NEURODEVELOPMENTAL PROCESSES IN HEALTH AND DISEASE: BRIDGING BASIC AND CLINICAL RESEARCH

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NEURODEVELOPMENTAL PROCESSES IN HEALTH AND DISEASE: BRIDGING BASIC AND CLINICAL RESEARCH

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Prediction Analysis for Transition to Schizophrenia in Individuals at Clinical High Risk for Psychosis: The Relationship of *DAO*, *DAOA*, and *NRG1* Variants with Negative Symptoms and Cognitive Deficits

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Jagannath V, Theodoridou A, Gerstenberg M, Franscini M, Heekeren K, Correll CU, Rössler W, Grünblatt E and Walitza S (2017) Prediction Analysis for Transition to Schizophrenia in Individuals at Clinical High Risk for Psychosis: The Relationship of DAO, DAOA, and NRG1 Variants with Negative Symptoms and Cognitive Deficits. Front. Psychiatry 8:292. doi: 10.3389/fpsyt.2017.00292

Schizophrenia is characterized by positive and negative symptoms and cognitive dysfunction. The glutamate hypothesis of schizophrenia has been hypothesized to explain the negative symptoms and cognitive deficits better than the dopamine hypothesis alone. Therefore, we aimed to evaluate whether glutamatergic variants such as D-amino acid oxidase (*DAO*), DAO activator (*DAOA*)/*G72*, and neuregulin 1 (*NRG1*) single-nucleotide polymorphisms (SNPs) and their mRNA levels predicted (i) transition to schizophrenia spectrum disorders and (ii) research domain criteria (RDoC) domains, mainly negative valence and cognitive systems. In a 3-year prospective study cohort of 185 individuals (age: 13–35 years) at high risk and ultra-high risk (UHR) for psychosis, we assessed *DAO* (rs3918347, rs4623951), *DAOA* (rs778293, rs3916971, rs746187), and *NRG1* (rs10503929) SNPs and their mRNA expression. Furthermore, we investigated their association with RDoC domains, mainly negative valence (e.g., anxiety, hopelessness) and cognitive (e.g., perception disturbances, disorganized symptoms) systems. *NRG1* rs10503929 CC + CT versus TT genotype carriers experienced significantly more disorganized symptoms. *DAOA* rs746187 CC versus CT + TT genotype, *DAOA* rs3916971 TT versus TC + CC genotype, and *DAO* rs3918347 GA + AA versus GG genotype carriers experienced nominally more hopelessness, visual perception disturbances, and auditory perception disturbances, respectively. The schizophrenia risk G-allele of *DAO* rs3918347 nominally increased risk for those UHR individuals with attenuated positive symptoms syndrome. No association between *DAO*, *DAOA*, *NRG1* SNPs, and conversion to schizophrenia spectrum disorders was observed. Our findings suggest that *DAO*,

DAOA, and *NRG1* polymorphisms might influence both RDoC negative valence and cognitive systems, but not transition to schizophrenia spectrum disorders.

Keywords: D-amino acid oxidase/DAO/DAAO, D-amino acid oxidase activator/G72/DAOA, neuregulin 1/NRG1, attenuated positive symptoms syndrome/APSS, single-nucleotide polymorphism/SNP, research domain criteria/ RDoC

INTRODUCTION

Schizophrenia is a chronic and debilitating disorder, preceded by a broad range of symptoms. The emergence of psychotic features in schizophrenia is typically between the late teens and mid-30s (1). Early recognition of individuals at-risk for psychosis and the provision of early intervention is likely to be associated with improved outcomes (2). Individuals at clinical risk for psychosis are identified by two complementary approaches: the high risk (HR) and the ultra-high risk (UHR) criteria. The HR concept is based on basic symptoms and comprises two partially overlapping risk constellations, the cognitive-perceptive (COPER) basic symptoms and the cognitive disturbances (COGDIS) (3, 4). The UHR criteria comprise attenuated positive symptoms syndrome (APSS), brief limited intermittent psychotic symptoms (BLIPS), and a combination of a risk factor for psychosis and a recent functional decline (5). A meta-analysis of 27 studies showed that 18, 21, 27, and 32% of individuals at-risk for psychosis (HR + UHR) transitioned to psychotic disorders at 6, 12, 24, and 36 months of follow-up, respectively (6). This meta-analysis also showed that the mean transition risk was 49, 28, and 22% using the HR approach, UHR approach, and when combining both HR and UHR approaches, respectively (6). One study reported that about one-third of individuals at UHR for psychosis transitioned to psychosis (7). Our recent study, using a multivariable prediction model, demonstrated that as expected, UHR criteria predicted conversion to psychosis but combining HR and UHR criteria in this help-seeking at-risk population did not improve the predictive accuracy of UHR alone (8). Therefore, it is important to optimize the identification of individuals at HR/UHR for psychosis by minimizing the false positive rate and improving the true positive prediction rate of conversion to psychosis.

The estimated heritability in schizophrenia is around 60–80% (9). Studies have demonstrated an association between schizophrenia and D-amino acid oxidase (*DAO*), DAO activator (*DAOA*)/G72, and neuregulin 1 (*NRG1*) single-nucleotide polymorphisms (SNPs) (10, 11). *DAOA* and *NRG1* polymorphisms were shown to predict the transition to schizophrenia in individuals at HR/UHR for psychosis (12–14). Genetic studies in schizophrenia have shown the association of neurocognitive endophenotypes with several glutamatergic gene polymorphisms including *NRG1* (15–17). These studies suggest that genetic variations and neurocognitive endophenotypes may help to improve the prediction accuracy of clinical symptoms and HR/UHR criteria for transition in an at-risk population.

The glutamate hypothesis of schizophrenia originated from the observation that *N*-methyl-D-aspartate (NMDA) receptor blockers like ketamine induced schizophrenia-like symptoms. As antipsychotics (dopamine D2 receptor antagonists) have little effect on negative symptoms and cognitive deficits, the glutamatergic system is an attractive therapeutic target (18). Meta-analyses have reported that addition of NMDA receptor agonist D-serine and glycine transporter type 1 inhibitor sarcosine as an adjunct to antipsychotics reduce total and negative symptoms (19, 20). Based on these observations, the glutamate hypothesis is thought to describe the pathophysiology underlying negative symptoms and cognitive deficits better than the dopamine hypothesis (21–23). NMDA receptor hypofunction might lead to decreased dopamine activity in the mesocortical pathway, which may manifest as negative symptoms and cognitive dysfunction in schizophrenia (24). The NMDA receptor hypofunction theory proposed in schizophrenia might be partly explained by increased DAO activity modulated by DAOA leading to decreased D-serine, a co-agonist of NMDA receptors (25). The function of NRG1 is mediated by binding to receptor tyrosine kinases called ErbB (ErbB3 and ErbB4), and an altered NRG1/ErbB4 signaling is thought to result in NMDA receptor hypofunction (26, 27). These studies highlight the potential pathogenic link between NMDA receptor hypofunction and dysregulation of *DAO*, *DAOA*, and *NRG1* genes.

The National Institute of Mental Health started the research domain criteria (RDoC) initiative to guide and organize research in psychiatric disorders beyond the typical diagnostic classification approach (28). This initiative provides a non-disease-based structured conceptual framework to understand the dimensional range of human behavior from normal to abnormal by integrating multiple levels of information (from genomics to self-reports). The RDoC represents a paradigm shift from Diagnostic and Statistical Manual of Mental Disorders (DSM)/International Classification of Diseases (ICD) to dimensional approaches with an aim to integrate basic research and psychopathology (29).

In this study, we aimed (1) to identify predictive glutamatergic genetic polymorphisms in at-risk individuals for transition to schizophrenia spectrum disorders, (2) to identify endophenotypes potentially linked to the glutamatergic system in at-risk individuals using RDoC constructs, and (3) to evaluate differences in *DAO*, *DAOA*, and *NRG1* mRNA levels across clinical and RDoC domains.

MATERIALS AND METHODS

Study Population

Participants were recruited by the "Early Recognition and Intervention Program for Psychosis and Bipolar Disorder" project as part of the Zurich Program for Sustainable Development of Mental Health Services (ZInEP)[1] in the Canton of Zurich,

[1] http://www.zinep.ch.

Switzerland. The detailed design, inclusion, and exclusion criteria of the study were described in our previous studies (30, 31). A total of 185 individuals aged 13–35 years at HR/UHR for psychosis were assessed at baseline and were subsequently followed-up at 6, 12, 24, and 36 months for the transition to schizophrenia. At 36 months follow-up, 50% of individuals ($n = 93$) dropped out of the study. The dropouts were due to refusal to participate in the study in most of the cases and due to non-response after contacting in few cases. However, we are unable to give exact numbers of individuals who refused to participate or who did not respond due to missing information in several study participants. Individuals at HR for psychosis were assessed using the Schizophrenia Proneness Instrument-Child and Youth version (SPI-CY) (age < 18 years) (32, 33) or Schizophrenia Proneness Instrument-Adult version (SPI-A) (age ≥ 18 years) (34) and were included when they had one COPER basic symptom or at least two COGDIS. Individuals at UHR for psychosis were assessed using the Structured Interview for Prodromal Syndromes (SIPS) (35, 36) and were included when they met criteria for the APSS or the BLIPS or the state-trait criteria (>30% reduction in global assessment of functioning in the past year plus either schizotypal personality disorder or a first-degree relative with psychosis). The transition to schizophrenia was defined according to ICD-10 criteria (37). The severity of positive and negative symptoms was assessed using the Positive and Negative Syndrome Scale (PANSS) (38), severity of depressive symptoms with the Calgary Depression Rating scale for Schizophrenia (CDSS) (39), and anxiety symptoms with the Beck Anxiety Inventory (BAI) (40). The demographic and diagnostic characteristics of the study population are shown in Table S1 in Supplementary Material. This study was approved by the Cantonal Ethics Commission of Zurich (Ref. Nr. EK: E-63/2009) and complies with the Declaration of Helsinki. Informed written consent was obtained from adult participants and legal guardians of minors, and written assent was obtained from minors.

