMR1*) constitutes the genetic hallmark of premutation carriers (OMIM#300623), whereas >200 repeats give rise to Fragile X syndrome (FXS; OMIM#300624), the leading inherited form of cognitive impairment (Kogan et al., 2008; Tassone et al., 2012; Battistella et al., 2013). Later in life, premutation carriers may have an increased risk of developing a neurodegenerative disorder known as Fragile X-associated tremor/ataxia syndrome [FXTAS (Kogan et al., 2008; Hagerman and Hagerman, 2013)]. Initially, this prevalent allelic variant was thought to be free of phenotypic traits; however, neurodevelopmental problems like autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), anxiety, and other types of psychopathologies (Farzin et al., 2006; Tassone et al., 2012; Winarni et al., 2012; Wong et al., 2012; Battistella et al., 2013; Chonchaiya et al., 2013) have been reported in some young carriers, but not as often as in Fragile X syndrome [i.e., incidence of ASD in FXS is ∼4-fold of that in premutation carriers (Farzin et al., 2006; Garcia-Nonell et al., 2008; Harris et al., 2008; D'Hulst et al., 2009; Zingerevich et al., 2009; Hagerman et al., 2010; Chonchaiya et al., 2013)].

Unlike the full mutation alleles, which undergo repeat-mediated gene silencing (Pieretti et al., 1991; Sutcliffe et al., 1992), premutation alleles are active and actually show normal or elevated *FMR1* mRNA levels (Tassone et al., 2000a,b, 2007). It is still not clearly understood how the premutation pathology arises and research to date has focused on how premutation alleles might trigger neurodegeneration through a gain-of-function (toxicity) RNA mechanism (Tassone et al., 2004; Hagerman and Hagerman, 2013). It has been suggested that the "excess" of premutation transcript may bind and sequester factors important for cell function (Sellier et al., 2010, 2013). Previous work from our laboratory conducted on fibroblasts from premutation individuals has shown that zinc (Zn) might also be included among these factors (Napoli et al., 2011). It has been shown that while *FMR1* gene expression could be high in some carriers, its product FMRP can be low (Tassone et al., 2000c; Tassone and Hagerman, 2003) which has been attributed to a reduced translation efficiency (Tassone and Hagerman, 2003). However, critical aspects of the pathology cannot be explained purely by an RNA-mediated process but rather to a protein-mediated neurodegeneration (Todd et al., 2013). Recently, the paradox of the RNA- mediated vs. protein-mediated toxicity in FXTAS, although still controversial (Banez-Coronel et al., 2012), has been partly explained by demonstrating that CGG repeats trigger repeat-associated non-AUG-initiated (RAN) translation (Todd et al., 2013) of a cryptic polyG-containing protein, FMRPolyG. Accumulation of FMRPolyG has been seen in ubiquitin-positive inclusions in *Drosophila*, mammalian cell cultures carrying the expansion, and brains of patients that died of FXTAS (Todd et al., 2013) and this lengthy polyglycine tract seems to be toxic to a number of cell types (Todd et al., 2013; Oh et al., 2015). So far, the relative contribution of RNA sequestration (of essential factors), low FMRP expression or RAN translation (of the toxic FMRPolyG) to the premutation pathology is unknown.

Adding to these mechanisms, bioenergetic deficits with increased oxidative stress biomarkers have been observed in post-mortem brain samples (Ross-Inta et al., 2010) and fibroblasts from premutation carriers (Ross-Inta et al., 2010; Napoli et al., 2011) and altered mitochondrial dynamics have been noted in neurons from a knock-in (KI) mouse model of *FMR1* premutation (Kaplan et al., 2012). These deficits seem to precede the occurrence of ubiquitin-positive intranuclear inclusions [considered a hallmark of FXTAS; (Greco et al., 2006)], and correlate with both CGG repeat expansion and severity of the phenotype (Ross-Inta et al., 2010; Napoli et al., 2011). Fibroblasts from >60 years old asymptomatic premutation carriers presented altered protein expression of the Zn transporters ZnT6/ZnT4 (Napoli et al., 2011) accompanied by mitochondrial dysfunction (MD; Ross-Inta et al., 2010; Napoli et al., 2011). This MD was mainly evidenced by an accumulation of precursor over mature mitochondrial proteins encoded by the nuclear DNA. This scenario was significantly reversed upon Zn supplementation, which allowed Zn-dependent import/processing pathways of nuclearly-encoded mitochondrial proteins to occur (Napoli et al., 2011).

Abnormal behavior (i.e., over-responsivity and hyperactivity-like behavior with acute Zn deficiency; ASD-like behavior secondary to prenatal Zn deficiency) was reported in young Zn-deficient mice with altered scaffolding elements within the postsynaptic density of excitatory synapses (Grabrucker et al., 2014). Indeed, it has been proposed that the ∼50% incidence of Zn deficiency in children with ASD (Yasuda et al., 2011) has the potential to contribute to the etiology and/or morbidity of ASD via dysregulation of the synaptic Shank scaffolding (Grabrucker et al., 2014). Furthermore, some symptoms of ASD seem to lessen with Zn supplementation (Russo and Devito, 2011).

Collectively these studies suggest that the *FMR1* premutation affects Zn homeostasis, and that Zn deficits have a detrimental effect on behavior, opening the door for evaluating the effect of environmental stressors (such as Zn deficits) at compounding or initiating MD early in life and, possibly, predisposing young carriers to develop ASD, ADHD, and/or FXTAS at older age (Tassone et al., 2012; Winarni et al., 2012; Wong et al., 2012; Battistella et al., 2013).

Thus, we hypothesized that *FMR1* premutation would alter brain Zn homeostasis, bioenergetics, and protein expression of Zn-dependent scaffolding protein SH3 and multiple ankyrin

repeat domains 3 (Shank3), outcomes which could be further affected by nursing on milk from premutation carriers. To test our hypothesis, *FMR1* gene expression, Fragile X mental retardation protein 1 (FMRP) protein expression, bioenergetics, Zn levels, and Shank3 protein expression were evaluated at post-natal days (PND) 0, 9 (or 7), 21, and 210 in cerebellum, hippocampus (both areas rich in Zn-containing neurons) as well as in cortex from a KI premutation mouse model. This murine model recapitulates some of the molecular, histological, and neurobehavioral deficits observed in premutation carriers (i.e., elevated *FMR1* mRNA, FMRP protein levels reduced or normal), and with age they develop intranuclear inclusions in neurons and astrocytes, ataxia-like mild motor dysfunctions, anxiety, and cognitive impairments (Hunsaker et al., 2009, 2011, 2012; Wenzel et al., 2010). To evaluate the effect of carriers' milk on the offspring's brain bioenergetics, a cross-fostering experiment was designed in which brain mitochondrial function was tested in suckling WT and KI pups at PND21. To complement the mouse model studies, Zn and Zn-associated outcomes were evaluated in breast milk from control and premutation nursing mothers.

Given the key role of Shank3 in post-synaptic neuron scaffolding and the contribution of mitochondria to the regulation of synaptic transmission, brain function, and cognition, we propose that early nutritional Zn interventions may represent a new preventive strategy in newborns of premutation mothers with the potential of lowering the risk of developing emotional or neurological symptoms later in life, emphasizing the interdependence between genetics and nutrition.

MATERIALS AND METHODS

Animals

This study was approved by the IACUC Committee at the University of California Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care (IACUC-approved protocol number 17896). All animals were treated accordingly to the guidelines established by the NIH and the UCD animal welfare committee. Mice were monitored daily during the length of the experiments. Mice with signs of stress, weight loss >20%, paralysis, or any other serious disease, would have been euthanized immediately to avoid unnecessary pain or discomfort. However, none of the animals showed these signs of distress or were euthanized. No animal procedure was attempted without prior approval from the NIH and the UCD animal welfare committee, as well as all personnel was trained to handle animals under the current regulations. Wild-type female C57BL/6J mice were obtained commercially (Charles River, Wilmington, MA) and the knock-in (KI) mouse model of the premutation in the same genetic background (Wenzel et al., 2010) was from Dr. Robert Berman (University of California, Davis). The mice were housed in polycarbonate cages and fed *ad libitum*. Zn content of the diet used (Purina Pico chow 5058) was 1200 ppm, which with an average adult body weight of 30 g/mouse and a food intake of 2–5 g/mouse equals to 10–20 mg Zn/kg body weight *per diem*. Female mice during pregnancy increase their food intake by about two-fold and during lactation by about four-fold resulting in 20–40 mg Zn/kg body weight *per diem* during pregnancy and 40–60 mg Zn/kg body weight per day during lactation [reference values = 10 mg/kg and 30 mg/kg for adult and pregnant/lactating mice, respectively (Knapka et al., 1974; Luecke and Fraker, 1979; Beach et al., 1982)]. Mice were maintained on a 12 h light/dark cycle under controlled temperature and humidity. Mice were bred and allowed to deliver naturally. At PND0, 9 (or 7), 21, and 210, mitochondria were isolated from hippocampus, cerebellum and cortex from WT and KI male mice (n = 9–13 at each time point for a total of 54 WT and 55 KI). For the cross-fostering experiments (performed twice), at birth KI pups (males; n = 12; CGG repeats = 196 ± 6) and WT pups (males; n = 12; CGG repeats = 9.3 ± 0.2) were randomly foster-nursed either on KI dams (173 ± 4 CGG repeats) or on WT dams, with six pups on each dam. The KI pups were fed with the same frequency as WT animals. At PND21, WT, and KI dams were removed from pups for 2 h to control for effects of suckling on Zn transporter expression and localization, and subsequently were euthanized by CO_2 asphyxiation. Mammary glands from lactating dams were removed and either snap-frozen in liquid nitrogen or stored in RNA later. At PND21 pups were also euthanized by CO_2 asphyxiation. Euthanasia was performed by an experienced technician by way of inhalation of 30% CO_2 (compressed gas cylinder). This method is considered acceptable by the 2000 Report of American Veterinary Medical Association (AVMA) Panel on Euthanasia and it has been approved by IACUC. The cortex, cerebellum, and hippocampus were removed post-mortem and processed immediately to isolate mitochondria.

Isolation and Purification of Mitochondria from Brain Regions

Enriched mitochondrial fractions were obtained from cortex, cerebellum and hippocampus at PND0 (n = 13 WT and 13 KI), PND7 (n = 9 WT and 9 KI), PND21 (n = 12 WT and 12 KI), and PND210 (n = 11 WT and 12 KI). Intact, highly purified, non-synaptosomal mitochondria from the same brain tissues were isolated through a Percoll gradient as previously described in detail (Napoli et al., 2012, 2013), resuspended in iso-osmotic 150 mM KCl and immediately used for oxygen consumption measurements. Western blots to actin (cytosolic protein) and beta-ATPase (mitochondrial protein) showed a cytosolic contamination of the mitochondrial fraction of less than 2% [see (Napoli et al., 2013)].

Primary Neuronal Cultures

To assess the contribution of glia to brain mitochondrial outcomes [as previously shown in Napoli et al. (2012)], isolated, intact primary neurons were obtained from WT and KI pups at PND0, using an established methodology (Brand, 1990; Jekabsons and Nicholls, 2004). Cultures of dissociated neurons were prepared as described in Kaplan et al. (2012) and kindly provided by Dr. Eitan Kaplan. Intact (non-permeabilized) neurons suspended in PBS supplemented with 10 mM glucose were used for evaluation of oxygen consumption followed by sequential additions of 5 μg/ml oligomycin and 20 μM FCCP

(Napoli et al., 2011). The RCR under uncoupling conditions or RCRu in intact cells was calculated as the oxygen uptake ratio of State 3u (with FCCP) over that of oligomycin-induced State 4.

Mitochondrial Outcomes

Enriched mitochondrial fractions or in intact, purified mitochondria were used for evaluation of oxygen consumption using a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) as described (Napoli et al., 2013). An aliquot (0.1–0.3 mg protein/ml) of mitochondria was added to the oxygen chamber in a buffer containing 0.22 M sucrose, 50 mM KCl, 1 mM EDTA, 10 mM KH_2PO_4, and 10 mM HEPES, pH 7.4. Oxygen consumption rates were evaluated in the presence of (i) 1 mM ADP plus 1 mM malate–10 mM glutamate followed by the addition of 5 μM rotenone; (ii) 10 mM succinate followed by the addition of 10 mM malonate; (iii) 10 mM α-glycerophosphate followed by addition of 3.6 μM antimycin A; and (iv) 10 mM ascorbate and 0.2 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine followed by the addition of 1 mM KCN. The activities of mitochondrial NADH oxidase, succinate oxidase, and cytochrome *c* oxidase were evaluated as the difference of oxygen uptake recorded before and after the addition of rotenone, malonate, antimycin A, and KCN respectively, and normalized by the activity of citrate synthase (a marker of mitochondrial mass). Citrate synthase activity was evaluated spectrophotometrically as described elsewhere (Napoli et al., 2013) using 1–2 μg of mitochondrial protein. The respiratory control ratio (RCR) with malate/glutamate as a substrate was calculated as the ratio between oxygen uptake rates in State 3 (with ADP or under phosphorylating conditions) and State 4 [rotenone-resistant oxygen uptake or non-phosphorylating conditions; (Napoli et al., 2013)].

Western Blots

For quantification of FMRP, Shank3, cytochrome *c* oxidase subunit IV (CCOIV), ATPase β-subunit (ATPB), β-actin, GAPDH, and tubulin expression levels, brain regions (hippocampus, cerebellum, and cortex) at PND9, 21, and 210 from WT and KI mice were homogenized in cold 20 mM HEPES, pH 7.4, added with protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO), and centrifuged at 13,000 × *g* for 10 min to eliminate particulate matter. A second set of Shank3 immunoblots (shown in Supplementary Figure 1) was carried out by using acetone precipitation to concentrate and partly delipidate the samples as previously described (Fujisawa et al., 2015). Thirty to fifty microgram of proteins were solubilized in SDS sample buffer (Life Technologies, Grand Island, NY) and loaded onto a 4–12% bis-tris gel (Life Technologies) as previously described (Napoli et al., 2013). After transferring proteins with an iBlot apparatus (Life Technologies), membranes were blocked with LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and subsequently probed with anti-FMRP antibody (Sigma, St. Louis, MO; 1:700 dilution), anti-Shank3 antibody (Santa Cruz Biotechnologies, Dallas, TX; 1:500 dilution), anti-CCOIV antibody (Cell Signaling, Danvers, MA, 1:1000 dilution), anti ATPB (BD Biosciences, San Jose, CA, 1:3000 dilution) overnight at 4°C, and with anti-β-actin (Sigma, St. Louis, MO; 1:20,000 dilution) for 1 hour at room temperature. Tubulin (Proteintech, Chicago, IL; 1:1,000 dilution) and GAPDH (Santa Cruz Biotechnology, Dallas, TX, 1:300 dilution) were used as loading controls. Secondary antibodies were from LI-COR (Lincoln, NE; 1:10,000 dilution). Membranes were visualized with the use of the Odyssey Infrared Imaging System (LI-COR) and densitometry analysis carried out with the Carestream software (Napoli et al., 2013).