Phenotypic Domains

The participants were grouped into clinical phenotypes (cases versus controls) namely, converters to schizophrenia spectrum disorders ($n = 27$), i.e., schizophrenia, schizophreniform disorder, and acute psychotic disorder, versus non-converters ($n = 65$) at 36 months follow-up [dropouts ($n = 93$)], and APSS ($n = 98$) group versus all other help-seeking individuals (BLIPS, state-trait criteria, COGDIS, COPER; $n = 87$) at baseline. The transition to schizophrenia was defined according to ICD-10 criteria (37).

First, we grouped our cohort as per the factor structure of the PANSS, concentrating on negative symptoms and general psychopathology (38). As we did not find any significant differences in negative symptoms (sum score of negative (N1–N7) PANSS subscale) and general psychopathology (sum score of general psychopathology (G1–G16) PANSS subscale) across *DAO*, *DAOA*, and *NRG1* SNPs, we then decided to use an exploratory approach by focusing on subgroups of psychopathology constructs defined according to the RDoC domains. The RDoC framework consists of five domains namely, negative valence systems, positive valence systems, cognitive systems, systems for social process, and arousal/regulatory systems (41). In this study, we decided to concentrate on two RDoC domains: negative valence systems and cognitive systems, due to the potential role of *DAO*, *DAOA*, and *NRG1* polymorphisms in the glutamate hypothesis of schizophrenia, as it appears to explain the pathogenesis of negative symptoms and cognitive deficits better than the dopamine hypothesis (22, 23, 42) and unavailability of relevant neuropsychological scales in our study to create the positive valence domain. Negative valence systems focus on responses to aversive situations, such as fear, anxiety, and loss. Cognitive systems concentrate on various cognitive processes, such as perception, language, memory, and cognitive control. Within negative valence systems, we focused on the constructs of threat (acute and sustained) and loss. The negative valence system construct of threat was assessed by the total score of BAI with higher BAI scores pointing to increased severity of anxiety (40). The negative valence system construct loss was assessed by the response to the CDSS item 2 "hopelessness," which can be scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) (39). Within cognitive systems, we chose the constructs of perception (visual and auditory) and cognitive control. The cognitive system construct visual perception was assessed by summing the following four SPI-A (and equivalent SPI-CY) items O4 (other visual perception disturbances), F1 (hypersensitivity to light), F2 (photopsia), and F3 (micropsia/macropsia), with higher scores pointing to more frequent disturbances in visual perception. The cognitive system construct of auditory perception was assessed by summing the following three SPI-A (and equivalent SPI-CY) items O5 (other acoustic perception disturbances), F4 (hypersensitivity to sounds/noise), and F5 (changed intensity/quality of acoustic stimuli), with higher scores pointing to more frequent disturbances in auditory perception. The cognitive system construct of cognitive control was obtained by summing the following four SIPS disorganization items D1, D2, D3, and D4 (35, 36), with higher scores indicating more severe disturbance in disorganized symptoms.

The above-mentioned scales used to tap into RDoC domains were assessed at baseline and last-available follow-up until 36 months. As there was a dropout rate of 50% at 36 months, if there were no data available at 36 months, we took the scores from the last follow-up that the individual attended (i.e., 6 or 12 or 24 months).

TaqMan SNP Genotyping

The study population was genotyped for *DAO* (rs3918347, rs4623951), *DAOA* (rs778293, rs3916971, rs746187), and *NRG1* (rs10503929) SNPs. These SNPs were selected based on previously reported significant association with schizophrenia (10, 43). In our recent meta-analysis, we found a significant association of *DAO* rs4623951 [odds ratio (OR) = 0.88; minor allele: C], *DAOA* rs778293 (OR = 1.17; minor allele: G), *DAOA* rs3916971 (OR = 0.84; minor allele: T), and *NRG1* rs10503929 (OR = 0.89; minor allele: C) with schizophrenia (11). In this study, the carriers of the risk allele of *DAO*, *DAOA*, and *NRG1* SNPs were anticipated to have worse psychopathology scores in RDoC-negative valence and cognitive system constructs. DNA was isolated from whole blood ethylenediaminetetraacetic acid tubes collected from the study population using QIAamp DNA Blood Maxi Kit (Qiagen) as per manufacturer's protocol. A spectrophotometer (NanoVue

Plus, GE) was used to measure DNA concentrations, A260/A280, and A260/A230 ratios. The study population was genotyped for *DAO* (rs3918347 assay number: C_27937201_10, rs4623951 assay number: C_32177440_10, both from Applied Biosystems, USA), *DAOA* (rs778293 assay number: C_8704507_10, rs3916971 assay number: C_27495752_10, rs746187 assay number: C_1925241_10, all from Applied Biosystems, USA), and *NRG1* (rs10503929, assay number: C_2870393_10, Applied Biosystems, USA) SNPs (44). DNA (10 ng/μl); TaqMan® Genotyping Master Mix (Applied Biosystems, USA); and above-mentioned *DAO*, *DAOA*, and *NRG1* SNP Genotyping Assays (Applied Biosystems, USA) were combined in a 384-well plate. Real-time polymerase chain reaction (PCR) was performed in a C1000™CFX384™ Thermal cycler (Bio-Rad) using TaqMan® SNP Genotyping Assay PCR standard protocol. The allelic discrimination program of Bio-Rad CFX Manager™ Software version 2.1 was used to determine genotypes (44). Samples were run in duplicates to ensure correct results. In case of ambiguity in duplicates, genotyping was repeated in a separate run to ensure correct results. No-template controls were included in every run to exclude impurities.

Quantification of *NRG1*, *DAO*, and *DAOA* mRNA Levels Using Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from whole blood collected from the study population using PAXgene Blood RNA Kit (Qiagen) according to manufacturer's protocol. A spectrophotometer (NanoVue Plus, GE) was used to measure RNA concentrations, A260/A280, and A260/A230 ratios. RNA integrity was analyzed using Experion automated electrophoresis system (Bio-Rad) in a subset of samples to ensure RNA integrity number/RNA quality indicator >7. RNA (500 ng) was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad) as per manufacturer's protocol. In a subset of samples, negative controls were prepared with RNA using iScript™ cDNA Synthesis Kit (Bio-Rad) without reverse transcriptase enzyme as per manufacturer's protocol. qRT-PCR was performed using cDNA, QuantiFast SYBR Green PCR kit (Qiagen), 1 μM *NRG1* primer (QT00061964, Qiagen), and reference genes [β-actin (*ACTB*) (QT01680476), aminolevulinate synthetase (*ALAS1*) (QT00073122), ribosomal protein L13a (*RPL13A*) (QT00089915), alanyl-tRNA synthetase (*AARS*) (QT00054747), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (QT01192646), peptidyl prolyl isomerase A (*PPIA*) (QT00866137), and X-prolyl aminopeptidase1 (*XPNPEP1*) (QT00051471); all from Qiagen]. *NRG1* mRNA levels were normalized to the references genes (44). PCR efficiencies were calculated using LinReg PCR program (45), and mean PCR efficiencies for all studied amplicons were found to be between 91 and 93%. Normalized *NRG1* mRNA levels were quantified using qBASE plus software (Biogazelle), which utilizes gene-specific amplification efficiencies and allows normalization with multiple reference genes (46).

We performed qRT-PCR to detect *DAO* mRNA using *DAO* primers described by Verrall et al. (47) and predesigned primers [qHsaCID0011122 and qHsaCEP0058247 (Bio-Rad), Hs.PT.58.3248433 and Hs.PT.58.45768871 (IDT)]. We performed qRT-PCR to detect *DAOA* mRNA using primers for *DAOA* gene described by Benzel et al. (48), Cheng et al. (49), and pre-designed primers [QT00058863 (Qiagen), Hs.PT.58.555086 (IDT), 4331182 (ThermoFisher scientific), qHsaCEP0024792 (Bio-Rad)]. QuantiTect Whole Transcriptome Kit (207043, Qiagen) followed by qRT-PCR was also used to detect *DAO* and *DAOA* mRNA levels (44). However, we were unable to quantify *DAO* and *DAOA* mRNA with the aforementioned methods in the whole blood as either no signal was observed or genomic DNA was amplified concomitantly.

Statistical Analysis

The results from SNP genotyping was analyzed using PLINK software (50). The minor allele frequency (MAF) and deviation from Hardy-Weinberg Equilibrium (HWE) was computed using PLINK software, and $p < 0.05$ was considered as statistically significant. The *DAO* (rs3918347), *DAOA* (rs3916971, rs778293, rs746187), and *NRG1* (rs10503929) SNPs were in HWE ($p > 0.05$), and the MAF of *DAO*, *DAOA*, and *NRG1* SNPs were similar to HapMap CEU MAF (Table S5 in Supplementary Material). The *DAO* rs4623951 SNP deviated from the HWE ($p < 0.05$; Table S4 in Supplementary Material). The differences in allele and genotype frequencies across clinical phenotypes (cases versus controls) were assessed using Chi-square test, and $p < 0.05$ was considered as statistically significant. The OR, 95% CI, and *p* value for SNP models (allelic, dominant, and recessive) across clinical phenotypes were calculated from allele/genotype frequencies using an online OR calculator,[2] and *p* value was adjusted based on the number of analyzed SNPs (Bonferroni correction, $p < 0.008$). The *post hoc* power analyses for association of *DAO*, *DAOA*, and *NRG1* SNPs with converters to schizophrenia spectrum disorders versus non-converters (Table S5 in Supplementary Material) and APSS versus all other help-seeking group were conducted using Fisher's exact test of independence in G*Power software (51), the calculated OR was used, and the alpha level was set at 0.05.

IBM® SPSS® Statistics (version 21) software was used for statistical analysis. Shapiro–Wilk test with Lilliefors significance correction was used to assess the normality of the distribution of *NRG1* gene expression and clinical scales (BAI, CDSS, SPI-A/SPI-CY, SIPS, and PANSS). *NRG1* gene expression and clinical scales showed both normal and non-normal distribution. We used non-parametric tests even for normally distributed data to maintain consistency between statistical evaluations. The differences in RDoC domains (negative valence and cognitive systems) across models (genotypic, dominant, recessive) were assessed using Mann–Whitney test (for two groups) or Kruskal–Wallis test (for >2 groups), and *p* values were adjusted based on the number of constructs analyzed (Bonferroni correction, $p < 0.008$). The differences in *NRG1* mRNA levels across clinical phenotypes were assessed using Mann–Whitney test, and $p < 0.05$ was set as statistically significant. The *post hoc* power analyses for RDoC-negative valence and cognitive systems across *DAO*, *DAOA*, and

[2]https://www.medcalc.org/calc/odds_ratio.php.

NRG1 SNPs were conducted using analysis of variance test for three groups or *t*-test for two groups in G*Power software (51), the effect sizes were determined from means of neuropsychological scales used in RDoC-negative valence and cognitive systems, and the alpha level was set at 0.05 (Table S7 in Supplementary Material). The differences in *NRG1* mRNA levels across *NRG1* rs10503929 SNP genotypes was assessed using Kruskal–Wallis test, and across dominant (CC + CT, TT) and recessive models (CC, CT + TT) were assessed using Mann–Whitney test, with $p < 0.05$ being set as statistically significant. The correlation between *NRG1* gene expression and RDoC domains (negative valence and cognitive systems) was assessed using Spearman's rank correlation test, and $p < 0.05$ was considered statistically significant.

RESULTS

The *DAO* (rs3918347), *DAOA* (rs3916971, rs778293, rs746187), and *NRG1* (rs10503929) SNPs were in HWE ($p > 0.05$), and the MAF of *DAO*, *DAOA*, and *NRG1* SNPs were similar to HapMap CEU MAF (Table S2 in Supplementary Material).

DAO, *DAOA*, and *NRG1* Polymorphisms across Clinical Phenotypes

There were no significant associations between *DAO*, *DAOA*, and *NRG1* SNPs with converters to schizophrenia spectrum disorders compared to non-converters at 36 months follow-up (power range: 0.05–0.31; Table S3 in Supplementary Material). However, there was a nominal association ($p > 0.008$) of *DAO* rs3918347 with APSS compared to all other help-seeking group at baseline, and the G-allele had a tendency to be a risk allele for APSS (OR = 1.84, 95% CI = 1.13–3.01, $p = 0.01$), with a power of 0.76 (Table S4 in Supplementary Material). There were no significant associations between the rest of the *DAO*, *DAOA*, *NRG1* SNPs and APSS (power range: 0.05–0.89; Table S4 in Supplementary Material).

DAO, *DAOA*, and *NRG1* Polymorphisms across RDoC Domains

D-amino acid oxidase activator rs746187 recessive (CC) genotype carriers experienced nominally more hopelessness (higher item 2 score, CDSS) than CT + TT genotype carriers at the last-available follow-up time point (LA) until 36 months ($p = 0.04$) analyzed by Mann–Whitney test (RDoC-negative valence system: loss; **Figures 1A–C**; power = 0.62; effect size = 0.55; Table S8 in Supplementary Material). *DAOA* rs3916971 recessive (TT) genotype carriers experienced nominally more disturbances in visual perception (higher sum of O4 + F1 + F2 + F3, SPI-A) than TC + CC genotype carriers at LA until 36 months ($p = 0.009$) analyzed by Mann–Whitney test (RDoC cognitive system: visual perception; **Figures 2A–C**; power = 0.28; effect size = 0.34; Table S8 in Supplementary Material). Individuals with *DAOA* rs3916971 TC + CC versus TT genotype improved and had fewer visual perceptual disturbances at LA until 36 months compared to baseline, but at LA until 36 months versus baseline, they continued to have less visual perceptual disturbances than individuals with TT genotype (**Figure 2C**). There were no significant differences in negative valence (threat; Table S5 in Supplementary Material; power range: 0.05–0.72, Table S8 in Supplementary Material), auditory perception disturbances (Table S6 in Supplementary Material; power range: 0.05–0.56, Table S8 in Supplementary Material), and cognitive control (Table S7 in Supplementary Material; power range: 0.05–0.31, Table S8 in Supplementary Material) in individuals with *DAOA* SNP genotypes (rs3916971, rs778293, rs746187).

D-amino acid oxidase rs3918347 GA + AA genotype carriers experienced nominally ($p = 0.04$) more disturbances in auditory perception (higher sum of O5 + F4 + F5, SPI-A) than GG

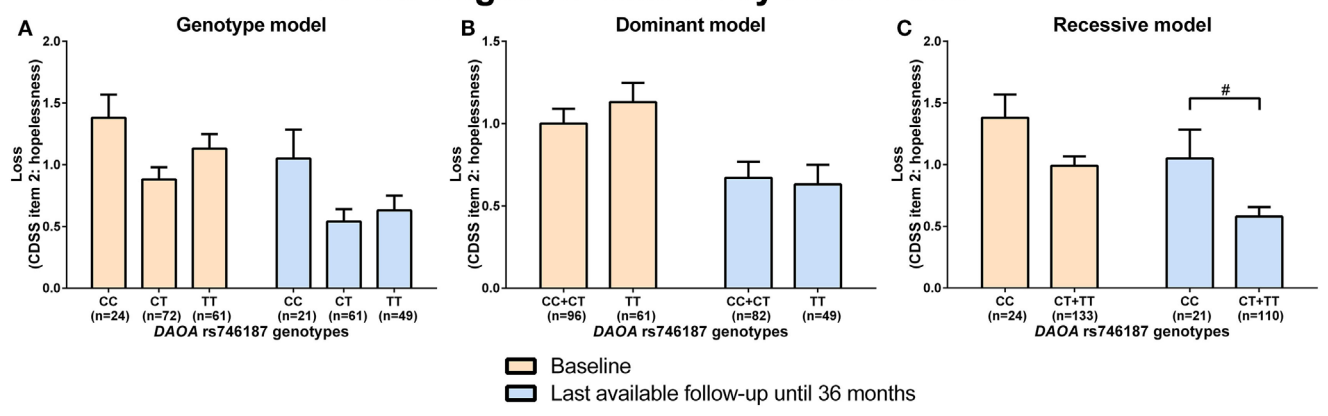

FIGURE 1 | Differences in research domain criteria (RDoC) negative valence system loss across D-amino acid oxidase activator (*DAOA*) rs746187 genotypes. Differences in Calgary Depression Scale for Schizophrenia (CDSS) item 2: hopelessness scale across *DAOA* rs746187 genotypes in genotype **(A)**, dominant **(B)**, and recessive **(C)** model at baseline and last-available follow-up time point until 36 months. Data are presented as mean ± SEM; $^{\#}0.008 < p < 0.05$ (significant without Bonferroni correction).

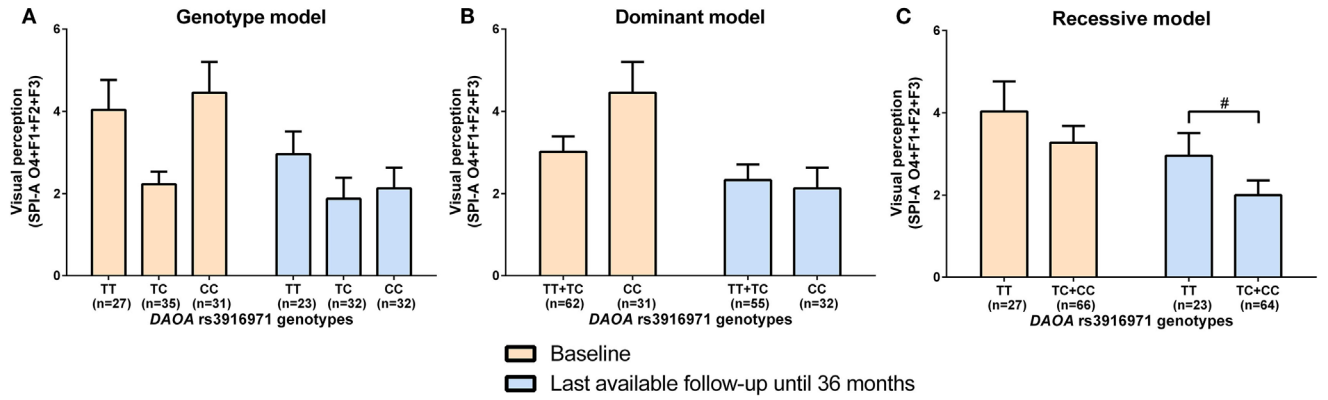

FIGURE 2 | Differences in research domain criteria (RDoC) cognitive system visual perception across D-amino acid oxidase activator (*DAOA*) rs3916971 genotypes. Differences in Schizophrenia Proneness Instrument-Adult version (SPI-A) O4 + F1 + F2 + F3 sum score across *DAOA* rs3916971 genotypes in genotype **(A)**, dominant **(B)**, and recessive **(C)** model at baseline and last-available follow-up time point until 36 months. Data are presented as mean ± SEM; #$0.008 < p < 0.05$ (significant without Bonferroni correction).