Gene Expression of *ZnT4/T6* In Mammary Glands from Lactating WT and KI Dams

RT-qPCR performed with three different sets of commercially available primers from Life Technologies showed low specificity and efficiency. Thus, the gene expression of *ZnT4* and *ZnT6* was assessed by PCR. Total RNA was isolated from homogenized mammary gland following the manufacturer's instructions (Qiagen). Concentration and purity of RNA was measured at an absorbance of 260 nm and 280 nm using the Tecan i-control 1.6 software (v.1.6.19.2) on Tecan infinite M200 Nanoquant (Tecan, Austria). Following RNA isolation, 2 μg of the RNA was used to make cDNA with the Qiagen Quantitech RT Kit following the manufacturer's instructions. Following cDNA synthesis, *ZnT4,* and *ZnT6* gene expression was performed by Touchdown PCR in a 96-well plate in a 25 μl reaction volume containing: 100 ng of cDNA, 2.5 μl of 10x Advantage 2 PCR buffer (Clontech, Mountain View, CA), 0.5 μl of 50X Advantage 2 Polymerase Mix (Clontech), 1 μl of 10 mM dNTPs (Life Technologies, Grand Island, NY), and 0.5 μl of each 10 mM forward and reverse primer. Mouse primers for *ZnT4* were: forward 5′-CTCCAGGCCGACGATGACT-3′; reverse 5′-GTGTCCACTAGATACCATGCTTGG-3′. Primers for *ZnT6* were: forward 5′-CAGACCTTAGCCGCAGCTTG-3′; reverse 5′-GGTCTGAGAAGTTTAGGACGTTGG-3′. PCR cycling parameters were: 95°C for 3 min; 10 cycles of: 94°C for 15 s, 65°C for 30 s (with 1°C decrease at each cycle), and 72°C for 40 s; 30 cycles of: 95°C for 15 s, 55°C for 30 s, and 72°C for 40 s; 5 min at 72°C, 15°C hold step. PCR was performed on a Mastercycler EP Realplex thermocycler (Eppendorf, Westbury, NY). PCR products were separated in a 1.3% agarose gel in the presence of ethidium bromide, and the fragments had the expected sizes of 585 bp (*ZnT4*) and 537 bp (*ZnT6*). Gene expression of *ZnT4* and *ZnT6* was normalized by that of *GAPDH*. Expression of *GAPDH* was performed in a 96-well PCR plate by Real Time RT PCR with 100 ng of cDNA, TaqMan Universal PCR Mastermix (Life Technologies, Grand Island, NY), and 0.5x *GAPDH* primer-probe mix (Life Technologies, Grand Island, NY). Amplification was performed using the default cycling parameters of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 60 s at 60°C. PCR product was separated in a 3% agarose gel electrophoresis in the presence of ethidium bromide with an expected fragment size of 107 bp.

Human Subjects and Breast Milk Samples

Control breast milk samples were obtained from 25 healthy women who gave birth to healthy term infants enrolled in the UC Davis Foods for Health Institute Lactation Study (ClinicalTrials.gov Identifier: NCT01817127). Donors filled out

detailed health history questionnaires regarding demographics, anthropometrics, pregnancy history, current, and prior health history, current dietary intake habits and restrictions, physical activity level, as well as medication and supplementation intake history during the third trimester of pregnancy and 1 year postpartum. Half of the control donors reported intake of prenatal vitamins and minerals during the post-partum period. Breast milk was also obtained from one control and five premutation lactating mothers seen through the Fragile X Treatment and Research Center at the M.I.N.D. Institute at the University of California Davis Medical Center. All milk samples (5–30 ml) were obtained between 8 and 20 lactation weeks. These women delivered their infants between 38 and 41 weeks of gestation. Fresh milk samples were collected using hand expression at the conclusion of a morning breastfeed (between 09.00 and 12.00 h). All milk samples were frozen within 1 h of expression at −20°C, and transferred to −80°C until analyzed. Genotyping for these individuals was performed by extracting gDNA from milk samples and following the procedure described before (Tassone et al., 2012). The UC Davis Institutional Review Board approved all aspects of the study (IRB # 200917212-1) and informed consent was obtained from all donors.

Activity of Milk Alkaline Phosphatase

ALP activity was measured in breast milk and the method optimized using unpasteurized cow's milk (from Trader Joe's, Davis, CA). ALP activity was evaluated in 6 μl (correspondent to ∼12–13 μg of protein) of breast milk samples using the QuantiChrom™ Alkaline Phosphatase Assay Kit (BioAssay Systems, Hayward, CA). The assay utilizes *p*-nitrophenyl phosphate that is hydrolyzed by ALP into a color product, which is measured at $\lambda = 405$ nm for 5 min at 37°C. Total protein concentration of breast milk was measured with the Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA). ALP activity was expressed either as $\mu mol \times (min \times l\,milk)^{-1}$ or $nmol \times (min \times mg\ protein)^{-1}$. The detection limit of this assay was (mean ± SEM) $0.050 \pm 0.001 \mu mol \times (min \times l)^{-1}$ ($n = 16$) and the inter-assay CV was 8.1% ($n = 32$).

Milk Lactose Determination

Lactose was determined by an enzymatic spectroscopic method according to the manufacturer's instructions (Biovision, Milpitas, CA). The recovery of a known amount of lactose added to the milk samples was (mean ± SEM) 101 ± 0.9 % ($n = 12$). The detection limit of this assay was 0.025 ± 0.002 nmol galactose ($n = 12$) and the inter-assay CV was 10.2% ($n = 42$).

Determination of Zn Levels in Murine Brain and Human Milk

Measurements of Zn in murine brain regions and human milk were carried out with the Zn fluorophore zinquin (Sigma-Aldrich, St. Louis, MO) essentially as previously described (Zalewski et al., 2006) with some modifications. To evaluate total Zn levels in mouse brain regions, homogenized samples for Western blot analysis were used. One-hundred microgram of brain protein was added to each well of a 96-well-microplate to a final volume of 100 μl in the presence of 10 μM zinquin dissolved in Zn-free Hank's balanced salt solution (HBSS, in mM: 0.03 Na_2HPO_4, 0.4 KH_2PO_4, 4.2 $NaHCO_3$, 5.4 KCl, 5.6 D-glucose, 137 NaCl; pH 7.4) following the addition of Zn-free ovalbumin to a final concentration of 0.3 mg/ml (to prevent precipitation of the lipophilic zinquin from aqueous solutions). Samples were incubated in the dark for 40 min at 22°C, and fluorescence was evaluated at excitation and emission wavelengths of 365 and 510 nm, respectively.

For the determination of Zn in milk, 5 μl of milk were used and the assay carried out essentially as described for brain samples, but with and without 10 mM EGTA. EGTA-containing samples were used to obtain the non-labile Zn fluorescence, as EGTA removes any free Zn-related fluorescence. Free Zn concentrations were evaluated by subtracting the fluorescence in the presence of EGTA by the total fluorescence without EGTA, and converting fluorescence values into Zn concentrations using the linear part of a calibration curve performed with 1 to 25 μM $ZnSO_4$.To confirm that milk or brain samples did not display any intrinsic fluorescence at the excitation and emission wavelengths used, blanks without zinquin were prepared. No unspecific fluorescence was recorded in any of the samples in the absence of zinquin.

Human Fibroblasts Collection and Outcomes

Skin biopsies from controls, premutation and full mutation carriers were obtained from subjects recruited through the Fragile X Treatment and Research Center at the M.I.N.D. at the University of California Davis Medical Center. The UC Davis Institutional Review Board approved all aspects of the study and informed consent was obtained from the parents of the children. All fibroblasts were grown in Minimum Essential Medium (MEM) supplemented with 15% FBS, 2 mM glutamine and 1 mM sodium pyruvate. FMRP levels were evaluated by Western blots upon lysis of whole cells in RIPA (25 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% SDS, and 1% DOC, pH 7.5), and centrifugation at 12,000 × *g* for 10 min. For *FMR1* gene expression, RNA was isolated from 10^6 fibroblasts using the RNeasy Plus Mini Kit from Qiagen (cat. no. 74134) following the manufacturer's protocol. cDNA was synthesized from the RNA using Qiagen's Quantitect RT kit following manufacturers recommendations. RNA and cDNA concentrations were determined using the Tecan Infinite M200 Nanoquant plate reader (Tecan, Austria). Primer/probe mix was purchased from Life Technologies (Grand Island, NY, USA) for *FMR1* and *XRCC5* (housekeeping gene) were also obtained by Life Technologies. Sequences of commercial primers and probes are proprietary. cDNA was diluted to 5 ng/μl and served as stock template for QRTPCR. QRTPCR was performed in a Mastercycler EP Realplex thermocycler (Eppendorf, Westbury, NY). Amplification was performed using the following cycling parameters: 2 min at 50°C, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min of at 60°C. The mean cycle time was obtained by double derivatives (CalqPlex algorithm; Eppendorf, Westbury, NY) and designated as Ct. Each sample was analyzed in triplicates, and positive and negative controls

were run on each plate. Coefficient of variation (CV) was 0.3% or less on average. The gene expression of *FMR1* was determined by the comparative Ct method using the following equation: $2\Delta Ct$, where $\Delta Ct = Ct_{Target} - Ct_{Housekeeper}$. The gene expression fold change (premutation or full muation/Control) was determined using the $\Delta\Delta Ct$ method using the following equation: $2^{(\Delta CtFXTAS - \Delta CtControl)}$. Fold changes were calculated based on their age matched controls. Values were converted to positive or negative values to indicate up or down gene regulation. Coupling between electron transport and ATP production were tested in whole, intact cells as previously described (Napoli et al., 2011).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed either by Student's *t*-test (for comparisons between WT and KI), or by Two-way ANOVA, followed by the Tukey's HSD *post-hoc* test for multiple comparisons with the GraphPad Prism 6.0 software (San Diego, CA). Significance was set at $p \leq 0.05$. Unless otherwise noted, experiments were run in triplicates (technical replicates) and performed at least in 2–3 separate occasions (biological replicates). To determine the minimum number of mice/group, we used an a priori G test (software STATsimple; v. 2.0.5) with selected mitochondrial outcomes that indicated 4 mice as optimal given an alpha of 0.05 with an actual power of 0.993. Considering the yield of mitochondria from mouse brain (a limiting step), sample needed for assay in triplicates, and the assay parameter (polarography) that requires the largest amount of sample, 5 mice/group were used at a minimum for each time point, unless otherwise stated.

RESULTS

Decreased FMRP Protein Levels in Brain from Young KI Mice

Male, hemizygous KI mice were used as a murine model of the *FMR1* premutation [CGG repeat expansion = 196 ± 6; (Wenzel et al., 2010)]. This model recapitulates most deficits observed in premutation carriers (Hunsaker et al., 2009, 2011, 2012; Wenzel et al., 2010). Despite the relatively high CGG expansions, the expression of the *FMR1* gene product FMRP, was still detectable at PND9, PND21, and PND210 in hippocampus, cerebellum, and cortex and not absent as observed in models of Fragile X syndrome [(Zalfa et al., 2003); **Figures 1A,B**]. Consistent with other reports (Ludwig et al., 2014), FMRP protein expression decreased with age in all brain areas in WT (**Figure 1B**); conversely, no age-dependent effect was observed on FMRP expression of KI mice in any of the brain regions tested. At PND9, 40-50% of FMRP control values were observed in hippocampus, cerebellum, and cortex of KI mice, effect that lasted into PND21 for the first two tissues (**Figure 1B**).

Interestingly, in all three-brain regions the expression levels of FMRP were followed by those of β-actin (**Figure 1C**). The correlation between actin and FMRP protein levels is consistent with the role of FMRP as regulator of actin filaments organization and dynamics (Castets et al., 2005; Nolze et al., 2013), a key process in the morphogenesis of dendritic spines (Castets et al., 2005), influencing the effect of actin on mitochondria distribution and function (Kusano et al., 2000; Xu et al., 2001; Dugina et al., 2009), mitochondrial fission (Korobova et al., 2013; Hatch et al., 2014; Li et al., 2015), short-distance mitochondrial movements (Boldogh and Pon, 2006), mitochondria quality control (Higuchi et al., 2013), mitochondria clustering and reactive oxygen species (ROS) generation (Li et al., 2004).

Deficits in Bioenergetics in Brain Regions from Young KI Mice

Different segments of the electron transport chain from brain mitochondria (hippocampus, cerebellum, and cortex) were tested for their capacity to generate ATP in WT and KI mice: NADH oxidase (using NAD-linked substrates such as glucose and comprising Complex I, III, IV, and V), succinate oxidase (using FAD-linked substrates such as fatty acids and comprising Complex II, III, IV, and V), as well as citrate synthase activity as a normalizing marker of mitochondrial mass. Complex IV, or cytochrome *c* oxidase (CCO), activity, and coupling between ATP synthesis and electron transfer (as judged by the RCR) were also tested.

At PND0-7, no statistically significant differences were observed in mitochondrial outcomes between WT and KI pups in any of the brain regions tested (**Figure 2**). At PND21, hippocampus was the most affected brain area in KI pups, with significant decreases in NADH oxidase, succinate oxidase, and CCO activities, as well as increased uncoupling between ATP production and electron transfer, relative to WT (**Figure 2**). At PND21, uncoupling was also evident in cerebellum of KI pups, but to a lesser extent than hippocampus (**Figure 2**), whereas in adult age (PND210) succinate oxidase, and coupling (hippocampus) were still significantly lower in KI hippocampus than age-matched WT mice. No difference was observed in any of the outcomes in cortex at any of the time points (**Figure 2**).

Mitochondrial protein levels (relative to total protein) were evaluated by measuring the expression of the abundant protein ATPase β-subunit of Complex V [ATPB; (Elfering et al., 2004; Haynes et al., 2010), **Figure 3**]. Lower levels of ATPB were noted at PND21 and lasting into adulthood (PND210) in hippocampus of KI mice, while in cerebellum significant ATPB deficits were observed only at PND21 (**Figure 3**). No statistically significant differences were observed in cortex at any time point (**Figure 3**).

Taken together, these results point toward a generalized OXPHOS deficit in premutation pups' hippocampus, characterized by lower ATP production with both NAD- and FAD-linked substrates, accompanied by lower expression of ATPB. These deficits were less evident in cerebellum (uncoupling and lower ATPB expression were evident at PND21 only), with no apparent involvement of cortex.