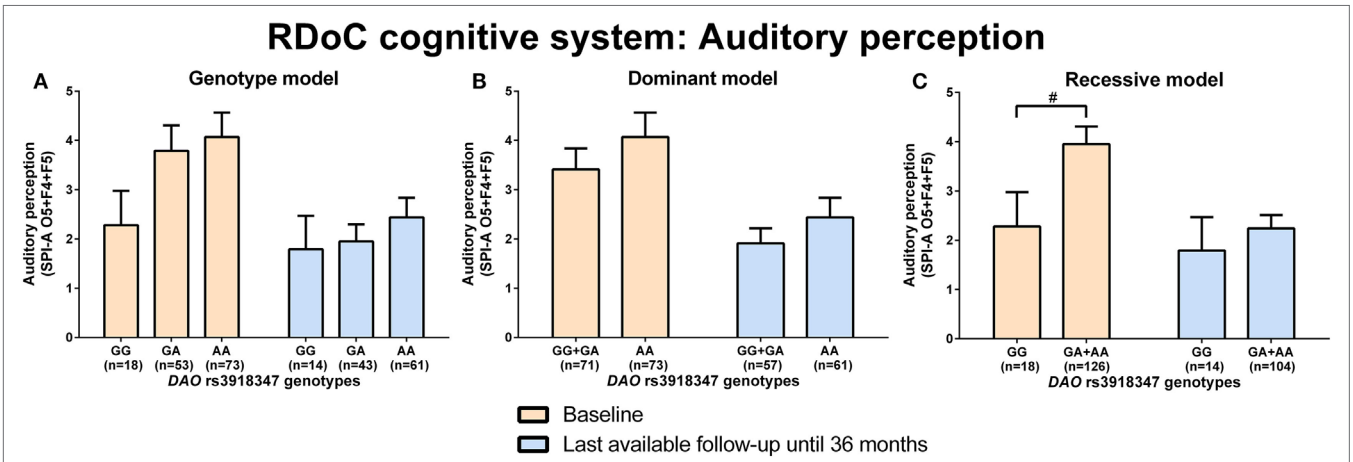

FIGURE 3 | Differences in research domain criteria (RDoC) cognitive system auditory perception across D-amino acid oxidase activator (*DAO*) rs3918347 genotypes. Differences in Schizophrenia Proneness Instrument-Adult version (SPI-A) O5 + F4 + F5 sum score across *DAO* rs3918347 genotypes in genotype **(A)**, dominant **(B)**, and recessive **(C)** model at baseline and last-available follow-up time point until 36 months. Data are presented as mean ± SEM; #$0.008 < p < 0.05$ (significant without Bonferroni correction).

genotype carriers at baseline analyzed by Mann–Whitney test (RDoC cognitive system: auditory perception; **Figures 3A–C**; power = 0.41; effect size = 0.44; Table S8 in Supplementary Material). Individuals with *DAO* rs3918347 GA + AA versus GG genotype improved and had lesser auditory perceptual disturbances at LA until 36 months compared to baseline, but at LA until 36 months versus baseline, they continued to experience more auditory perceptual disturbances than individuals with CC genotype (**Figure 3C**). We did not find significant differences in negative valence systems (anxiousness and hopelessness; Table S5 in Supplementary Material; power range: 0.05–0.92, Table S8 in Supplementary Material), visual perception disturbances (Table S6 in Supplementary Material; power range: 0.05–0.57, Table S8 in Supplementary Material), and cognitive control (Table S7 in Supplementary Material; power range: 0.05–0.22, Table S8 in Supplementary Material) in individuals with *DAO* (rs3918347, rs4623951) SNP genotypes.

Neuregulin 1 rs10503929 CC + CT genotype carriers had significantly ($p = 0.001$) more disorganized symptoms (higher sum of the D1–D4 score, SIPS) than TT genotype carriers at baseline analyzed by Mann–Whitney test (RDoC cognitive system: cognitive control; **Figures 4A–C**; power = 0.99; effect size = 0.59; Table S8 in Supplementary Material). There were no significant differences in negative valence systems (anxiousness and hopelessness; Table S5 in Supplementary Material; power range: 0.07–0.51, Table S8 in Supplementary Material) and visual and auditory

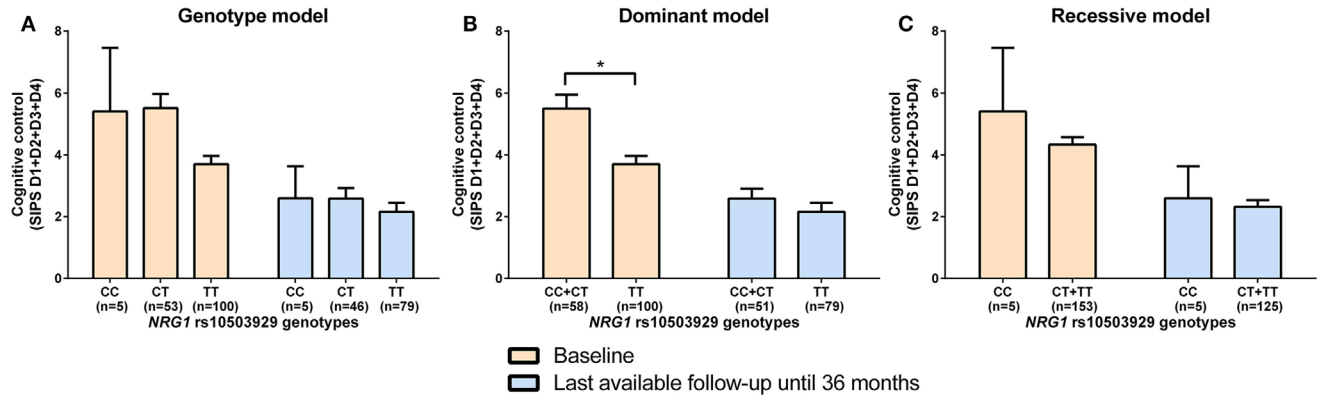

FIGURE 4 | Differences in research domain criteria (RDoC) cognitive system cognitive control across neuregulin 1 (*NRG1*) rs10503929 genotypes. Differences in Structured Interview for Prodromal Syndromes (SIPS) sum score of D1-D4 across *NRG1* rs10503929 genotypes in genotype **(A)**, dominant **(B)**, and recessive **(C)** model at baseline and last-available follow-up time point until 36 months. Data are presented as mean ± SEM; $^*p < 0.008$ (significant with Bonferroni correction).

perception disturbances (Table S6 in Supplementary Material; power range: 0.05–0.36, Table S8 in Supplementary Material) in individuals with *NRG1* (rs10503929) SNP genotypes.

To analyze the effect of age on RDoC domains across *DAO*, *DAOA*, and *NRG1* SNPs, we performed an analysis of covariance with age as a covariate after transformation of not normally distributed BAI, SPI-A, and SIPS data, as described previously (52). We did not find a significant effect of age on differences in RDoC domains across *DAO*, *DAOA*, and *NRG1* SNPs (data not shown).

NRG1, *DAO*, and *DAOA* mRNA Expression across RDoC and Clinical Domains

There was a significant positive correlation between increased *NRG1* mRNA levels and higher scores on the RDoC-negative valence system loss at LA until 36 months (Table S9 in Supplementary Material). Apart from this, there were no significant correlations between *NRG1* mRNA levels and RDoC domains at baseline and at LA until 36 months (Table S9 in Supplementary Material). There were no statistically significant differences in *NRG1* mRNA levels across clinical phenotypes (Table S10 in Supplementary Material). We did not find significant differences in *NRG1* mRNA levels across *NRG1* rs10503929 SNP genotypes, dominant, and recessive models analyzed by Mann–Whitney and Kruskal–Wallis tests (Table S11 in Supplementary Material). We were unable to quantify *DAO* and *DAOA* mRNA levels in the whole blood of at-risk population.

DISCUSSION

In this 3-year follow-up study of 185 at-risk individuals, *NRG1* rs10503929 CC + CT versus TT genotype carriers experienced significantly more disorganized symptoms, *DAOA* rs746187 CC versus CT + TT genotype, *DAOA* rs3916971 TT versus TC + CC genotype, and *DAO* rs3918347 GA + AA versus GG genotype carriers experienced nominally more hopelessness, visual perception disturbances, and auditory perception disturbances, respectively. Moreover, we found no significant association between *DAO*, *DAOA*, *NRG1* SNPs, and conversion to schizophrenia spectrum disorders; however, we did find a nominally increased risk for APSS with the G-allele of *DAO* rs3918347 carriers.

The *DAO*, *DAOA*, and *NRG1* SNPs did not predict conversion to schizophrenia spectrum disorders at 36 months follow-up. This lack of association may be due to a low conversion rate of 14.6%, a high dropout rate of 50% at 36 months follow-up, and that the comparison group of non-converters was not a healthy control group but rather a heterogeneous group of HR and UHR individuals. In this heterogeneous at-risk population, individuals are likely to be on different developmental trajectories of/toward various neuropsychiatric disorders, which might have further complicated the genetic prediction of transition to schizophrenia in our study. Furthermore, our conversion group not only contained patients with schizophrenia but also patients with other schizophrenia spectrum disorders. Thus, recruiting a more homogeneous at-risk population has been more appropriate (53, 54). In contrast to previous meta-analyses showing conversion rates of 29–32% at 36 months follow-up (4, 6), our study had a low conversion rate of 14.6%. A study of 82 UHR individuals showed that 100% of the *DAOA* rs1341402 CC genotype carriers ($n = 4$) compared to 50% of the *DAOA* rs778294 AA genotype carriers ($n = 10$; A-allele protective against schizophrenia) progressed to psychosis within 24 months (13). However, a recent study with 225 UHR individuals did not replicate these findings (14). Furthermore, another study of 67 UHR individuals showed that 100% of TT genotype carriers ($n = 25$) of *NRG1* rs62510682 developed psychosis within 12-months (12), but this finding was not replicated in the aforementioned study with 225 UHR individuals (14). About 46% of the *NRG1* rs4281084 AA genotype UHR carriers ($n = 13$) and 44% of the T-allele UHR carriers ($n = 45$) transitioned to psychosis within a 15-year follow-up

period (14). Thus, there is ambiguity regarding the association of *DAOA* and *NRG1* polymorphisms with the transition to psychosis. We did not assess the aforementioned *DAOA* and *NRG1* SNPs in this study as we only focused on the SNPs associated with schizophrenia (10, 43).

Our finding that *NRG1* rs10503929 TT genotype carriers had significantly less disorganized symptoms than the dominant (CC + CT) genotype carriers at baseline (RDoC cognitive control) is consistent with previous finding of a significant association of *NRG1* rs10503929 with cognitive domains (abstraction and mental flexibility, attention, and verbal memory) in schizophrenia patients, in which the C-allele (protective against schizophrenia) was associated with decreased cognitive performance (55). We found that *NRG1* rs10503929 (CT + TT) versus CC genotype carriers had nominally more auditory perception disturbances. To our knowledge, there are no previous studies on associations between the *NRG1* rs10503929 (exon 8/9/10) and perceptual disturbances. However, a study in adolescents demonstrated that *NRG1* rs3924999 (exon 2) was associated with perceptual disturbances (56).