The OXPHOS changes observed in brain mitochondria could not be ascribed to a specific subcellular localization (e.g., synaptosomal mitochondria derived only from the termini of neurons vs. free, non-synaptosomal mitochondria) because the majority of brain mitochondria [88% of all mitochondria

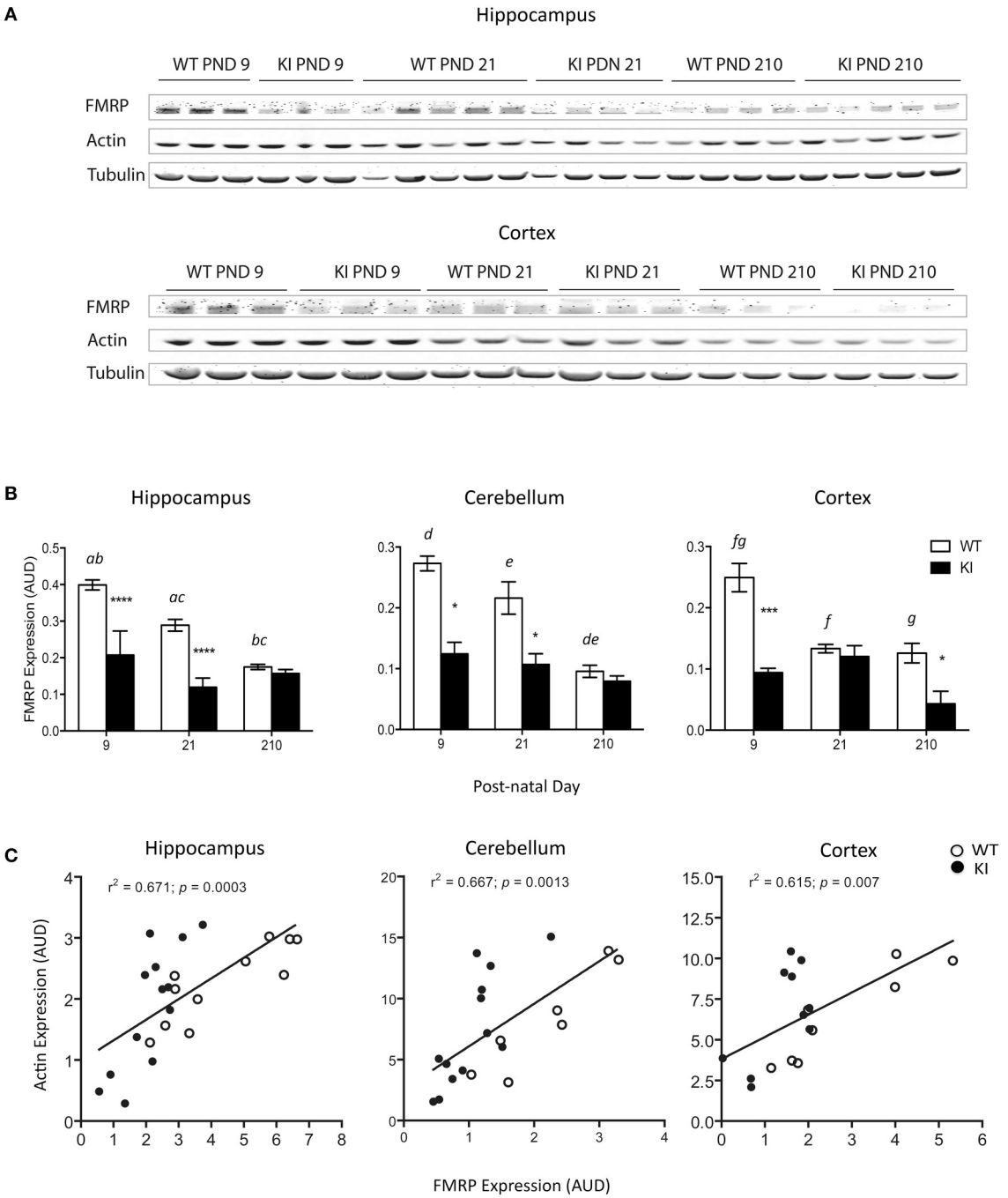

FIGURE 1 | Changes in FMRP protein expression in brain from WT and KI mice. (A) Representative Western blots of FMRP and actin protein expression levels in hippocampus and cortex of WT and KI mice. Tubulin was used as loading control. FMRP protein levels of KI mice hippocampus and cerebellum were 37 and 45% of WT at post-natal day (PND) 9, and 41 and 50% of WT at post-natal day 21. In cortex, FMRP protein levels in KI mice were 38% of WT at post-natal day 9. **(B)** Time-dependent changes in FMRP protein levels in hippocampus, cerebellum, and cortex respectively. Data are reported as mean ± SEM, $n = 3$–5 per genotype per time point. Statistical analysis was performed by Two-way ANOVA. *Post-hoc* analysis performed by Tukey's HSD test revealed significant differences between WT and KI, indicated in the figure by asterisk as follows. Hippocampus: $^{****}p < 0.0001$; Cerebellum: $^{*}p = 0.0110$; Cortex: $^{**}p = 0.0003$, $^{*}p = 0.0363$, $^{***}p = 0.0001$. Statistically significant differences among time points are indicated by letters with the following p values. $p = 0.0029$ (*a*), $p < 0.0001$ (*b*), $p = 0.0009$ (*c*), $p = 0.0260$ (*d*), $p = 0.0101$ (*e*), $p = 0.0033$ (*f*), $p = 0.0020$ (*g*). For more statistical details on the genotype, age, and genotype × age effect see **Table 3**. **(C)** Correlation between FMRP and actin in the same brain areas. AUD, Arbitrary Units of Densitometry.

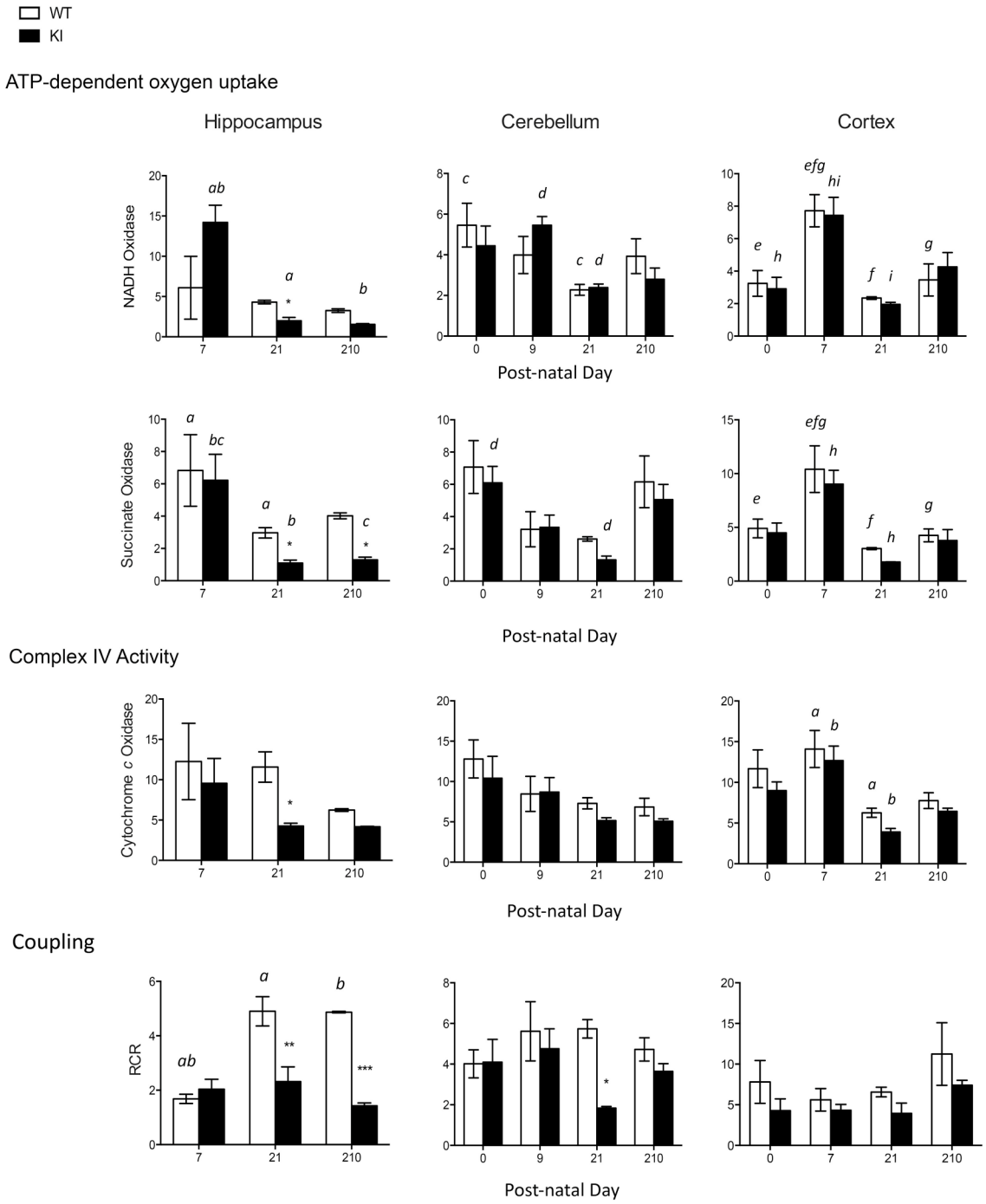

FIGURE 2 | Brain bioenergetics of KI mice during neurodevelopment and adulthood. Mitochondria were isolated from cortex, cerebellum, and hippocampus of WT and KI pups as described in the Methods section. Activities of NADH oxidase, succinate oxidase, and cytochrome *c* oxidase (CCO), and respiratory control ratio (RCR) were evaluated at PND0 (cerebellum and cortex only, due to the scarcity of hippocampal tissue), PND7, PND21, and PND210. Data are reported as mean ± SEM, $n = 3$–7 per genotype per time point. Statistical analysis was performed by Two-way ANOVA. *Post-hoc* analysis performed by Tukey's HSD test revealed significant differences between WT and KI indicated by asterisks as follows NADH oxidase: $^{*}p = 0.0468$; Succinate oxidase: $^{*}p = 0.0466$ at PND21, $^{*}p = 0.0423$ at PND210; Cytochrome *c* oxidase: $^{*}p = 0.0484$; Coupling: $^{**}p = 0.001$, $^{***}p = 0.0004$, $^{*}p = 0.0496$. Letters indicate statistically significant differences amongst time points as follows. NADH oxidase: $p = 0.0291$ (*a*), $p = 0.0033$ (*b*), $p = 0.0166$ (*c*), $p = 0.0320$ (*d*), $p = 0.0036$ (*e*), $p = 0.0001$ (*f*), $p = 0.0113$ (*g*), $p = 0.0032$ (*h*), $p = 0.0004$ (*i*); Succinate oxidase: $p < 0.0001$ (*a*), $p = 0.0002$ (*b*), $p = 0.0240$ (*c*), $p = 0.0434$ (*d*), $p = 0.0283$ (*e*), $p = 0.0009$ (*f*), $p = 0.0250$ (*g*), $p = 0.0013$ (*h*); Cytochrome *c* oxidase: $p = 0.0053$ (*a*), $p = 0.0035$ (*b*). Coupling: $p = 0.0003$ (*a*), $p = 0.0010$ (*b*). Further statistical details on the genotype, age, and genotype × age effect can be found in the legend of **Table 3**.

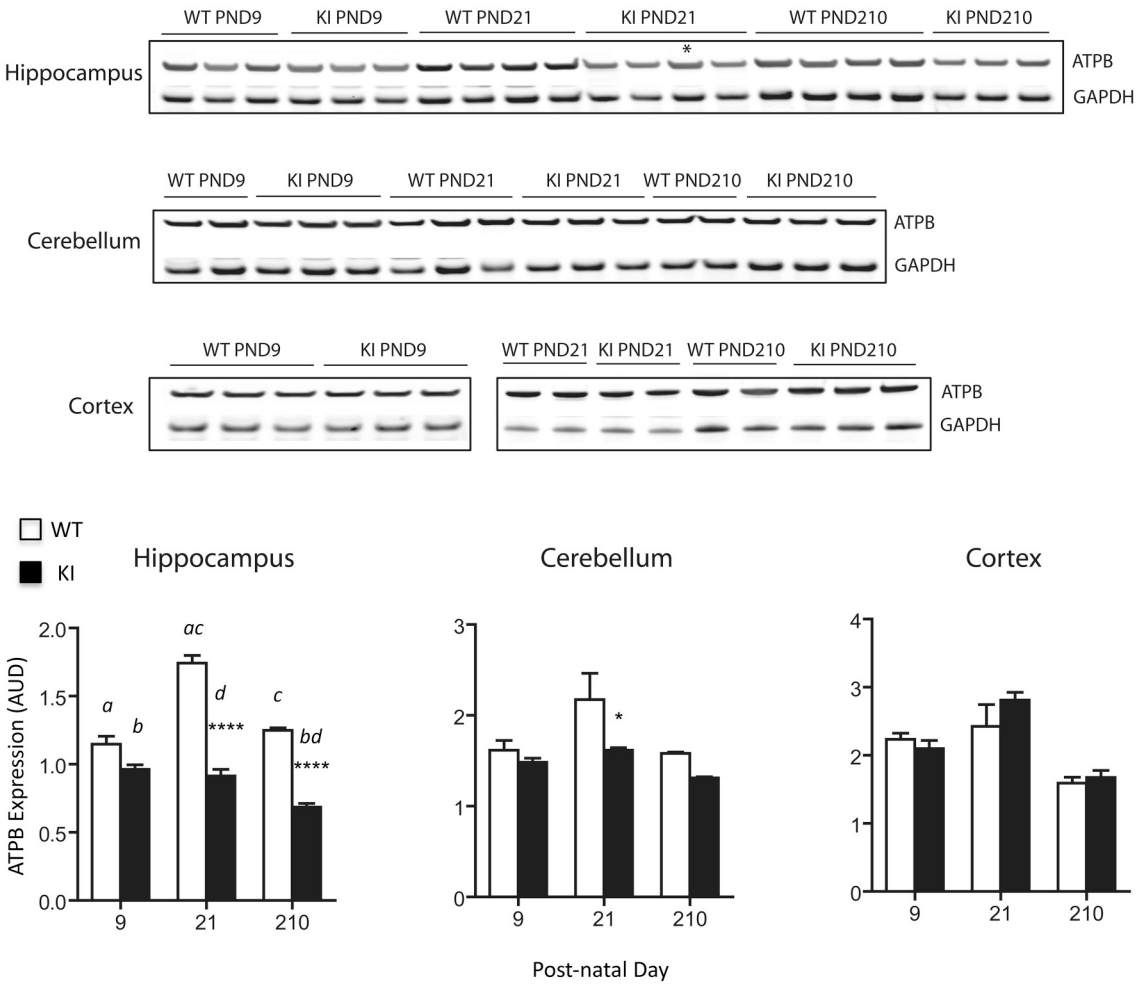

FIGURE 3 | Protein expression levels of mitochondrial ATPase β-subunit in brain from KI mice. ATPase β-subunit protein expression (normalized to GAPDH) was evaluated at PND9, 21 and 210 in hippocampus, cerebellum, and cortex lysates. Asterisk in the immunoblot image of hippocampus denotes a 90 CGG KI sample. Densitometry data for this sample have not been taken into account for averages calculation of KI. Cortex samples were ran in two different gels (PND9 and PND21-210) and are shown separately. Data are reported as mean ± SEM, $n = 3$–5 per genotype per time point, ran in triplicates. Statistical analysis was performed by Two-way ANOVA. *Post-hoc* analysis was performed by Tukey's HSD test for multiple comparisons. Significant difference between WT and KI are indicated by asterisks as follows. ****$p < 0.0001$; *$p < 0.0492$. Statistically significant differences among time points are indicated by letters as follows: $p < 0.0001$ (*a, c*), $p = 0.0087$ (*b*), $p = 0.0222$ (*d*). Further, statistical details on the genotype, age, and genotype × age effect can be found in **Table 3**. AUD, Arbitrary Units of Densitometry.

(Rendon and Masmoudi, 1985)] are non-synaptosomal (Napoli et al., 2012). As such, no OXPHOS differences were observed between mitochondria-enriched fractions and highly purified, non-synaptosomal mitochondria at PND21 in any brain region from WT and KI (**Table 1**).

Isolated Neurons Recapitulate the Mitochondrial Deficits Observed in KI Brain Regions

Non-synaptosomal mitochondria as well as mitochondria-enriched fractions are, by necessity, removed from their cytoplasmic environment with the potential of altering their function through the purification process. To address this issue, in parallel we tested mitochondrial function in a system retaining cellular specificity and with an intact intracellular milieu. OXPHOS was directly evaluated in isolated, intact hippocampal, cerebellar, and cortical neurons obtained from 4–6 pooled WT and KI mice at PND0 (**Table 2**). Glucose-sustained basal respiration of intact neurons, followed by the addition of the ATPase inhibitor oligomycin, was recorded to establish the ATP production linked to oxygen uptake. Subsequent addition of FCCP, an uncoupler of ATP production and electron transfer, allowed evaluating the maximum mitochondrial oxygen uptake capacity. ROS-mediated oxygen uptake and membrane proton leak (oligomycin-resistant oxygen uptake rates), oxygen uptake linked to ATP production (oligomycin-sensitive), and spare respiratory capacity (FCCP-mediated maximal respiration rate) were expressed as a fraction of the basal oxygen consumption rate for both WT and KI neurons (**Table 2**). At PND0, a deficiency in the ATP-driven oxygen uptake was already evident in isolated KI

TABLE 1 | Comparison of outcomes in mitochondria-enriched fractions vs. purified, non-synaptosomal mitochondria from cerebellum and cortex at PND21 in WT and KI mice.