The nominal associations between *DAOA* rs746187 and the RDoC-negative valence system: loss, and that between *DAOA* rs3916971 and the RDoC cognitive system: visual perception, points to the possible role of *DAOA* variations in modulating endophenotypes underlying psychosis risk. A recent study found a nominal association of *DAOA* rs3916971 with a psychotic disorder (57). Another study conducted in healthy male controls found that *DAOA* rs3916971 schizophrenia risk C-allele carriers had worse visual-spatial skills (58, 59). In our study, *DAO* rs3918347 GA + AA genotype carriers experienced nominally more auditory perception disturbances than GG genotype carriers at baseline (RDoC cognitive system). Another study found a negative association of *DAO* rs3918346 with neurocognitive functioning in schizophrenia patients (60). Therefore, the association of *DAO* and *DAOA* SNPs with hopelessness and perception disturbances of our study needs further confirmation.

We further found that *NRG1* mRNA levels increased with higher CDSS hopelessness scores. Previous postmortem studies have shown increased *NRG1* mRNA levels in the hippocampus (61) and prefrontal cortex (62, 63) of schizophrenia patients compared to that of healthy controls. Moreover, studies have shown dysfunctions in these regions might lead to hopelessness (64–66).

We examined associations of *DAO*, *DAOA*, and *NRG1* SNPs with risk profiles in individuals at risk for psychosis. For the sake of higher homogeneity, we also focused on the APSS subsample, a classification that the DSM-5 working group included under "conditions for further study" (67). We found that the schizophrenia risk G-allele of *DAO* rs3918347 had a tendency to be a risk allele for APSS compared to the remaining help-seeking group. This result has to be interpreted cautiously because the help-seeking group is not a healthy control group, but a mixed group of BLIPS, state-trait risk criterion, and HR.

We did not find any significant differences in *NRG1* mRNA levels between converters to schizophrenia spectrum disorders versus non-converters, and APSS versus all other help-seeking non-converters, which might be due to small subsample size leading to modest power (Tables S3 and S4 in Supplementary Material) and heterogeneous subgroups. Our results are in contrast to a study, which showed that *NRG1* (type I and II isoforms) mRNA expression was significantly lower in blood of UHR individuals who transitioned to psychosis ($n = 31$) compared to non-converters ($n = 66$) and controls ($n = 50$) (68). The discrepancy in the results of our study and the aforementioned study might also be due to the differences in isolation method, number of reference genes, stability of the reference genes, and normalization method used to normalize *NRG1* mRNA levels to reference genes. A study conducted on immortalized lymphocytes showed that there was no difference in *NRG1* mRNA levels between schizophrenia patients and healthy controls (69).

We were unable to detect *DAO* and *DAOA* mRNA using qRT-PCR in the whole blood of individuals at-risk for psychosis, which is in line with a study that used RNA sequencing to detect *DAO* and *DAOA* mRNA in healthy participants (70). As qRT-PCR can detect low copy number genes (71), undetectable *DAO* and *DAOA* mRNA levels might suggest either very low expression below the detection limit of qRT-PCR or extremely localized expression (44). The reasons for very low or no expression of *DAO* and *DAOA* mRNA in blood might be highly methylated (75–90%) Illumina CpG sites of *DAO* and *DAOA* genes in healthy individuals (72). Another reason for this low or no expression might be the expression of these genes specifically in the brain (47, 73) because of their role in glutamatergic neurotransmission via NMDA receptors (25).

In our study, *DAO* rs4623951 genotype data showed that the study population deviated from the HWE. Since we controlled for genotyping errors, this deviation from HWE might be due to the observed excess of CT heterozygotes (54%). This excess heterozygosity might be caused by "selection favoring heterozygotes, outbreeding, and negative assortative mating" (74). As deviation from the HWE creates bias in the associations reported (75), the association results of *DAO* rs4623951 SNP should be interpreted with caution. As this study did not have all the instruments suggested by RDoC for negative valence and cognitive systems, we used an exploratory approach, using the instruments available in this study to create the respective constructs. Thus, future studies are needed to confirm our RDoC findings.

Our study has several limitations, which must be acknowledged. Although a total of 185 individuals at risk for psychosis were recruited, sample sizes within genotypes across clinical and RDoC domains were small and the power of the study was modest. In this study, only a small percentage (14.6%) of at-risk individuals converted to schizophrenia at 36 months follow-up, 50% of individuals dropped out of the study before 36 months, and there was no healthy control group. The conversion status of the dropouts is unknown, and thus, it is not possible to reliably determine the conversion rate. The group of all other help seeking individuals was at heterogeneous risk (BLIPS, state-trait criteria, COPER, COGDIS). The sample size at 36-month follow-up for different psychopathology scales was small because of the high dropout rate. To circumvent this problem, we used the last psychopathology assessment available from each individual. Therefore, the results of LA until 36 months should be interpreted with caution, as they are not based on a homogeneous 36-month follow-up score. We studied only few genes (three

genes) and a relatively small number of their polymorphisms (six SNPs), which might be the reason for not finding markers for predicting conversion to schizophrenia spectrum disorders. However, these genes and their polymorphisms are still of interest due to their importance in glutamatergic neurotransmission. This study also has strengths, which needs to be highlighted. We recruited individuals from a broad age range (13–35 years) and used age-specific scales (e.g., SPI-A/SPI-CY). Most of the published literature in at-risk population has focused on clinical phenotypes. This study used both clinical phenotypes and RDoC domains, especially negative valence and cognitive systems, due to the role of the studied genes in the glutamate hypothesis of schizophrenia.

In summary, although *DAO*, *DAOA*, and *NRG1* SNPs did not emerge as predictive markers for conversion to schizophrenia spectrum disorders, future association studies with larger cohorts, and longer follow-ups are needed to confirm the role of these genes in transition to schizophrenia spectrum disorders in the at-risk population. We also identified an association between the studied glutamatergic variants and RDoC-negative valence and cognitive systems, which indirectly implicates the role of these genetic variants in the glutamate hypothesis of schizophrenia. Future studies using RDoC domains might help to determine specific endophenotypes within at-risk populations. This might provide clinically useful, genetically informed risk prediction for dimensional and categorical outcomes among populations who maybe at-risk for developing psychosis.

ETHICS STATEMENT

This study was approved by the Cantonal Ethics Commission of Zurich (Ref. Nr. EK: E-63/2009) and complies with the Declaration of Helsinki. Informed written consent was obtained from adult participants and legal guardians of minors, and written assent was obtained from minors.

AUTHOR CONTRIBUTIONS

WR, AT, KH, and SW designed the genetic part of the ZInEP study; AT, MG, SW, and EG designed the present genetic glutamatergic study. AT, MG, and MF collected the data and blood samples. VJ performed experiments, analyzed data, and drafted the manuscript. AT, MG, MF, KH, CUC, WR, SW, and EG reviewed the manuscript. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fpsyt.2017.00292/full#supplementary-material.

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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Disrupted-in-schizophrenia 1 (DISC1) is a key protein involved in behavioral processes and various mental disorders, including schizophrenia and major depression. A transgenic rat overexpressing non-mutant human DISC1, modeling aberrant proteostasis of the DISC1 protein, displays behavioral, biochemical and anatomical deficits consistent with aspects of mental disorders, including changes in the dorsal striatum, an anatomical region critical in the development of behavioral disorders. Herein, dorsal striatum of 10 transgenic DISC1 (tgDISC1) and 10 wild type (WT) littermate control rats was used for synaptosomal preparations and for performing liquid chromatography-tandem mass spectrometry (LC-MS)-based quantitative proteomics, using isobaric labeling (TMT10plex). Functional enrichment analysis was generated from proteins with level changes. The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling. DISC1-regulated proteins proposed in the current study are also highly associated with neurodevelopmental and mental disorders. Bioinformatics analyses from the current study predicted that the following biological processes may be activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation. Our findings demonstrate that the effects of overexpression of non-mutant DISC1 or its misassembly has profound consequences on protein networks essential for behavioral control. These results are also relevant for the interpretation of previous as well as for the design of future studies on DISC1.

Keywords: DISC1, proteomics, synapses, animal model, dopaminergic system, axon guidance, striatum

INTRODUCTION

Disrupted-in-schizophrenia 1 (DISC1) is a gene originally identified as a translocation mutation in an extended Scottish pedigree where carriers suffered from diverse mental disorders comprising schizophrenia and affective disorders (Millar et al., 2000). Similarly, the DISC1 haplotype was associated with schizophrenia in a Finnish cohort (Hennah et al., 2003). A second family was later identified with a missense mutation and associated diverse clinical phenotypes (Sachs et al., 2005), and genetic association studies have supported association of DISC1 with mental disorders (Chubb et al., 2008). A role of the DISC1 gene for adaptive behavior was also suggested by various animal studies (Brandon and Sawa, 2011; Dahoun et al., 2017).

The DISC1 protein has features of a scaffold protein (Yerabham et al., 2013) and several subdomains have an intrinsic tendency to form high molecular multimers (Yerabham et al., 2017). Insoluble DISC1 protein has been identified in human post mortem brains with mental disorders (Leliveld et al., 2008), indicating that the DISC1 protein can be subject to aberrant proteostasis *in vivo*. For modeling the effects of aberrant proteostasis *in vivo*, a transgenic rat model overexpressing (approximately 11-fold) the full length, non-mutant human DISC1 gene (transgenic DISC1, tgDISC1 rat) was generated that exhibited perinuclear aggregates throughout the brain, accentuated in dopamine-rich regions such as in the striatum (Trossbach et al., 2016). The tgDISC1 rat exhibited phenotypes such as amphetamine supersensitivity, an increase in D2Rhigh receptors, and dopamine transporter mislocalization and dysfunction consistent with phenotypes observed in schizophrenia (Trossbach et al., 2016). Also, at the neuroanatomical level fewer dopaminergic neurons and projections into the dorsal striatum, as well as aberrant interneuron positioning was observed indicating subtle neurodevelopmental disturbance (Hamburg et al., 2016).

These findings, induced by aberrant proteostasis of the DISC1 protein, leading to its misassembly and perinuclear deposition, suggest an important role of the DISC1 protein and its correct assembly for protein networks involved in adaptive behavior. Such protein networks have been described both, at the protein and the genetic level. At the genetic level, Teng et al. (2017) carried out targeted sequencing of 59 DISC1 interactome genes and 154 regulome genes in psychiatric patients, identifying altered regulation of schizophrenia candidate genes by DISC1. In an attempt to dissect DISC1 function through protein-protein interactions based upon a yeast two-hybrid system along with bioinformatic methods, a comprehensive network around DISC1 was generated (Camargo et al., 2007). Using this iterative yeast two-hybrid system, a framework was provided to explore the function of DISC1, and interrogation of the proposed interactome has shown DISC1 to have protein-protein interactions consistent with that of an essential synaptic protein (Camargo et al., 2007). Current evidence suggests that DISC1 functions as a neuronal intracellular trafficking regulator that includes transport of neurotransmitter receptors, vesicles, mitochondria and mRNA, rendering synaptic regulation vulnerable to DISC1 dysfunction (Devine et al., 2016).

The objective of this study was to identify the proteomic signatures of the tgDISC1 rat model vs. its littermate wild type (WT) control to gain insights onto the DISC1-regulated proteins and downstream synaptic processes and to identify molecular circuitry regulated by relatively modest changes in expression level leading to DISC1 misassembly. Identification of changes in protein networks relevant for behavioral processes would raise the possibility for the DISC1 protein to represent a non-genetic interface with exogenous influences for mental disorders.

There is mounting evidence for a focal role of the DISC1 protein in striatal functions, and particularly on dopamine homeostasis in relation to behavioral changes (Trossbach et al., 2016; Wang et al., 2017). Therefore we chose to select proteins from the synapse-enriched membrane fractions (synaptosomes) from the dorsal striatum for this study. Differential proteomics by isobaric labeling (TMT10plex) enable multiplexed protein identification and quantitative analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This allows the unbiased analyses of approximately 6000 proteins and targets synaptic proteins including receptors, transporters and channels that have been implicated in psychiatric disorders. Combining proteomics and bioinformatics approaches enabled a comprehensive view on the *in vivo* protein changes and the biological functions of DISC1.

MATERIALS AND METHODS

Animals

Previously described tgDISC1 Sprague-Dawley rats and WTs were used in this study (Trossbach et al., 2016). Briefly, full-length, non-mutant human DISC1 as transgene with the polymorphisms F607 and C704 were integrated into the pronuclei of Sprague Dawley rats. Ten male tgDISC1 rats and 10 male WT littermate control rats, aged 14–15 months (ZETT, Heinrich Heine University, Düsseldorf, Germany) were used. One WT rat and one tgDISC1 rat were derived from each pair of parents. The study was carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985), and the German Law on the Protection of Animals. It was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW.

Preparation of Synaptosomal Fractions

Dorsal striata from fresh brains were dissected and stored at −80°C. Synaptosomal fractions from bilateral regions were prepared for individual animals (for tgDISC1 and WT; $n = 10$ each), using a microscale discontinuous sucrose gradient modified from previous protocols (Hahn et al., 2009; Sialana et al., 2016). Collected synaptosomes from 1.25/1.0 M sucrose interface were diluted with 10 mM HEPES, divided into two and pelleted at 15,000× *g* for 30 min. Pelleted synaptosomal samples were reconstituted in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 50 mM TEAB supplemented with protease inhibitors) for LCMS analyses and SDS buffer

(1.5% SDS, 100 mM NaCl, 20 mM Tris supplemented with protease inhibitors) for WB analyses and were sonicated for 1 h. Protein amounts were estimated using the Pierce 660 protein assay or BCA protein assay (ThermoFisher Scientific).

Proteolytic Digestion and Isobaric Labeling

Fifty micrograms of samples were digested with a Trypsin-LysC enzyme mixture (1:100 w/w, Promega) using the filter-aided sample preparation (FASP), as previously described, with minor modifications (Wisniewski et al., 2009). The resulting peptide samples were purified with reversed-phase C18 and labeled with TMT 10-plex according to the instructions supplied by the manufacturer. Two TMT-10plex experiments were performed, with each experiment consisting of five tgDISC1 and five WT animals (n = 10 biological replicates per group). For each TMT experiment, ten isobarically labeled peptide samples were pooled, the peptides separated by high pH reversed-phase LC into 100 time-based fractions and pooled into 25 samples (Gilar et al., 2005). The peptides were vacuum concentrated and reconstituted in 5% formic acid. Details of the procedure are essentially as described previously (Sialana et al., 2016) and in the Supplementary Figure S1.

Liquid Chromatography and Tandem Mass Spectrometry

Samples were injected onto a Dionex Ultimate 3000 system (ThermoFisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Schwerte, Germany). Software versions used for the data acquisition and operation of the Q-Exactive were Tune 2.8.1.2806 and Xcalibur 4. HPLC solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostated autosampler, 10 μL that correspond to 1 μg of the peptide mixture were automatically loaded onto a trap column (PM100-C18 3 μm, 75 μm × 20 mm, ThermoFisher Scientific, Austria) with a binary pump at a flow rate of 5 μL/min using 2% acetonitrile in 0.1% TFA for loading and washing the pre-column. After washing, the peptides were eluted by forward-flushing onto a 50 cm analytical column with an inner diameter of 75 μm packed with 2 μm-C18 reversed phase material (PepMap-C18 2 μm, 75 μm × 500 mm, ThermoFisher Scientific, Austria). For label free quantification (LFQ), the LCMS analyses was performed using a single-shot LCMS approach with 4-h gradient with LCMS parameters as described previously (Stojanovic et al., 2017).

The fractionated TMT10plex labeled peptides were eluted from the analytical column with a 120 min gradient ranging from 5% to 37.5% solvent B, followed by a 10 min gradient from 37.5% to 50% solvent B and finally, to 90% solvent B for 5 min before re-equilibration to 5% solvent B at a constant flow rate of 300 nL/min. The LTQ Velos ESI positive ion calibration solution (Pierce, IL, USA) was used to externally calibrate the instrument prior to sample analysis and an internal calibration was performed on the polysiloxane ion signal at m/z 445.120024 from ambient air. MS^1 scans were performed from m/z 375–1400 at a resolution of 70,000. Using a data-dependent acquisition mode, the 15 most intense precursor ions of all precursor ions with +2 to +7 charge were isolated within a 1.2 m/z window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher-energy collisional dissociation (HCD) cell at 32% normalized collision energy with a fixed first mass at 100 m/z and detected in an Orbitrap mass analyzer at a resolution of 35,000. The dynamic exclusion for the selected ions was 30 s. Maximal ion accumulation time allowed in MS and MS^2 mode was 50 and 100 ms, respectively. Automatic gain control was used to prevent overfilling of the ion trap and was set to 3×10^6 ions and 1×10^5 ions for a full Fourier transform MS and MS^2 scan, respectively.

Protein Identification and Quantification

All MS-MS^2 spectra were searched against UniProtKB/Swiss-Prot rat protein database version v 2016.04.14 (27,815 sequences, including isoforms). In addition, sequences of the human DISC1 protein and 11 isoforms produced by alternative splicing with the polymorphisms F607 and C704 were appended to the rat database. All spectra files were processed in Proteome Discoverer 2.1 (Thermo Scientific, Germany) platform with Mascot using mass tolerances of ±10 ppm and ±0.02 Da for precursor and fragment ions. One missed tryptic cleavage site was allowed. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues, TMT 10-plex labeling of peptide N-termini and lysine residues were set as fixed modification. Thresholds were determined via the target-decoy approach using a reversed protein database as the decoy by imposing 1% false discovery rate (FDR). Label-free quantitation was implemented using the Minora feature of Proteome Discoverer 2.2. The following parameters are used: maximum retention time alignment of 10 min with minimum of S/N of 5 for feature linking mapping. Abundance were based precursor/peptide area intensities. Normalization was performed such that the total sum of the abundance is the same for all sample channels. Imputation was performed by replacing the missing values with random values from the lower 5% of the detected values. For TMT 10-plex labeled samples, relative abundances of proteins were determined from the TMT reporter ions without imputation. Protein abundance ratios were calculated based on unique and razor peptides. Relative protein levels were determined from the sum of the reporter ion intensities per quantitative channel that correspond to each biological animal replicate.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset identifier PXD008123.

Bioinformatics

Quantitative data were analyzed using Perseus statistical package (version 1.5.1.6; Tyanova et al., 2016). Statistical significance of differences in protein levels between the groups were evaluated using a two-sided T-test with $P < 0.05$ (either Student's or Welch's as required). Enrichment of GO annotations were

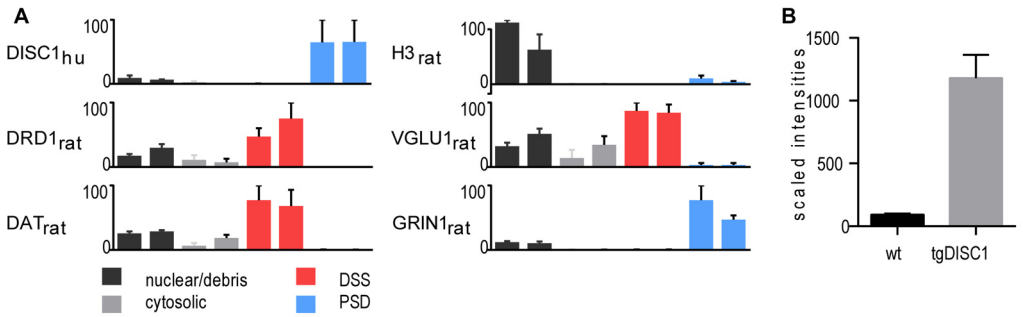

FIGURE 1 | Proteomic profile of the transgenic DISC1 (tgDISC1) fractions. **(A)** Protein levels of representative proteins for the following biochemical fractions of the dorsal striatum: nuclear/debris, cytosolic, detergent-soluble synaptosomal preparation (DSS), postsynaptic density (PSD) preparation. Protein levels of representative synaptic markers were estimated from label-free LCMS analyses. Protein levels of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers are enriched in nuclear/debris, DSS and PSD preparations, respectively. The majority of the human Disrupted-in-schizophrenia 1 (DISC1) protein was enriched in the PSD preparations. The level of overexpression is approximately 10-fold higher than the endogenous DISC1 protein in the whole synaptosomes **(B)**.

performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Bindea et al., 2009; Huntley et al., 2015). To reduce redundancy of GO terms the fusion option was selected. Enriched GO terms (Benjamini–Hochberg P-value < 0.05) are functionally grouped into networks linked by their kappa score level ($\geq$0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled. Pathway analyses on the significant proteins were performed through the use of IPA (Ingenuity® Systems[1]). The differentially expressed genes were categorized to related canonical pathways. Only those experimentally observed or highly predicted molecules and/or relationships from tissues and cells from the nervous system were considered. The top enriched categories of canonical pathways with a P-value $< 10^{-3}$ as well as representative differentially expressed proteins in each canonical pathway is reported. Curated gene-disease annotations were obtained from Comparative Toxigenomics database (Davis et al., 2015). The IPA regulation z-score algorithm was used to predict biological functions that are expected to be activated (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset.