Outcomes	Cerebellum				Cortex			
	Total mitochondria		Non-synaptosomal mitochondria		Total mitochondria		Non-synaptosomal mitochondria	
	WT	KI	WT	KI	WT	KI	WT	KI
OXYGEN UPTAKE RATES								
NADH oxidase	5.38 ± 0.01	4.5 ± 0.3	52.4 ± 1.3	58.3 ± 5.6	8.8 ± 3.3	8.4 ± 1.0	50 ± 11	45 ± 6
Succinate oxidase	6.31 ± 0.02	2.33 ± 0.01**	47.1 ± 1.0	26 ± 2**	8.1 ± 0.8	6.5 ± 1.0*	74 ± 15	45 ± 2*
ACTIVITIES								
Cytochrome *c* oxidase	14.3 ± 0.1	10 ± 1**	168 ± 28	116 ± 19**	27 ± 1	16 ± 1**	149 ± 17	64 ± 4**
Citrate synthase	274 ± 52	293 ± 31	2314 ± 305	2400 ± 320	263 ± 27	282 ± 39	2258 ± 360	2324 ± 199

*All activities were expressed as nmol oxygen consumed × (min × mg protein)$^{-1}$. Citrate synthase was expressed as nmol × (min × mg protein)$^{-1}$. Statistical analysis was carried out with the Student's t-test. The p-values are as follows: *< 0.05, **< 0.01.*

TABLE 2 | Mitochondrial coupling and respiratory capacity in intact, isolated neurons from WT and KI mice at PND0.

Outcomes	Hippocampus		Cerebellum		Cortex	
	(% Basal rate)					
Oxygen consumption rates	**WT**	**KI**	**WT**	**KI**	**WT**	**KI**
Oligomycin-sensitive	90 ± 1	85 ± 1**	89 ± 3	80 ± 6	84 ± 2	77 ± 3
Oligomycin-resistant	0.33 ± 0.33	6.9 ± 0.5***	-0.22 ± 0.22	0.9 ± 0.2*	3 ± 5	19 ± 3*
Spare respiratory capacity	128 ± 12	62 ± 2**	102 ± 30	74 ± 42	110 ± 17	67 ± 12
Coupling	**Hippocampus**		**Cerebellum**		**Cortex**	
RCRu	11 ± 2	3.4 ± 0.8*	9 ± 1	1.7 ± 0.4**	7 ± 1	0.9 ± 0.3***

*Oxygen consumption rates under basal conditions (in the presence of glucose only) were not statistically significant different between WT and KI in any of the brain regions [average of WT and KI = 6.5 ± 0.7; 3.4 ± 0.9; and 3.1 ± 0.5 nmol oxygen consumed × (min × 10^6 cells)$^{-1}$ for cortex, cerebellum and hippocampus, respectively]. Oxygen uptake linked to ATP production (oligomycin-sensitive mitochondrial oxygen uptake), ROS-mediated oxygen uptake and membrane proton leak (oligomycin-resistant mitochondrial oxygen uptake), and spare respiratory capacity (FCCP-mediated maximal respiration rate) were expressed as a fraction of the basal oxygen consumption rate. The RCRu represents the ratio between FCCP and oligomycin-resistant oxygen uptake rates. Data (from three separate preparations run in duplicates) are shown as mean ± SEM. Statistical comparison between WT and KI was performed with the Student's t-test. The p-values are as follows: *< 0.05; **< 0.005; ***< 0.0005.*

hippocampal neurons, consistent with this area being the most affected.

Interestingly, KI neurons show a dramatically lower spare respiratory capacity than WT, capacity defined as the mitochondrial ability to meet increased energy demand with increased respiration (**Table 2**). This may indicate a lower capacity to adapt to stressful situations with increased ATP need. The coupling (as judged by the RCRu) and the oligomycin-resistant oxygen uptake were significantly different (3–5-fold lower and 4–20-fold higher, respectively, than controls) in KI compared to WT in all brain areas (**Table 2**). The lower RCRu and spare respiratory capacity of hippocampal KI cells are of significant biological relevance because energy deficits generated by an imbalance between bioenergetic reserve and demand, play a critical role in the survival of neurons under stress conditions (Nicholls, 2008) and can lead to disrupted synaptic network (Selkoe, 2002; Yadava and Nicholls, 2007).

The apparent discrepancy between the marked deficits observed at PND0 in KI isolated neurons (**Table 2**) relative to the ones at PND7 in KI non-synaptosomal mitochondria (**Table 1**) or total mitochondrial fractions (**Figure 2**) can be bridged taking into account the absence of supporting cells (glia) in a neuron-only culture system (Iwata-Ichikawa et al., 1999; Bélanger and Magistretti, 2009), which could be even more critical for neurons with an already compromised genetic background. Alternatively, neuronal mitochondria might be more affected than those of glia, masking the effect when both are tested in mitochondria-rich fractions from brain.

Coupling Between Electron Transport and ATP Synthesis Correlates with FMRP But Not *FMR1* Expression Levels In Human Fibroblasts

As a proof of concept, to test whether *FMR1* gene or FMRP protein expression influence mitochondrial outcomes, we turned to human primary skin fibroblasts from premutation and full mutation carriers, as commercially available *FMR1* KO mice are

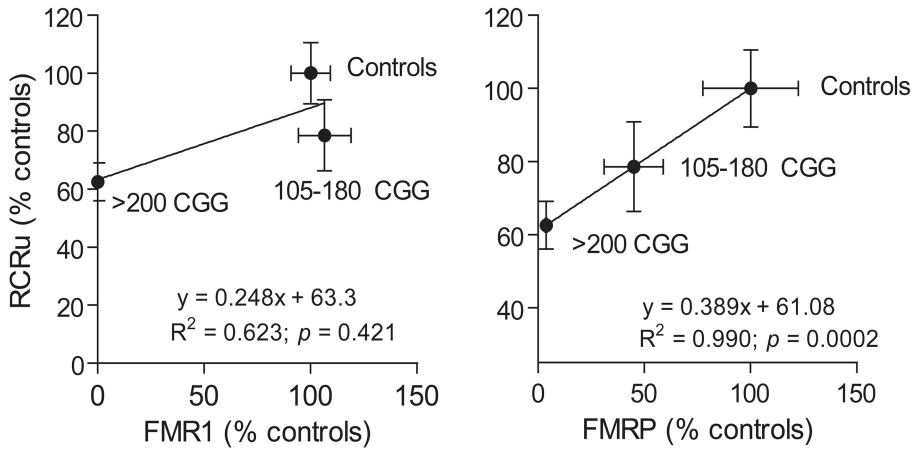

FIGURE 4 | Correlation between mitochondrial outcomes and FMRP or *FMR1* expression levels. The correlation between FMRP and *FMR1* expression with a mitochondrial outcome (i.e., coupling between ATP synthesis and electron transfer or RCRu) was carried out using human primary dermal from controls, premutation carriers (105–180 CGG), and full mutation carriers (>200 CGG repeats). RCRu, *FMR1*, and FMRP levels were expressed as percentage of control values. Data are shown as mean ± SEM for controls, permutation, and full mutation carriers.

on a different genetic background (FVB129 or FVB129/NJ) than the ones used in this study (C57BL/6J) making the comparison invalid [see as an example for Huntington's disease, another triplet-nucleotide repeat disease (Menalled et al., 2009)]. In addition, KO mice may not recapitulate the full mutation seen in humans, since humans show no expression of FMRP over time as a result of a repeat-mediated gene silencing sometime during early development (Pieretti et al., 1991; Sutcliffe et al., 1992). To this end, *FMR1* and FMRP expression were assessed in fibroblasts from controls, premutation (105–180 CGG matching the range of CGG in KI mice), and full mutation (>200 CGG repeats) carriers (**Figure 4**). Fibroblasts from premutation carriers showed normal or marginally elevated *FMR1* transcript levels with ∼50% FMRP levels of controls; in contrast, fibroblasts from full mutation carriers showed no gene or protein expression. When the link between *FMR1* and FMRP expression and bioenergetics (as judged by coupling between ATP synthesis and electron transfer, RCRu) was investigated in these samples, a strong direct correlation was observed between RCRu and FMRP levels but not *FMR1* mRNA levels (**Figure 4**).

Further studies are warranted by including either a wider array of outcomes or more subjects, however, these results are consistent with the association between bioenergetics and FMRP protein expression rather than to *FMR1* transcript levels.

Zn Concentrations, Bioenergetics, and Shank3 Deficiencies in KI Brain

The bioenergetic deficits observed in brains from KI mice were similar to those reported in previous studies performed on primary dermal fibroblasts from older premutation carriers (Napoli et al., 2011). That study ascribed the bioenergetic deficits to altered ZnT6 protein level and lower transport of cytoplasmic Zn (Napoli et al., 2011), which resulted in deficient Zn-mediated import/processing of nuclearly-encoded mitochondrial subunits (Napoli et al., 2011). As a result of the defective Zn-dependent processing/import of nuclear DNA (nDNA)-encoded mitochondrial proteins, higher ratios of precursor-to-mature mitochondrial proteins were observed in fibroblasts and brain samples of premutation carriers.

To test whether the import/processing of nDNA-encoded mitochondrial proteins was also affected in the KI mouse model, providing an explanation for the observed defects in OXPHOS, we evaluated the protein expression levels of mature and precursor cytochrome *c* oxidase subunit IV (CCOIV, **Figure 5**) in brains from WT and KI mice. The CCOIV Precursor-to-Mature ratios (P:M) were significantly increased in hippocampus (PND21 and PND210) and cerebellum (PND21) of KI mice (**Figure 5**), consistent with our previous reports.

To test for Zn homeostasis, Zn levels in brain were evaluated in parallel. Lower Zn concentrations were observed in hippocampus and cerebellum of KI mice at PND21 and lasting into PND210 for both brain regions (**Figure 6**). Interestingly, these regions are the ones with the highest Zn concentrations (average two-fold of cortex; **Figure 6**), consistent with their higher fraction of Zn-containing neurons (Sawashita et al., 1997) and neuron-to-glia ratios (Napoli et al., 2012). In agreement with the findings previously obtained with fibroblasts from older premutation carriers (Napoli et al., 2011), a positive correlation was also observed between Zn levels and mitochondrial outcomes (namely, activities of Complex IV and citrate synthase and coupling; **Figure 7**). Taken together, these results confirmed the lower import/processing capacity of nDNA-encoded proteins to mitochondria in premutation and the occurrence of altered Zn bioavailability.

Zn deficits are linked not only to altered mitochondrial protein import (Tokatlidis et al., 2000; Napoli et al., 2011) but also to dysregulation of Shank2/3 scaffolding (Grabrucker

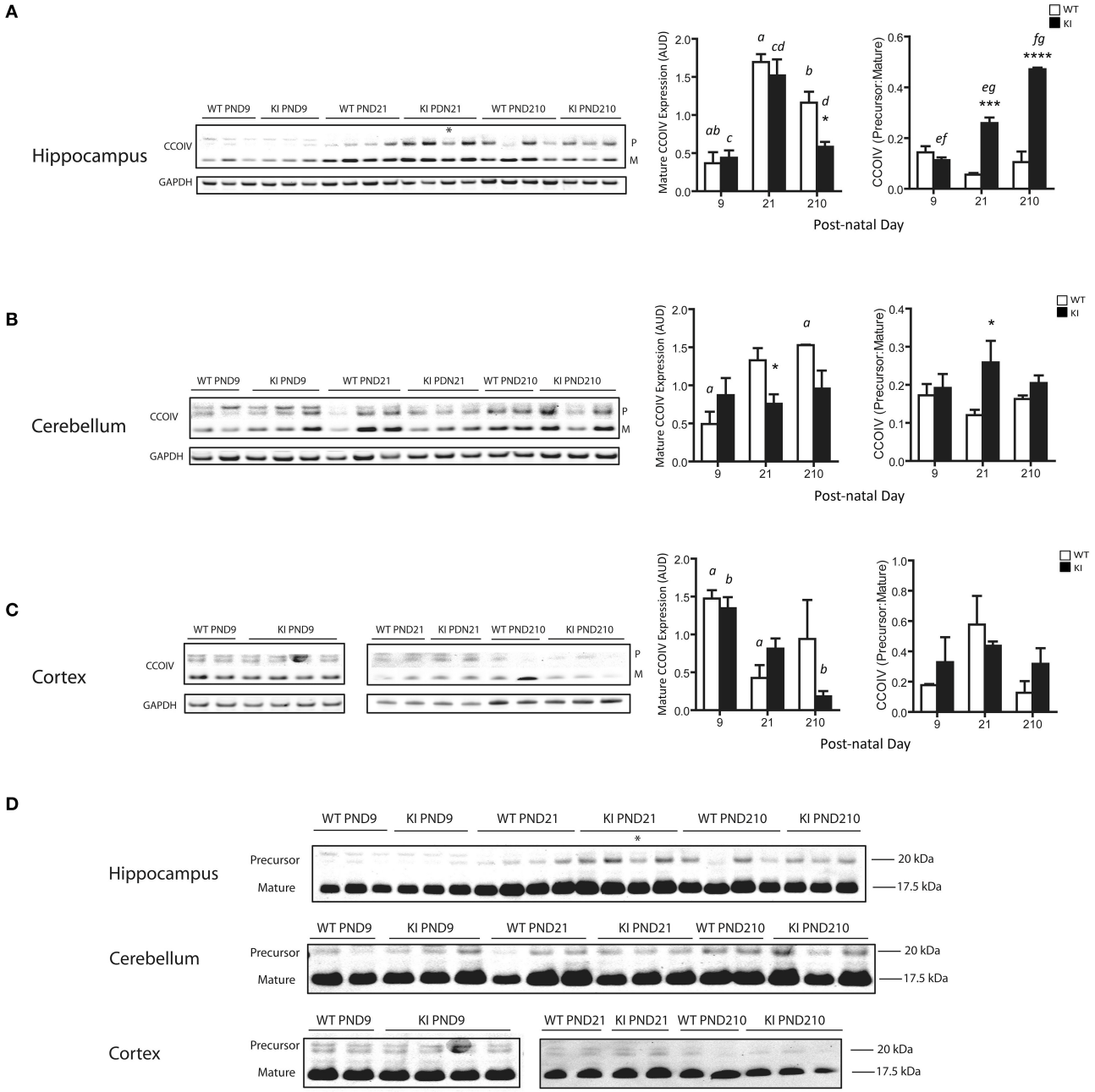

FIGURE 5 | Protein expression of precursor and mature CCOIV in brains from KI mice. Representative Western blots of CCOIV (precursor and mature proteins) in hippocampus **(A)**, cerebellum **(B)** and cortex **(C)** of WT and KI mice. The densitometry for all the samples is also shown. Data were expressed as Arbitrary Densitometry Units and reported as mean ± SEM. Mature form of CCO4 was normalized by GAPDH. Data are reported as mean ± SEM, $n = 3$–5 per genotype per time point, ran in triplicates. Statistical analysis was performed by Two-way ANOVA, followed by Tukey *post-hoc* test for multiple comparisons. Statistically significant differences between WT and KI are indicated by asterisks as follows. Hippocampus: $^{*}p = 0.0416$; $^{***}p = 0.0002$; $^{****}p < 0.0001$. Cerebellum: $^{*}p = 0.0212$ for mature CCO at PND21; $^{*}p = 0.0197$ for P:M at PND21. Statistically significant differences among time points are indicated by letters as follows. Hippocampus: $p = 0.0002$ (*a*), $p = 0.0172$ (*b*), $p = 0.0013$ (*c*), $p = 0.0047$ (*d*), $p = 0.0086$ (*e*), $p < 0.0001$ (*f*), $p = 0.0003$ (*g*). Cerebellum: $p = 0.0459$ (*a*). Cortex: $p = 0.0391$ (*a*), $p = 0.0113$ (*b*). Further statistical details on the genotype, age, and genotype × age effect can be found in **Table 3**. **(D)** Uncropped version of the Western blot image showing the intensity and mobility of the precursor band relative to the mature protein. The observed molecular weights for the CCOIV precursor and mature forms were, respectively, 20.0 and 17.5 kDa, as extrapolated by the Molecular Weight markers with the use of the Carestream software. This 2.5 kDa difference was close to the theoretical calculated molecular weight (2.4 kDa) of the 22 residues of amino acids (MLATRVFSLVGKRAISTSVCVR) present in the precursor form, which is cleaved by mitochondrial matrix peptidases (Isaya et al., 1991) to produce the mature mitochondrial form of the protein (UniProtKB P13073). Asterisk in the immunoblot image of hippocampus (panels A and D) denotes a 90 CGG KI sample. Densitometry data for this sample have not been included in the averages for KI. For cortex, samples were run in two separate gels (PND9 and PND21-210). AUD, Arbitrary Units of Densitometry.