Immunoblotting

The following antibodies were used according to the instructions supplied by the manufacturer: mouse anti-PSD95 (124011, Synaptic Systems), mouse anti-SYP (sc-55507, Santa Cruz Biotechnology), rabbit anti-NMDAR1 (ab32915, Abcam), mouse anti-VGLUT1 (135311, Synaptic Systems), rabbit anti-GAPDH (ab9485, Abcam), rabbit anti-DAT1 (ab111468, Abcam) and mouse anti-huDISC1 (3D4, Korth lab; Ottis et al., 2011). Immunoblot data were normalized to corresponding whole-lane densitometric volumes of protein-stained membranes (Welinder and Ekblad, 2011). Immunoblotting conditions were as previously described (Sialana et al., 2016) and antibody dilutions are provided in the Supplementary Table S1.

[1]www.ingenuity.com

RESULTS

In the current study, a high-throughput proteomic approach was employed to generate a comprehensive view of the *in vivo* protein changes in striatal synaptosomes of the tgDISC1 rat model (experimental workflow, Supplementary Figure S1). Methodologically, tissue fractionation was initially performed on the dorsal striatum of tgDISC1 rats to determine the subcellular expression of tgDISC1 and which enrichment steps would be employed in this study (Phillips et al., 2001; Sialana et al., 2016). Dorsal striata of tgDISC1 rats were fractionated into nuclear/debris, cytosolic, detergent soluble synaptosome (DSS) and postsynaptic density (PSD) preparations. LCMS-based proteomic analyses of the biochemical fractions resulted in the identification and LFQ of 5002 protein groups (Supplementary Data 1). Distribution of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers enriched in nuclear/debris, DSS and PSD preparations is given in **Figure 1A**. Although DISC1 was observed in all preparations, the majority of the human DISC1 protein was enriched in the Triton-X100-resistant PSD fractions. This is in agreement with previous immunoblotting studies of DISC1 in adult rats (Hayashi-Takagi et al., 2010). We have previously shown that dopaminergic pathways are modulated in the striatum of the tgDISC1 rat (Trossbach et al., 2016). Taking into account that dopamine receptor 1 and the dopamine transporter were highly enriched in the DSS preparations (**Figure 1A**), it was decided to study the *whole synaptosome* for quantitative proteomics experiments. Immunoblots of postsynaptic (GRIN1 and PSD95) and presynaptic (VGLU1 and SYP) proteins show enrichment of synaptosomal proteins on the biochemical fraction (Supplementary Figure S2). The level of overexpression is approximately 10-fold higher than

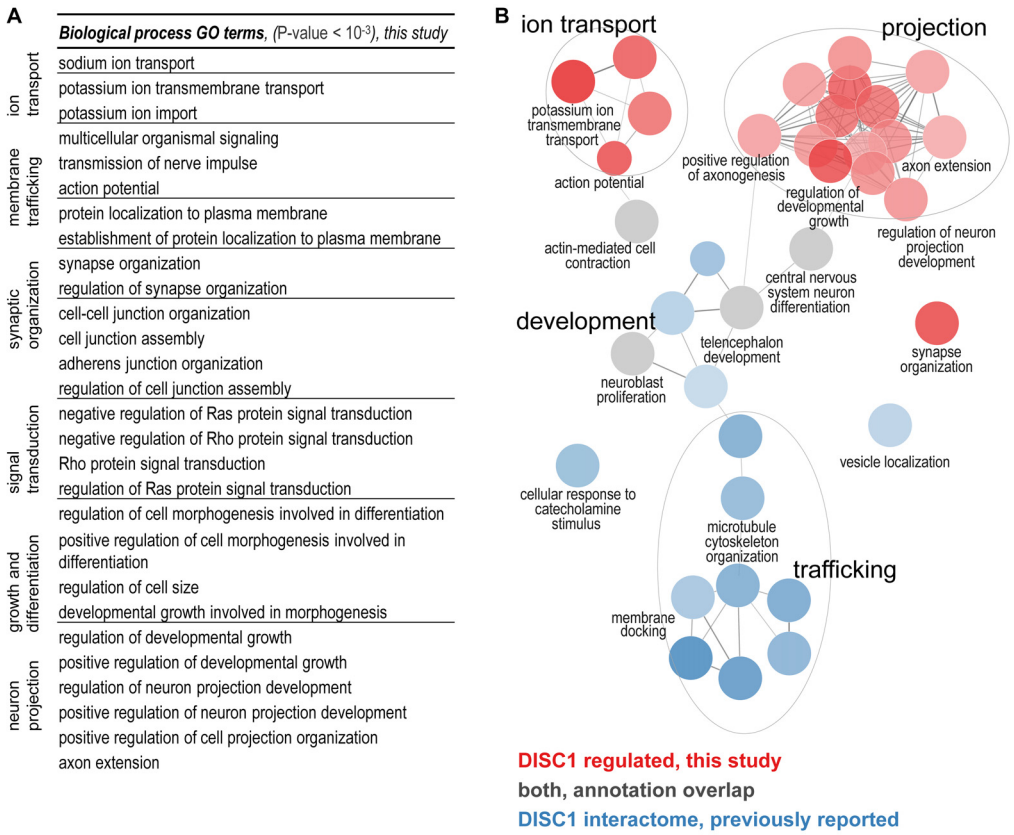

FIGURE 2 | (A) Biological processes regulated by DISC1. Enriched GO terms (*P*-value < 10^{-3}) are functionally grouped. The synaptic components terms as well as membrane trafficking, ion transport, synaptic organization and neurodevelopment processes are well represented. **(B)** Comparison of the DISC1-regulated proteins and the previously reported interacting proteins. In the current study ion transport, projections and synaptic organization were novel findings. Developmental processes from previous studies were confirmed (gray GO enrichment analyses was performed using ClueGO. Enriched GO terms (Benjamini–Hochberg *P*-value < 10^{-3}) are functionally grouped into networks linked by their kappa score level (≥0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled.

the endogenous DISC1 protein in the whole synaptosomes (**Figure 1B**).

DISC1 Regulated Proteins—Proteomic Profiling of Striatal Synaptosomes

An expression proteomics experiment was performed to identify the proteins potentially regulated by DISC1. Synaptosomal fractions of bilateral dorsal striata of 10 wt and 10 tgDISC1 rats using TMT10plex were analyzed in two separate 10-plex experiments (5 tgDISC1 and 5 WT). In total, 7227 protein groups were identified (Supplementary Data 2) including 252 receptors and 672 transporters/channels. Out of the 6153 quantifiable protein groups, 213 proteins were statistically different between the tgDISC1 and WT rats (Supplementary Table S2, Supplementary Data 3). Protein levels were considered statistically different between groups when $P \leq 0.05$ using a two-sided *T*-test (either Student's or Welch's as required). Given the large number of comparisons made and the possibility of Type 1 error, the *p* values given cannot be interpreted in terms of "significance", but rather as "measures of effect".

As we used a good number of biological replicates for TMT-based proteomics (10 animals per group), we opted to use *T*-test that performs "individual proteins-based" hypotheses test (*T*-test) rather than a background "all-proteins-based" hypothesis test (FDR). TMT-based proteomics experiments are sensitive and precise but quantification is known to undergo ratio compression (Ow et al., 2011). The values from FDR corrections depend on effect size; smaller differences yield higher P-corrected (q-values); thus only two proteins passed the corrected thresholds. An additional filter is applied when enrichment analyses (GO annotation, IPA) is employed. Slight differences in the levels of multiple proteins should cluster relevant processes and the proteins from the top enriched processes/pathways are of higher emphasis (Pascovici et al., 2016).

Immunoblotting analyses of DAT1, GRIN1 and DISC1 of WT and tgDISC1 indicated that the direction of fold differences measured by TMT-proteomics and western blotting (Supplementary Figure S3) was consistent.