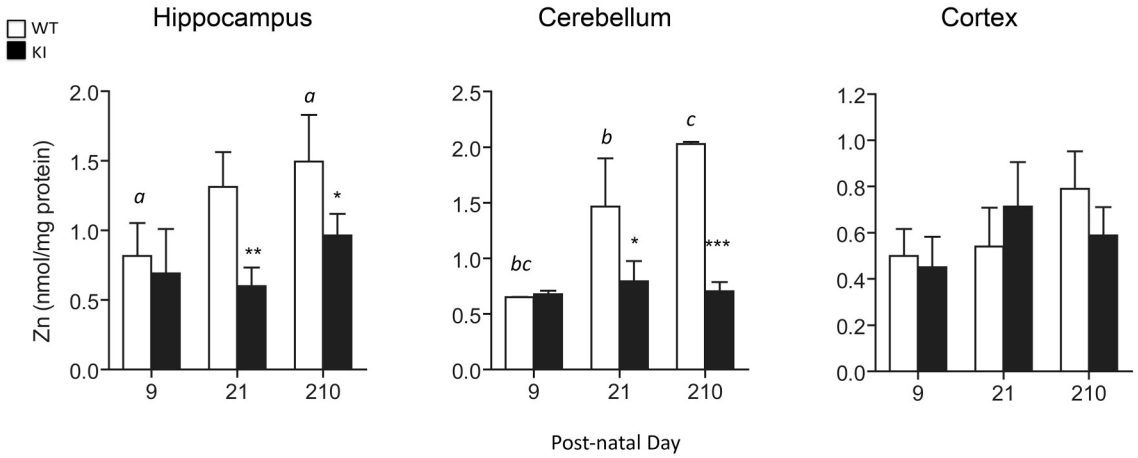

FIGURE 6 | Brain Zn concentrations in WT and KI mice. Total Zn levels were measured in whole hippocampus, cerebellum and cortex homogenates from WT and KI mice. Data are shown as mean ± SEM, $n = 3$–6 individuals *per* genotype, *per* time point, ran individually. Statistical analysis was performed by Two-way ANOVA, followed by Tukey's HSD post-hoc test for multiple comparisons. Statistically significant differences between WT and KI at individual time-points are indicated by asterisks as follows: Hippocampus: $^{*}p = 0.0425$; $^{**}p = 0.0070$; Cerebellum: $^{*}p = 0.0158$; $^{***}p = 0.0003$. Statistically significant differences among time points are indicated by letters as follows: $p = 0.0190$ (*a*), $p = 0.0144$ (*b*), $p = 0.0003$ (*c*). Further statistical details on the genotype, age, and genotype × age effect can be found in **Table 3**.

et al., 2014). Indeed, Shank3 transcript—as actin transcript—has been identified as one of the main mRNAs interacting with FMRP (Darnell et al., 2011) and both FMRP and *FMR1* mRNA are normally present in ribosomes associated to postsynaptic dendritic sites, location shared by Shank3 (Weiler et al., 1997).

Deficits in Shank3 protein expression—evaluated by Western blots—were noticeable at PND9, PND21, and PND210 in hippocampus and cerebellum from KI mice (**Figure 8** and Supplementary Figure 1). These results are consistent with other studies reporting Shank3 protein expression being brain-region/cell-type specific and developmentally regulated (Wang et al., 2014). Of note, although Shank3 is a postsynaptic density protein, several studies have shown that Shank3 expression pattern in whole brain areas mirrors that observed at the synaptic regions (Han et al., 2013; Kouser et al., 2013) providing support for the Shank3 expression in total homogenates from brain regions. Furthermore, the amount of tissue collected from each pup, especially at early time points—i.e., PND9 and PND21—would not have been sufficient as starting material for postsynaptic density preparations.

Given that Zn deficits were observed after those in Shank3, it could be inferred that the reduced levels of FMRP at early time points affects mainly Shank3 translation whereas later, both Shank3 protein expression and Zn-dependent scaffolding seem affected. In brains of WT and KI mice, statistically significant positive correlations were noted between FMRP and Shank3 (**Figure 8C**), FMRP and Zn levels (**Figure 7B**) and between Shank3 and Zn levels (**Figure 7C**), reinforcing the concept of the crosstalk between FMRP, Shank3, and Zn homeostasis.

Interaction between Genotype and Age on FMRP Levels, Bioenergetics, Zn, and Shank3 in Brains of WT and KI Mice

To test the putative interaction between genotype and age, a Two-way ANOVA analysis was performed for each of the outcomes tested at different time-points (**Table 3**). A statistically significant genotype × age interaction was observed in hippocampus for FMRP, most of the mitochondrial outcomes tested (five of eight; NADH oxidase activity, uncoupling, citrate synthase activity, and ATPB and COXIV expression), and Shank3, whereas in cerebellum the interaction was statistically significant for FMRP, only one mitochondrial outcome (COXIV), Zn levels and Shank3 (**Table 3**). In cortex, the interaction of age and genotype was statistically significant only for FMRP levels (**Table 3**). Thus, the tissue that showed the most evident gene × age interaction was hippocampus, followed by cerebellum and then cortex (**Table 3**). Simple main effect analysis showed that the overall changes observed in hippocampus and cerebellum could be equally attributed to genotype and age, whereas in cortex age had a more prominent effect (**Table 3**).

In terms of outcomes, FMRP expression was the only parameter that showed a strong interaction between genotype and age in all three-brain regions, with a bigger contribution of genotype than age (**Figure 1** and **Table 3**). Differences recorded in mitochondrial outcomes appeared to be determined by both genotype and age in hippocampus, while age played a bigger role in cerebellum and cortex (**Figure 2** and **Table 3**). Differences observed in Zn levels in hippocampus and cerebellum seemed mainly due to genotype more than age (**Figure 6** and **Table 3**). Finally, Shank3 expression seemed to be influenced by genotype and age in hippocampus and cerebellum (**Figure 8** and **Table 3**), and mainly by age in cortex. Taken together, this analysis

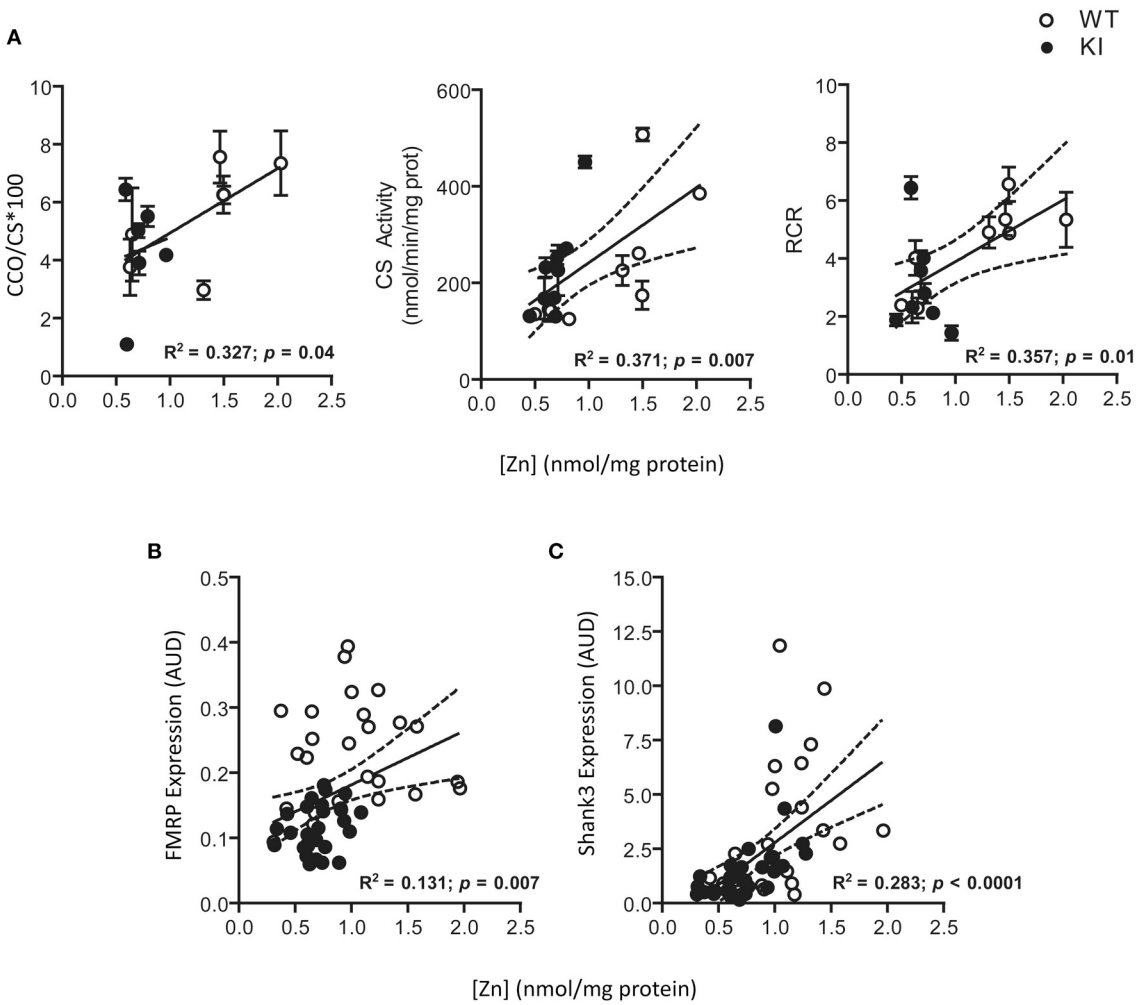

FIGURE 7 | Correlations among Zn levels, selected mitochondrial outcomes, and protein expression of FMRP and Shank3, in brain of WT and KI mice. A significant direct correlation was noted between average Zn levels and activities of CCO and CS and RCR for all the time points analyzed **(A)**. CCO and CS activities were expressed as nmol × (min × mg protein)$^{-1}$. CCO activity has been normalized to citrate synthase and multiplied by 100. All the other mitochondrial outcomes did not show a correlation with Zn. A direct correlation was also observed between Zn levels and both FMRP and Shank3 protein expression (individual values) in brains of WT and KI when all time points were combined **(B,C)**. Dotted lines illustrate the 95% CI obtained with WT and KI values.

indicates a stronger effect of the KI genotype than age on the outcomes evaluated in hippocampus, about equal for cerebellum and mostly age-dependent effects in cortex.

Altered ZnT Gene Expression in Lactating Mammary Glands from KI Dams

While some of the Zn, Shank3, and mitochondrial deficits lasted into PND210 in hippocampus and cerebellum (**Figures 2**–**8**), the finding that most of the mitochondrial outcomes were significantly different at PND21 (at the end of the nursing period) and that some amelioration was observed between PND21 and PND210 in cortex and cerebellum, suggested that lactation (nursing from KI dams vs. post-weaning diet constituted by vitamin- and mineral-balanced murine chow) might have compounded the early deficits. Due to the unsuccessful attempts to collect sufficient murine milk to evaluate Zn levels or any other biochemical analyses, as a surrogate for Zn homeostasis we tested the gene expression of *ZnT4* and *ZnT6* in lactating mammary glands from control and KI dams. *ZnT4* and *ZnT6* gene expression was evaluated by PCR, followed by separation of the products in a 1.3% agarose gel, visualized by using ethidium bromide. (Commercially available dual-labeled probes for these transporters resulted unspecific).

In lactating mammary glands from WT dams, both *ZnT4* and *ZnT6* (normalized to *GAPDH*) were expressed with a ratio of *ZnT6/ZnT4* equal to 15 (**Figure 9A**). The gene expression of both ZnTs was significantly higher (two-fold) in KI than WT, with no significant changes in the *ZnT6/ZnT4* expression ratio compared to WT (**Figure 9B**). These results suggested a disrupted ZnT expression, which may affect Zn efflux in milk, and as a consequence, the Zn status of the suckling pups.

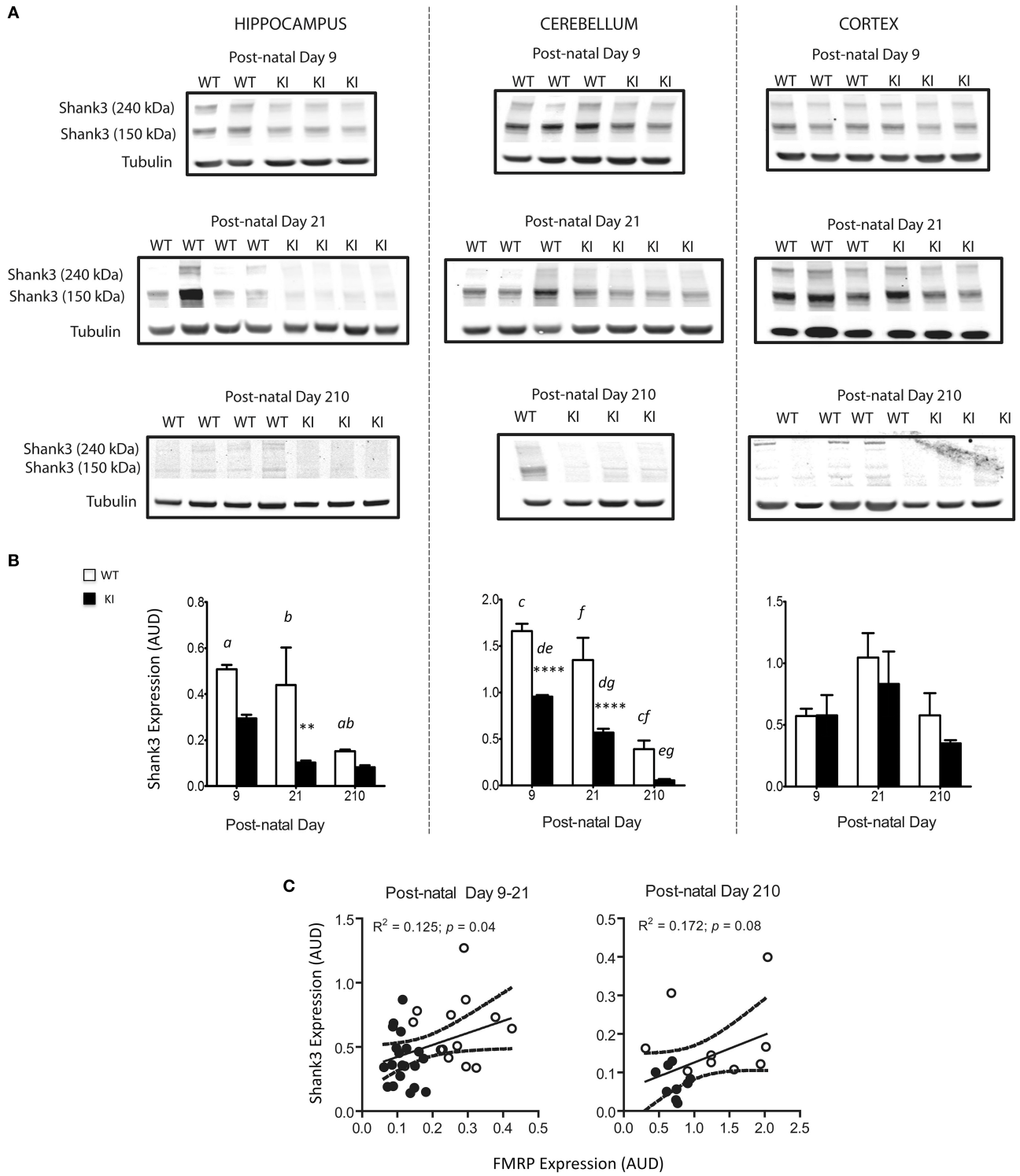

FIGURE 8 | Brain Shank3 protein levels in WT and KI mice. (A) Western blots for Shank3 protein expression levels in hippocampus, cerebellum, and cortex were performed at PND 9, 21, and 210 from WT (white) and KI (black) mice as described in details in the Materials and Methods Section. Further, images taken with acetone-precipitated samples and normalized by either tubulin or GADPH are found under Supplementary Figure 1. **(B)** Densitometry data is shown as mean ± SEM, $n = 4$–5 individuals *per* genotype, *per* time point, ran individually. Statistical analysis was performed by two-way ANOVA, followed by Tukey's HSD *post-hoc* test for multiple comparisons. Statistically significant differences between WT and KI are indicated by asterisks as follows: Hippocampus: $^{**}p = 0.0054$; Cerebellum: $^{****}p < 0.0001$. Statistically significant differences among time points are indicated by letters as follows: $p = 0.0074$ (*a*), $p = 0.0267$ (*b*), $p < 0.0001$ (*c, e, f*), $p = 0.0217$(*d*),

(Continued)

FIGURE 8 | Continued
$p = 0.0041$ (*g*). Further, statistical details on the genotype, age, and genotype × age effect can be found in **Table 3**. **(C)** Correlation between FMRP levels and Shank3 protein expression in brain of WT and KI mice. Both proteins have been normalized by tubulin, used as loading control. Shown are individual data points collected in cerebellum, hippocampus, and cortex of WT and KI mice at PND9, PND21, and PND210. PND210 is shown in a separate plot due to the difference in protein expression of both FMRP and Shank3 at this time point, relative to the previous ones. Dotted lines are 95% CI constructed with WT and KI values. AUD, Arbitrary Units of Densitometry.

TABLE 3 | Effect of genotype, age, and genotype × age interaction on outcomes evaluated in hippocampus, cerebellum, and cortex of WT and KI mice.

	Genotype × age interaction	Genotype effect	Age effect
HIPPOCAMPUS			
FMRP	$F_{(2, 17)} = 23.42$	$F_{(1, 17)} = 105.3$	$F_{(2, 17)} = 16.24$
	$p < 0.0001$	**$p < 0.0001$**	**$p = 0.0001$**
NADH oxidase	$F_{(2, 18)} = 8.466$	$F_{(1, 18)} = 2.435$	$F_{(2, 18)} = 17.12$
	$p = 0.0026$	**$p = 0.0464$**	**$p < 0.0001$**
Succinate oxidase	$F_{(2, 19)} = 0.6392$	$F_{(1, 19)} = 6.180$	$F_{(2, 19)} = 16.02$
	$p = 0.5387$	**$p = 0.0224$**	**$p < 0.0001$**
Cytochrome *c* oxidase	$F_{(2, 18)} = 1.099$	$F_{(1, 18)} = 5.245$	$F_{(2, 18)} = 2.915$
	$p = 0.3545$	**$p = 0.0343$**	$p = 0.0801$
RCR	$F_{(2, 22)} = 9.284$	$F_{(1, 22)} = 27.45$	$F_{(2, 22)} = 8.578$
	$p = 0.0012$	**$p < 0.0001$**	**$p = 0.0018$**
Citrate synthase	$F_{(3, 47)} = 1.307$	$F_{(1, 47)} = 2.162$	$F_{(3, 47)} = 8.239$
	$p = 0.283$	$p = 0.1481$	**$p = 0.0002$**
ATPB	$F_{(2, 15)} = 26.74$	$F_{(1, 15)} = 214.1$	$F_{(2, 15)} = 39.95$
	$p < 0.0001$	**$p < 0.0001$**	**$p < 0.0001$**
COXIV	$F_{(2, 15)} = 2.362$	$F_{(1, 15)} = 3.582$	$F_{(2, 15)} = 34.88$
	$p = 0.1283$	$p = 0.0779$	**$p < 0.0001$**
COXIV (P:M)	$F_{(2, 15)} = 30.14$	$F_{(1, 15)} = 78.45$	$F_{(2, 15)} = 23.12$
	$p < 0.0001$	**$p < 0.0001$**	**$p < 0.0001$**
[Zn]	$F_{(2, 17)} = 2.588$	$F_{(1, 17)} = 19.94$	$F_{(2, 17)} = 7.303$
	$p = 0.1045$	**$p = 0.0003$**	**$p = 0.0051$**
Shank3	$F_{(2, 20)} = 2.559$	$F_{(1, 20)} = 16.50$	$F_{(2, 20)} = 10.23$
	$p = 0.1024$	**$p = 0.0006$**	**$p = 0.0009$**
CEREBELLUM			
FMRP	$F_{(2, 15)} = 4.768$	$F_{(1, 15)} = 28.64$	$F_{(2, 15)} = 13.16$
	$p = 0.0250$	**$p < 0.0001$**	**$p = 0.0005$**
NADH oxidase	$F_{(3, 48)} = 1.471$	$F_{(1, 48)} = 0.08211$	$F_{(3, 48)} = 6.907$
	$p = 0.2341$	$p = 0.7757$	**$p = 0.0006$**
Succinate oxidase	$F_{(3, 44)} = 0.2040$	$F_{(1, 44)} = 1.196$	$F_{(3, 44)} = 8.347$
	$p = 0.893$	$p = 0.2802$	**$p = 0.002$**
Cytochrome *c* oxidase	$F_{(3, 42)} = 1.198$	$F_{(1, 42)} = 0.1964$	$F_{(3, 42)} = 2.490$
	$p = 0.3221$	$p = 0.6559$	$p = 0.0733$
RCR	$F_{(3, 40)} = 1.493$	$F_{(1, 40)} = 4.457$	$F_{(3, 40)} = 1.044$
	$p = 0.2312$	**$p = 0.0411$**	$p = 0.3835$
Citrate synthase	$F_{(3, 57)} = 7.890$	$F_{(1, 57)} = 0.9931$	$F_{(3, 57)} = 43.11$
	$p = 0002$	$p = 0.2323$	**$p < 0.0001$**
ATPB	$F_{(2, 12)} = 1.194$	$F_{(1, 12)} = 7.348$	$F_{(2, 12)} = 5.603$
	$p = 0.3427$	**$p = 0.0219$**	**$p = 0.0233$**
COXIV	$F_{(2, 12)} = 5.355$	$F_{(1, 12)} = 4.313$	$F_{(2, 12)} = 4.394$
	$p = 0.0262$	$p = 0.0645$	**$p = 0.0427$**
COXIV (P:M)	$F_{(2, 12)} = 3.523$	$F_{(1, 12)} = 8.816$	$F_{(2, 12)} = 0.3843$
	$p = 0.0695$	**$p = 0.0141$**	$p = 0.6906$

(*Continued*)

TABLE 3 | Continued

	Genotype × age interaction	Genotype effect	Age effect
[Zn]	$F_{(2, 11)} = 12.09$ **$p = 0.0017$**	$F_{(1, 11)} = 37.88$ **$p < 0.0001$**	$F_{(2, 11)} = 13.61$ **$p = 0.0011$**
Shank3	$F_{(2, 18)} = 4.293$ **$p = 0.0299$**	$F_{(1, 18)} = 85.27$ **$p < 0.0001$**	$F_{(2, 18)} = 95.15$ **$p < 0.0001$**
Cortex			
FMRP	$F_{(2, 12)} = 9.336$ **$p = 0.0036$**	$F_{(1, 12)} = 38.50$ **$p < 0.0001$**	$F_{(2, 12)} = 14.01$ **$p = 0.0007$**
NADH oxidase	$F_{(3, 41)} = 0.2289$ $p = 0.8757$	$F_{(1, 41)} = 0.0008$ P = 0.9286	$F_{(3, 41)} = 19.52$ **$p < 0.0001$**
Succinate oxidase	$F_{(3, 46)} = 0.09088$ $p = 0.9647$	$F_{(1, 46)} = 1.045$ $P = 0.3120$	$F_{(3, 46)} = 15.24$ **$p < 0.0001$**
Cytochrome *c* oxidase	$F_{(3, 46)} = 0.09514$ $p = 0.9623$	$F_{(1, 46)} = 3.203$ $p = 0.0801$	$F_{(2, 46)} = 12.30$ **$p < 0.0001$**
RCR	$F_{(3, 45)} = 0.2112$ $p = 0.8881$	$F_{(1, 45)} = 4.890$ **$p = 0.0321$**	$F_{(3, 45)} = 2.388$ $p = 0.0813$
Citrate synthase	$F_{(3, 55)} = 1.983$ $p = 0.1272$	$F_{(1, 55)} = 1.983$ $p = 0.3405$	$F_{(3, 55)} = 213.7$ **$p < 0.0001$**
ATPB	$F_{(2, 11)} = 1.249$ $p = 0.3246$	$F_{(1, 11)} = 0.6066$ $p = 0.4525$	$F_{(2, 11)} = 15.49$ **$p = 0.0006$**
COXIV	$F_{(2, 12)} = 3.819$ $p = 0.0630$	$F_{(1, 12)} = 1.054$ $p = 0.3313$	$F_{(2, 12)} = 12.72$ **$p = 0.0024$**
COXIV (P:M)	$F_{(2, 12)} = 1.018$ $p = 0.3993$	$F_{(1, 12)} = 0.4778$ $p = 0.5069$	$F_{(2, 12)} = 3.047$ $p = 0.0976$
[Zn]	$F_{(2, 12)} = 1.913$ $p = 0.1979$	$F_{(1, 12)} = 0.1174$ $p = 0.7389$	$F_{(2, 12)} = 3.011$ $p = 0.0947$
Shank3	$F_{(2, 12)} = 0.4705$ $p = 0.6322$	$F_{(1, 12)} = 1.679$ $p = 0.2114$	$F_{(2, 12)} = 9.085$ **$p = 0.0019$**

Statistical parameters reported in the table were calculated by Two-way ANOVA. In bold are statistically significant effects. Between parentheses are values of F(DFn, DFd). Further, statistical details are reported in the figure legend of each outcome tested.

Studies have shown that Zn deficiency in rodents results in anorexia (with about 50% of the body weight gained under physiological conditions), poor hair coat, scaly paws, and reproductive defects (Brody, 1999), none of which have been observed in either KI dams or pups, supporting the idea of a localized Zn homeostasis deficit related to the premutation (probably linked to the tissue expression of FMRP), rather than a generalized one.

Detrimental Effect of KI Milk on Brain Bioenergetics

On the basis of the findings shown thus far we hypothesize that Zn-deficient milk of KI dams would be detrimental on the suckling pups' bioenergetics requirements, whereas milk from WT dams might rescue some of the deficits observed in the premutation mice. To test this hypothesis we designed a cross-fostering experiment in which KI pups nursed on WT dams (WT milk) and WT pups nursed on KI dams (KI milk; **Figure 10**). We tested mitochondrial outcomes at PND21 from WT and KI in the pups (male hemizygous) nursing on KI milk to elucidate the effect of KI milk on the brain bioenergetics of WT pups, or nursing on WT milk to elucidate the effect of WT milk on the brain bioenergetics of KI pups (**Figure 10**).

A detrimental effect on brain bioenergetics was observed in WT pups nursing on KI milk, characterized by significantly lower mitochondrial outcomes relative to WT pups nursing on WT milk in all three brain areas, with the highest effect observed in hippocampus showing, deficits in four of the five measured outcomes. On average, the most affected outcome was ATP-dependent oxygen uptake sustained by NAD-linked substrate (NADH oxidase activity), followed by ATP-dependent oxygen uptake sustained by an FAD-linked substrate (succinate oxidase activity), CCO activity, and coupling (**Figure 10**). Furthermore, the hippocampus of WT pups nursing on KI milk showed a significant decrease in mitochondrial mass. Of note, although the detrimental effects of the KI milk on the mitochondrial outcomes in WT pups were significant (–29 ± 8% average of all outcomes), they were not severe enough to match the deficits observed in KI pups nursed on KI milk (–46 ± 11%, mean ± SEM). KI pups nursing on WT milk compared to KI pups nursed on KI milk exhibited some mitochondrial improvement, as judged by a partial recovery of succinate oxidase activity in cerebellum (from 60 to 82% of WT) and complete recovery of succinate oxidase and

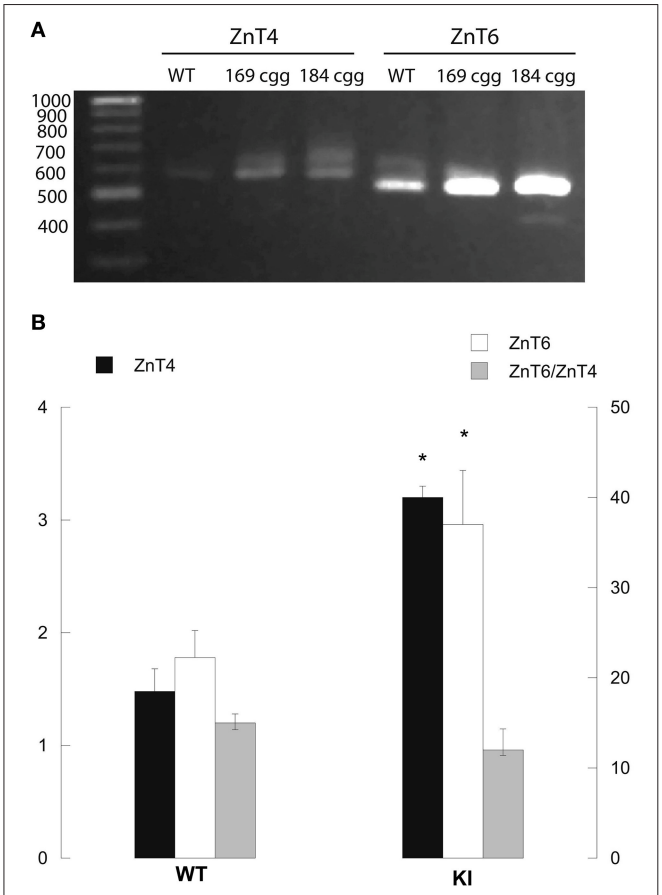

FIGURE 9 | *ZnT4* and *ZnT6* gene expression in mammary glands from lactating WT and KI dams. Representative image of the PCR products (obtained by RT-qPCR as described under Methods) that were separated in a 1.3% agarose gel and visualized with ethidium bromide **(A)**. The fragments exhibited the expected sizes of 585 bp (*ZnT4*) and 537 bp (*ZnT6*). *GAPDH* PCR product was separated in a 3% agarose gel electrophoresis with ethidium bromide resulting in a product of the expected of 107 bp (not shown). Intensities of *ZnT4* and *ZnT6* bands were obtained in a Kodak Imager were normalized by that of *GAPDH* **(B)**. Bars represent averages ± SEM of 4 individuals/genotype. Statistical analysis was carried out with the Student's *t*-test between WT and KI. $^*p < 0.05$.

coupling in cortex (**Figure 10**). None of the measured outcomes showed a significant recovery in hippocampus (**Figure 10**).

The effect of the diet (KI vs. WT milk) and genotype on mitochondrial outcomes was evaluated by Two-way ANOVA (**Table 4**). A significant milk type × genotype interaction on bioenergetics was observed in all brain regions, with the main effects being attributed to genotype in hippocampus and cerebellum, and to both genotype and milk type in cortex (**Table 4**).

Taken together these results suggest that, despite the presence of a susceptible genetic background conferred by the *FMR1* premutation, brain mitochondrial outcomes were modulated by a nutritional intervention (KI milk) mainly in cortex, the least Zn-enriched brain region. While the impact of cross-fostering on offspring's brain bioenergetics could not be explained by differences in caloric intake because there were no statistical differences in body weight gain or brain weight gain between the two groups (not shown), the changes in mitochondrial brain OXPHOS in both WT and KI mice were consistent with a compounding effect of an altered Zn homeostasis in milk from premutation carriers (**Table 4**). While WT pups nursing on KI milk resulted in MD (hippocampus and cerebellum), KI pups nursing on WT milk improved some of the mitochondrial outcomes (cortex). The lack of a significant improvement in all of the brain regions tested could be explained by their different brain Zn requirements (Sawashita et al., 1997), limitations of a non-optimized intervention on a specific genetic background, or inability of achieving a complete recovery of some of the components involved in post-synaptic scaffolding and bioenergetics beyond PND21.

Zn Deficits in Breast Milk from Premutation Carriers

To complement and further confirm the experiments performed with the premutation mouse model, experiments were extended to test the quality of milk from lactating women carrying the premutation. Zn concentrations were determined in mature human milk [i.e., 8–20 weeks of lactation (Worth et al., 1981)] from asymptomatic premutation women (milk CGG repeats = 63–119) and age-matched controls (**Table 5**). Furthermore, we tested selected Zn-associated outcomes, namely activity of milk alkaline phosphatase (ALP), a Zn-requiring enzyme that contributes with 20% of total milk Zn (Fransson and Lonnerdal, 1984), and concentrations of lactose, whose biosynthesis is dependent on the Zn-requiring enzyme β-1,4-galactosyltransferase (McCormick and Kelleher, 2012), the rate limiting enzyme in the lactose biosynthetic pathway (Jagoda and Rillema, 1991).

Milk from healthy donors had an average Zn concentration of 26 ± 2 μM (1.7 ± 0.1 mg/l), within the normal range reported before [1.0–1.7 mg/l at 3–5 months of lactation evaluated by atomic absorption spectrometry (Krebs et al., 1995)], with 86% of the Zn being in the labile form. In agreement with data previously reported (Nagra, 1989; Krebs et al., 1995), milk Zn concentrations from control and premutation women were reciprocally correlated with the postpartum period (**Figure 11A**), following a pseudo-first order kinetics with a biological half-life of 8 weeks (**Figure 11**). Although 10 of the 25 control donors were taking vitamins and mineral supplements (including Zn) during lactation, no significant differences in Zn concentrations were observed between these two groups, in agreement with the observed lack of correlation between milk or plasma Zn concentrations and maternal Zn intake (Krebs et al., 1995). In samples from premutation carriers, the average milk Zn concentration at 3–5 months of lactation was 56% of control values (14 ± 3 μM; $p = 0.020$). Specifically, milk Zn concentrations from two carriers at 8–9 lactation weeks and from one carrier at 19–20 weeks were significantly lower than the 95% CI (**Figure 11A**, **Table 6**). Zn levels in the remaining premutation milk samples, obtained after week 15, followed the decrease observed in control milk (**Figure 11A**, **Table 6**).

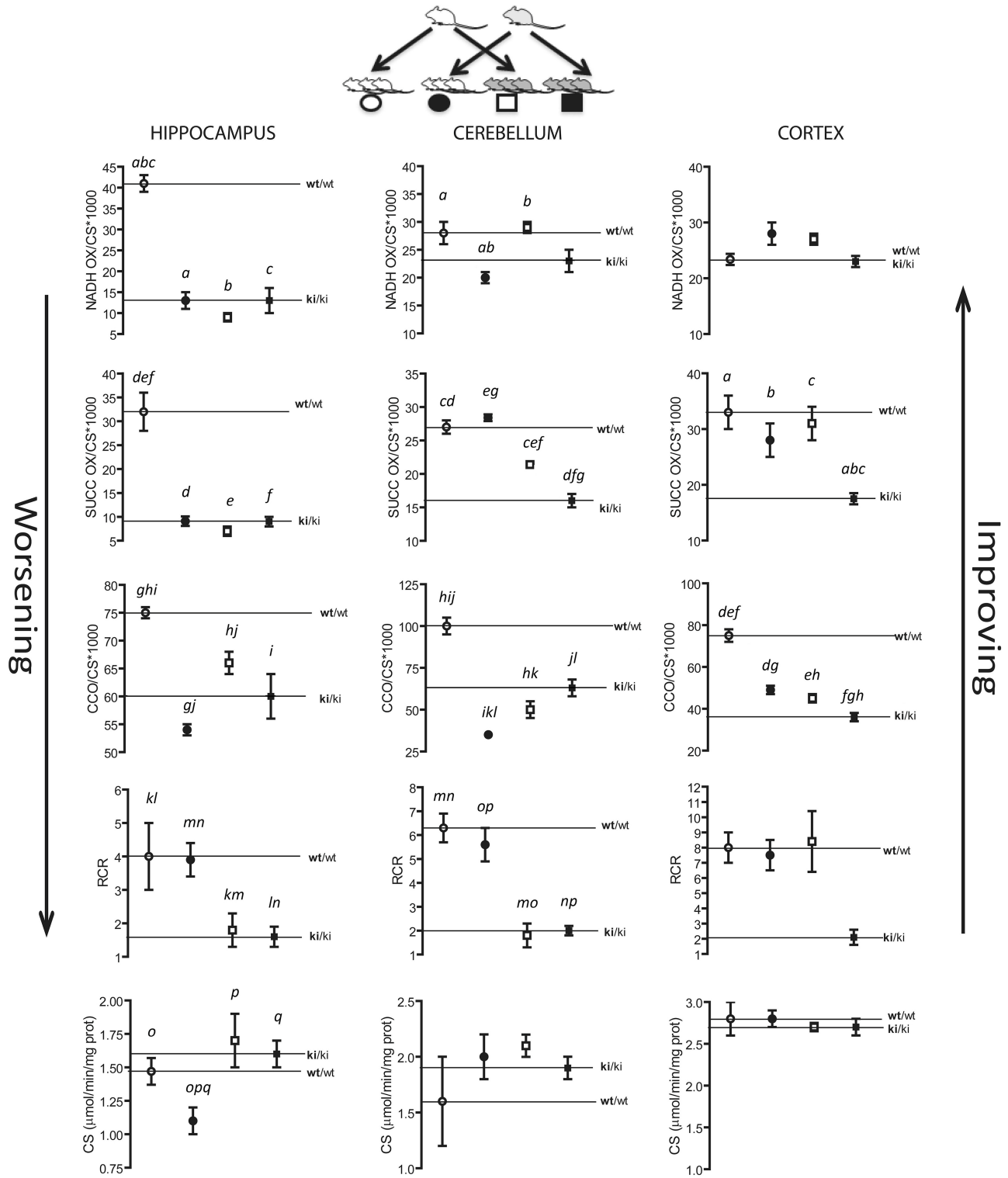

FIGURE 10 | Changes in mitochondrial outcomes in hippocampus, cerebellum and cortex of suckling WT and KI pups nursed on WT or KI dams. At birth, KI pups ($n = 24$) and WT pups ($n = 24$) were foster-nursed either on KI dams or WT dams, six pups on each dam. After 21 days, mitochondria were isolated from cortex, cerebellum, and hippocampus and activities of NADH oxidase, succinate oxidase, cytochrome *c* oxidase, and citrate synthase and RCR were evaluated as described in the Methods section. wt/wt = WT pups nursing on WT milk; ki/ki = KI pups nursing on KI milk. Circles represent WT pups, squares represent KI pups. White symbols represent WT milk and black symbols represent KI milk. Upward or downward arrows represent improvement or worsening, respectively, upon nursing on WT milk or KI milk. Activities (NADH oxidase, succinate oxidase, cytochrome *c* oxidase) were expressed as nmol × (min × mg protein)$^{-1}$, normalized by citrate

(Continued)

FIGURE 10 | Continued
synthase activity and multiplied by 1000. Data are shown as mean ± SEM (from technical replicates of pooled samples). Statistical analysis was performed by Two-way ANOVA, followed by Tukey's *post-hoc* test for multiple comparisons. The *p* values are as follows. Hippocampus: $p < 0.0001$ (*a, b, c, g, i, p, q*), $p = 0.0004$ (*d, o*), $p = 0.0002$ (*e, f*), $p = 0.0025$ (*h*), $p = 0.0008$ (*j*), $p = 0.0027$ (*k*), $p = 0.0006$ (*l*), $p = 0.0262$ (*m*), $p = 0.0082$ (*n*); Cerebellum: $p = 0.0296$ (*a*), $p = 0.0301$ (*b*), $p = 0.0039$ (*c*), $p < 0.0001$ (*d, g, h, i, j, k, l, m, n, o, p*), $p = 0.0023$ (*e*); $p = 0.0086$ (*f*); Cortex: $p < 0.0001$ (*a, b, c, d, e, f*), $p = 0.0047$ (*g*), $p = 0.0468$ (*h*). Further statistical details on the genotype, age, and genotype × age effect can be found in **Table 4**.

TABLE 4 | Effect of genotype, diet, and genotype × diet interaction on outcomes evaluated in hippocampus, cerebellum, and cortex of cross-fostered WT and KI pups.

	Genotype × diet interaction	Genotype effect	Diet effect
HIPPOCAMPUS			
NADH oxidase	$F_{(1, 11)} = 58.03$	$F_{(1, 11)} = 57.52$	$F_{(1, 11)} = 32.05$
	$p < 0.0001$	**$p < 0.0001$**	**$p = 0.0001$**
Succinate oxidase	$F_{(1, 11)} = 20.96$	$F_{(1, 11)} = 20.49$	$F_{(1, 11)} = 14.15$
	$p = 0.0008$	**$p = 0.0009$**	**$p = 0.0031$**
Cytochrome *c* oxidase	$F_{(1, 11)} = 29.96$	$F_{(1, 11)} = 1.501$	$F_{(1, 11)} = 97.53$
	$p = 0.0002$	$p = 0.2462$	**$p < 0.0001$**
RCR	$F_{(1, 11)} = 0.1242$	$F_{(1, 11)} = 39.29$	$F_{(1, 11)} = 1.039$
	$p = 0.7312$	**$p < 0.0001$**	$p = 0.3298$
Citrate synthase	$F_{(1, 11)} = 10.35$	$F_{(1, 11)} = 26.72$	$F_{(1, 11)} = 67.89$
	$p = 0.0082$	**$p = 0.0003$**	**$p < 0.0001$**
CEREBELLUM			
NADH oxidase	$F_{(1, 11)} = 0.6863$	$F_{(1, 11)} = 15.68$	$F_{(1, 11)} = 1.384$
	$p = 0.4250$	**$p = 0.0022$**	$p = 0.2642$
Succinate oxidase	$F_{(1, 11)} = 12.75$	$F_{(1, 11)} = 97.54$	$F_{(1, 11)} = 5.300$
	$p = 0.0044$	**$p < 0.0001$**	**$p = 0.0419$**
Cytochrome *c* oxidase	$F_{(1, 11)} = 905.1$	$F_{(1, 11)} = 69.16$	$F_{(1, 11)} = 403.2$
	$p < 0.0001$	**$p < 0.0001$**	**$p < 0.0001$**
RCR	$F_{(1, 11)} = 14.28$	$F_{(1, 11)} = 1146$	$F_{(1, 11)} = 4.768$
	$p = 0.0031$	**$p < 0.0001$**	$p = 0.0515$
Citrate synthase	$F_{(1, 11)} = 1.582$	$F_{(1, 11)} = 0.8701$	$F_{(1, 11)} = 0.3943$
	$p = 0.2290$	$p = 0.3667$	$p = 0.5401$
CORTEX			
NADH oxidase	$F_{(1, 11)} = 98.63$	$F_{(1, 11)} = 4.235$	$F_{(1, 11)} = 0.5049$
	$p < 0.0001$	$P = 0.0641$	$p = 0.4921$
Succinate oxidase	$F_{(1, 11)} = 34.47$	$F_{(1, 11)} = 61.96$	$F_{(1, 11)} = 149.0$
	$p = 0.0001$	**P < 0.0001**	**$p < 0.0001$**
Cytochrome *c* oxidase	$F_{(1, 11)} = 16.45$	$F_{(1, 11)} = 109.7$	$F_{(1, 11)} = 71.65$
	$p = 0.0019$	**$p < 0.0001$**	**$p < 0.0001$**
RCR	$F_{(1, 11)} = 60.13$	$F_{(1, 11)} = 44.72$	$F_{(1, 11)} = 84.89$
	$p < 0.0001$	**$p < 0.0001$**	**$p < 0.0001$**
Citrate synthase	$F_{(1, 11)} = 2.068$	$F_{(1, 11)} = 0.1195$	$F_{(1, 11)} = 0.0441$
	$p = 0.1724$	$p = 0.7347$	$p = 0.8367$

Statistical parameters reported in the table were calculated by Two-way ANOVA. Outcomes were measured in 24 mice (12 WT and 12 KI). Due to the scarce amount of tissue collected from hippocampus and cerebellum, material from four animals belonging to the same genotype and nursed from the same dam were pooled. The cross-fostering experiment was repeated twice, for a total of 48 mice used. In bold are statistically significant effects. Between parentheses are values of F(DFn, DFd). Further, statistical details are reported in the figure legend of each outcome tested.

Although the sample size is small, the incidence of low Zn concentration was higher in carriers compared to controls (60% vs. 24%; test for one proportion $p = 0.0595$; 95% CI = 0.2307–0.8824).

The ALP activity in human breast milk from control donors increased almost linearly from eight to 18–20 weeks, in agreement with other reports (Chanda et al., 1951; Stewart et al., 1958; Worth et al., 1981) and with published values (Worth et al., 1981; Coburn et al., 1992; **Figure 11B**, **Table 6**). As observed with milk Zn concentrations, ALP activities in controls were not affected by vitamin supplementation. Changes in milk ALP activity with lactation week from premutation donors paralleled those of controls, with only one carrier showing a 51% ALP activity of control values at 19 weeks (**Figure 11B**, **Table 6**).

TABLE 5 | Details of the study population.

Premutation carrier	Milk CGG repeats	Maternal age at sample collection (y)	Lactation week at sample collection
1	29, 63	38	20
2	20, 80	34	9
3	29, 91	34	16
4	30, 93	23	19
5	29, 119	34	8
		MEAN ± SEM	
Premutation women ($n = 5$)	89 ± 9	33 ± 2	14 ± 2
Control women ($n = 25$)	27 ± 2 ($p = 0.002$)	31 ± 1	13.1 ± 0.5

CGG repeats were evaluated by extracting total genomic DNA from milk samples and following the procedure described before (Tassone et al., 2012). The mean CGG for premutation patients represent that of the longer allele only.

In terms of protein and lactose concentrations, the mean nutrient composition of milk samples was not different between controls and premutation individuals and remained fairly constant throughout the evaluated period, consistent with published concentrations for controls [[protein] in g/l: this study = 12.8 ± 0.5, 95%CI 12-13, literature = 12 ± 1.5 (Tudehope, 2013); [lactose] in mM: this study = 205 ± 3 95%CI 199-210, literature = 196 ± 15 (Nagra, 1989; Mohammad et al., 2012; Tudehope, 2013)]. However, the incidence of either low protein or low lactose concentrations was higher in premutation carriers than controls (**Table 6**). Interestingly, these observations are consistent with the findings obtained in HC11 cells in which overexpression of ZnT4 lowers both β-1,4-galactosyltransferase activity (McCormick and Kelleher, 2012) and lactose concentration by compromising the incorporation of Zn into Zn-requiring proteins.

Taken together, these results show lower milk Zn concentrations in premutation women, consistent with an altered Zn homeostasis as observed in mammary glands from KI dams, supporting the idea that adequate gene expression of *ZnT4* and *ZnT6* may be required during the switch from the non-lactating to the lactating condition to support normal Zn efflux into milk.

DISCUSSION

Our study reports the contribution of FMRP protein expression to the development of brain bioenergetics, cytoskeleton structure, and post-synaptic scaffolding protein Shank3 in *FMR1* premutation carriers at early stages of life. In support of this concept, we found (i) mitochondrial deficits at PND0 in isolated neurons from hippocampus, cerebellum and cortex of KI pups likely reflecting an embryonic (pre-natal) FMRP-dependent dysfunction; (ii) deficits in bioenergetics, Zn concentrations and Shank3 protein, mainly in hippocampus and cerebellum (Zn-rich brain areas) of KI pups at PND21, with some of these deficits lasting into adulthood; and (iii) defective import/processing of nDNA-encoded mitochondrial subunits secondary to impaired Zn homeostasis. A strong genotype × age interaction was observed for most of the outcomes tested in hippocampus and cerebellum, whereas in cortex, age played a major factor.

The effect of maternal milk Zn on offspring bioenergetics highlights the influence of genetics × nutrition on the premutation supported by the altered gene expression of *ZnT6/T4* in lactating KI mammary glands, the KI milk-dependent detrimental effect on KI and WT brain bioenergetics, and the lower milk Zn content in breast milk from lactating premutation women. A highly significant milk type × genotype interaction was observed for all three-brain regions being cortex the most influenced.

Brain Zn concentrations change with age, reflecting its function as a neuromodulator and as a required element for brain development (Sawashita et al., 1997), especially in regards to synaptic transmission and bioenergetics (Ho et al., 2003; Kogan et al., 2008; Grabrucker et al., 2014; Hara et al., 2014; Picard and McEwen, 2014). Our study is consistent with the reported detrimental effect of milk Zn deficiency on perinatal outcomes in the lethal milk (*lm*) mice (Huang and Gitschier, 1997), and in agreement with the proposed role of FMRP as modulator of synaptogenesis through actions on cytoskeletal proteins, by interacting with specific mRNAs [such as Shank3 or actin (Brown et al., 2001; Darnell and Richter, 2012; Han et al., 2013)] or via the Rac1 pathway (Bardoni and Mandel, 2002). The lower milk Zn concentration found in premutation women and the altered *ZnT4/ZnT6* gene expression in lactating KI murine mammary gland may account for the effect of KI milk on the brain bioenergetics of both WT and KI offspring, but more evident in KI pups. Given the heavy reliance of brain on mitochondrial ATP as well as on *de novo* synthesis of Krebs cycle-associated neurotransmitter amino acids [i.e., Glu, Asp, and GABA (Butterworth and Heroux, 1989; Navarro et al., 2008)], mitochondrial and synaptic scaffolding deficits are likely to increase the risk for some of the neurological symptoms observed in pediatric carriers. Indeed, altered brain bioenergetics and those of the post-synaptic scaffolding protein Shank3 during perinatal periods may explain the abnormal behavior observed

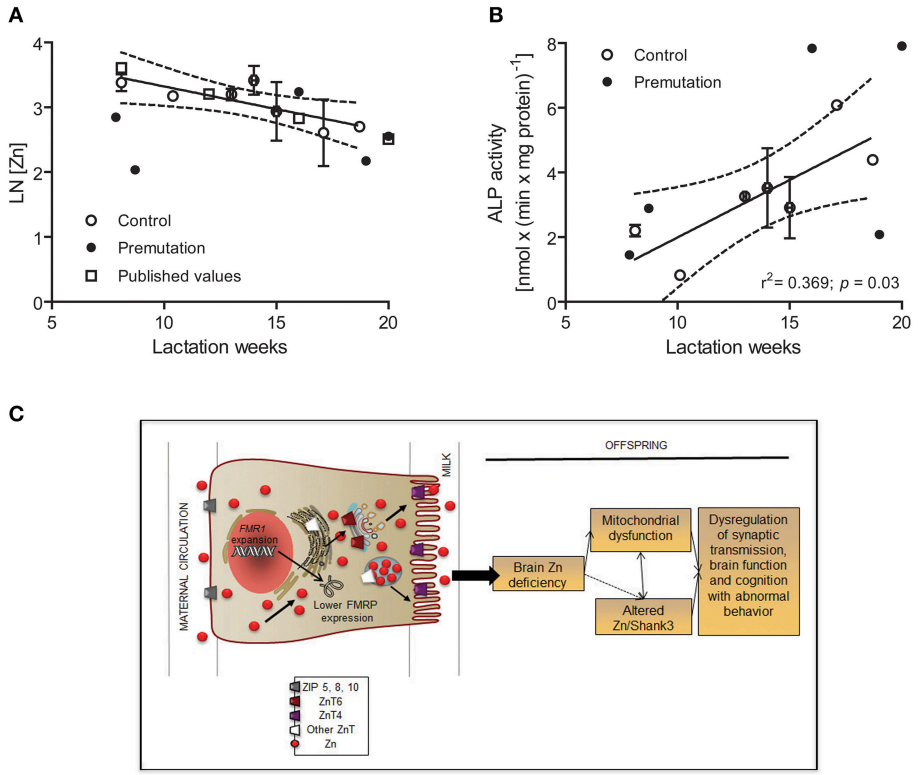

FIGURE 11 | Role of milk Zn in brain bioenergetics and synapse scaffolding in premutation carriers. Milk-Zn concentrations followed a pseudo-first order kinetics over the lactation period **(A)**. Milk Zn concentrations from control and premutation donors decreased with the post-partum period, ranging from 30 ± 3 μM at 6–8 weeks to 15 ± 2 μM at 18–20 weeks. The LN values of the Zn concentrations (in μM) were plotted against lactation week. The published values were obtained from Nagra et al. (Nagra, 1989) and shown as white squares. Regression parameters are as follows: for published [Zn], $r^2 = 0.997$, $y = -0.09x + 4.3$; for this study, $r^2 = 0.647$, $y = -0.07x + 4.0$. *P* value for slopes = 0.458; *P* value for intercepts = 0.986. ALP activity [expressed as nmol × (min × mg protein)$^{-1}$] in human breast milk from control donors increased linearly from 8 to 18–20 weeks **(B)**. The average ALP activity of controls was 3.3 ± 0.3 nmol × (min × mg protein)$^{-1}$ or 46 ± 1 μmol × (min × l)$^{-1}$ and within reported values [40 to 60 μmol × (min × l)$^{-1}$; (Worth et al., 1981; Coburn et al., 1992)]. Linear regression analyses were performed with control values (reported as mean ± SEM) for both outcomes ($r^2 = 0.638$ and 0.639 for Zn concentration and ALP activity, respectively). The 95% CIs performed with control values are shown with dotted lines. Individual premutation carriers' values for Zn concentrations and ALP activities are shown in **Table 5**. ALP activities in controls were not affected by vitamin and mineral supplementation [supplemented: 3.7 ± 0.4 vs. not supplemented: 3.2 ± 0.4 nmol × (min × mg protein)$^{-1}$; $p = 0.624$]. A model integrating experimental data based on altered Zn homeostasis, mitochondrial dysfunction [current study and (Ross-Inta et al., 2010; Napoli et al., 2011)], and the putative effect of defective synaptic scaffolding in premutation individuals is shown in **(C)**. During lactation, Zn uptake is exerted by ZIP5, 8 and 10, then excreted to milk via ZnT4/ZnT2 with a lower flux through ZnT6 (ZnT10) in the Golgi [Adapted from (Kelleher et al., 2012)]. The presence of the CGG expansion in *FMR1*, as it is the case with premutation carriers, may ensue in a lower expression of FMRP, which may affect the cytoskeleton (e.g., actin, Shank3), and the expression or function of membrane transporter such as ZnT4/T6. This situation would ensue in the suboptimal delivery of Zn into milk, mitochondrial dysfunction, and synaptic dysregulation. The arrows indicate the direction of Zn transport from maternal blood to milk.

in KI mice later in life [12 and 24 weeks; (Van Dam et al., 2005; Hunsaker et al., 2009, 2012)], similar to the spatial processing defects observed in humans with FXTAS (Hunsaker et al., 2012).

While the relative contribution of RNA sequestration of essential factors, low FMRP expression and RAN translation (of the toxic FMRPolyG) to the premutation pathology is still unknown, this study supports the notion that that lower levels of wild-type FMRP [as observed in this study and most premutation carriers (Kenneson et al., 2001), with or without the generation of aberrant FMRP isoforms (Todd et al., 2013)] may provide a disrupted cellular background that affects cytoskeleton/scaffolding (actin, Shank3) and Zn homeostasis allowing environmental factors (e.g., quality of the breast milk) to further affect synaptogenesis and mitochondrial function early in life (**Figure 11C**).

In support of this concept, FMRP protein expression—and not *FMR1* mRNA levels—correlates positively with mitochondrial outcomes (ATP-driven oxygen uptake and coupling) in both the KI mouse model and primary dermal fibroblasts from premutation and full mutation carriers. Furthermore, the incidence of neurodevelopmental disorders like ASD, ADHD, anxiety, and other types of psychopathologies (Farzin et al., 2006; Tassone et al., 2012; Winarni et al., 2012; Wong et al., 2012; Battistella et al., 2013; Chonchaiya et al., 2013) observed in young carriers seem to follow the FMRP protein expression [e.g., incidence of ASD in FXS is 60% (Garcia-Nonell et al., 2008; Harris et al., 2008; D'Hulst et al., 2009; Zingerevich et al., 2009; Hagerman et al., 2010) and in premutation carriers is ∼15% (Farzin et al., 2006; Chonchaiya et al., 2013)]. In this regard, and consistent with our findings, the expression levels of a subset of

TABLE 6 | Characteristics of mature breast milk from premutation individuals.

Premutation carrier	[Zn] (μM) [mg/l]	ALP activity [nmol × (min × mg)$^{-1}$]	[Protein] (mg/ml)	[Lactose] (mM)
1	13 ± 1	7.9 ± 0.6	12.2 ± 0.4	213 ± 3
	[0.84 ± 0.08]			
2	**8 ± 2**	2.9 ± 0.3	**6.0 ± 0.1**	**160 ± 4**
	[0.50 ± 0.05]			
3	25 ± 2	7.3 ± 1.0	**11.4 ± 0.4**	231 ± 1
	[1.67 ± 0.13]			
4	**9 ± 1**	**2.1 ± 0.3**	17.7 ± 0.7	**196 ± 2**
	[0.57 ± 0.09]			
5	**17.2 ± 4**	**1.5 ± 0.1**	19.9 ± 2.0	208 ± 1
	[1.13 ± 0.91]			
(Mean ± SEM)	14 ± 3	4 ± 2	13 ± 3	202 ± 13
	[0.94 ± 0.21]			
<95% CI	3 of 5	1 of 5	2 of 5	2 of 5
p-value	0.001	n.s.	0.054	0.054
Controls (mean ± SEM)	26 ± 2	4 ± 1	12.8 ± 0.5	205 ± 3
	[1.68 ± 0.12]			
95% CI			12–13	199–210

For samples from premutation carriers, each outcome was expressed as mean ± SD, reflecting technical variability of triplicates. In bold, values below the 95% CI calculated with control values. For Zn concentrations and ALP activities, outcomes that change with the lactation week, the 95% CIs are shown in ***Figures 9A,B****. No significant correlation was obtained between lactation week and lactose or protein concentrations, thus the 95%CIs were calculated using all control data regardless of lactation week. The p-values correspond to the test for one proportion. n.s., not significant. A post-hoc analysis to calculate the power of the analysis for the means of milk-Zn concentration from each group was 1.00, whereas for all others was 0.504 or lower (G*power software; v. 3.0.10).*

miRNAs involved in learning, memory and autistic behavior have been shown to be deregulated in FXTAS (Zongaro et al., 2013; Nguyen et al., 2016).

While additional studies will be necessary to estimate Zn requirements for breast-fed only premutation carriers' babies and assess potential benefits of Zn-fortified milk (while controlling for confounding factors and monitoring for potential adverse effects), our findings emphasize that early nutritional interventions to prevent MD seem critical for the management of premutation carriers at high risk of developing emotional and neurological/cognitive problems (including autism) and/or FXTAS later in life (Tassone et al., 2012; Winarni et al., 2012; Wong et al., 2012; Battistella et al., 2013; Kim et al., 2013). This is largely relevant considering the independence of milk-Zn concentrations on maternal diet (Moore et al., 1984; Krebs et al., 1995), and consistent with the fact that infants born to the lowest milk Zn-producing mothers (among other feeding factors) are more stunted than infants of women with higher milk Zn concentrations (Krebs et al., 1995; Umeta et al., 2000; Krebs and Hambidge, 2007).

AUTHOR CONTRIBUTIONS

EN measured mitochondrial outcomes in mice at PND21 and 210, Zn, FMRP, and Shank3 levels, Zn in milk, run all western blots from rodents and human cells, analyzed the data, helped drafting, and reviewed the manuscript; CI measured mitochondrial outcomes isolated neurons and in mice at PND0-9 and PND21; GS measured ALP activity, proteins, and lactose levels in human milk and reviewed the manuscript; SW evaluated the *FMR1* expression in fibroblasts and *ZnT*s gene expression, and reviewed the manuscript; LG recruited the patients, collected the medical history, and the premutation milk samples; JS provided all milk samples from controls; FT determined the CGG repeats in fibroblasts and milk samples from gDNA extracted by Wong; CG conceptualized the work, wrote, and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00159

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