A

Canonical Pathways	P-value	Proteins
Signaling by Rho Family GTPases	4.57E-06	MYL6, ARHGEF4, RAF1, ROCK1, GNAZ, FGFR3, DES, PIK3CA, ARPC1B, CDH20, GNAS, ITGA3
Axonal Guidance Signaling	1.07E-04	MYL6, LINGO1, RAF1, ABLIM2, EPHA6, ROBO2, ROCK1, GNAZ, FGFR3, SEMA7A, PIK3CA, ARPC1B, GNAS, ITGA3
RhoGDI Signaling	2.57E-04	MYL6, ARHGEF4, ROCK1, GNAZ, ARPC1B, CDH20, GNAS, ITGA3
Ephrin Receptor Signaling	2.69E-04	RAF1, EPHA6, ROCK1, GNAZ, ARPC1B, GNAS, ITGA3, GRIN1
Actin Cytoskeleton Signaling	3.47E-04	MYL6, ARHGEF4, RAF1, ROCK1, CSK, FGFR3, PIK3CA, ARPC1B, ITGA3
Gαq Signaling	8.71E-04	RAF1, ADRA1A, ROCK1, CSK, FGFR3, PIK3CA, GNAS
Dopamine-DARPP32 Feedback in cAMP Signaling	9.33E-04	CACNA1A, ADCY3, KCNJ4, KCNJ11, GNAS, KCNJ12, GRIN1

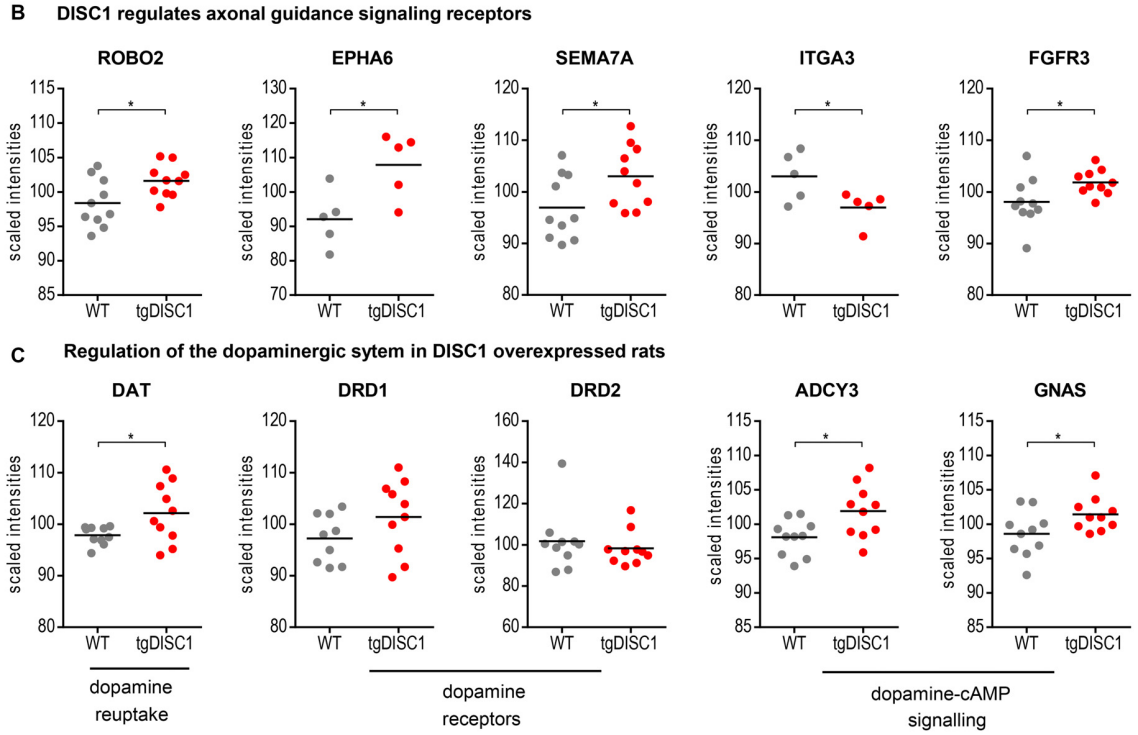

FIGURE 3 | Pathways regulated by DISC1. Significantly enriched canonical pathways (Fishers' exact test, $P < 10^{-3}$, IPA) of the proteins altered in by tgDISC1 rats in the dorsal striatum **(A)**. Representative proteins from the dopaminergic **(B)** and axonal guidance signaling pathway **(C)** are shown. Values represent $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared using two-sided *T*-tests.

Functional Classification of Proteins Modulated in tgDISC Rats

The biological functions of the 213 proteins with highly different protein level changes between wildtype and tgDISC1 rats were explored using GO enrichment analyses. Enrichment of synaptic components such as axons, dendritic spines, membrane rafts, neuron projection membrane, and the ion channel complex were revealed (Supplementary Table S3, Supplementary Figure S4). The voltage gated ion channels were the major protein classes represented (Supplementary Table S4). The results suggest that the modest overexpression of the full-length human DISC1 alters proteins linked to synaptic processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment (**Figure 2A**).

Functional Comparison of the DISC1 Regulated Proteins to Known Interacting Proteins

To determine the biological functions unique to DISC1 regulated proteins, we performed enrichment analyses for the

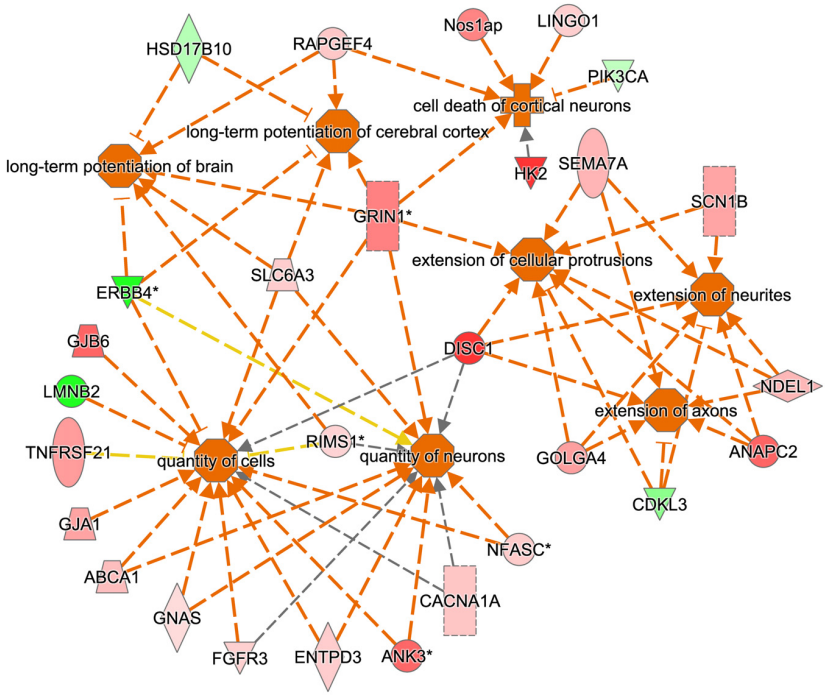

FIGURE 4 | Predicted biological functions of the tgDISC1 regulated proteins as evaluated by IPA. The IPA regulation *z*-score algorithm was used to predict the activation of biological functions in tgDISC1 rats relative to wild type (WT) according to our proteomics data. The network displays functional interactions between proteins (z-score ≥ 2 and p-value ≤ 0.05). Dashed lines indicate direct or indirect interactions. Proteins up-regulated in tgDISC1 rats are colored in shades of red; proteins down/regulated are colored in green.

DISC1 regulated proteins in comparison to previously reported interacting proteins (Camargo et al., 2007; Boxall et al., 2011; Bradshaw and Porteous, 2012; Thomson et al., 2013) as compiled by a recent study (Teng et al., 2017). Using ClueGO, 36 biological processes with strong enrichment ($P < 10^{-6}$) were revealed (**Figure 2B**; Supplementary Figure S5). The clusters of biological processes exclusive to the proteins regulated by DISC1 include: "regulation of neuron projection development", "positive regulation of axonogenesis", "action potential/potassium ion transport and synapse organization". Terms associated with microtubule development and neuronal transport were highly represented in the DISC1-interacting proteins. Biological processes such as "CNS differentiation" and "telencephalon development" were enriched in both, DISC1 regulated and interacting protein data sets.

Prediction of Canonical Pathways and Biological Function

To investigate the molecular mechanisms modulated by DISC1, data were analyzed through the use of Ingenuity Pathway analysis (IPA; Ingenuity® Systems[2]). The differentially expressed proteins were categorized to related canonical pathways. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling (Fisher's exact test, $P < 10^{-3}$, **Figure 3A**, Supplementary Figure S6). Only robustly predicted or experimentally observed molecules and/or relationships from tissues and cells from the nervous system were considered. Receptors from the axonal guidance signaling and the dopamine-DARPP32 feedback from the cAMP signaling canonical pathway are illustrated in **Figures 3B,C**).

The IPA regulation *z*-score algorithm was used to predict biological functions that are expected to be activated in tgDISC1 rats rather than in wildtype (positive *z*-score) according to own proteomics data (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset. From the expression data of the regulated proteins, the following processes are predicted to be activated: "activation regulation of cell quantities", "neuronal and axonal extension", "long term potentiation" and "apoptosis" (**Figure 4**, Supplementary Table S5).

Annotation of the DISC1 altered protein levels revealed that 54 proteins are associated with mental disorders and/or nervous system diseases as implemented by the Comparative Toxicogenomics Database (CTD; Davis et al., 2015). Disease-gene associations were based on genomic, transcriptomic and proteomic studies on the sequence variation

[2]www.ingenuity.com

and expression changes associated with brain diseases and disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) include: neurodevelopmental disorders, autistic disorders, schizophrenia spectrum, anxiety disorders, substance-related disorders (e.g., cocaine) and intellectual disability (**Table 1**). In particular, the schizophrenia-associated proteins including dopamine transporter 1 (SLC6A3), receptor tyrosine-protein kinase erbB-4 (ERBB4), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), membrane associated guanylate kinase WW and PDZ domain containing 2 (MAGI2) and regulator of G-protein signaling 12 (RGS12) were also regulated by DISC1 (Mateos et al., 2006; Silberberg et al., 2006; Xu et al., 2011; Koide et al., 2012; Guipponi et al., 2014; Jaros et al., 2015; Zhang et al., 2015; Li et al., 2017).

DISCUSSION

By the use of quantitative proteomics of synapse-enriched membrane (synaptosome) fractions of the dorsal striatum of the tgDISC1 rat, we have identified novel protein networks and signaling pathways regulated by an increase of non-mutant DISC1 expression or DISC1 misassembly. These results suggest that the DISC1 protein and its disturbed proteostasis can have an effect on mental disorder-relevant protein networks independent of genetic mutations. Likely, multiple exogenous or endogenous factors other than overexpression could lead to a failure of DISC1 proteostasis, such as exposure to high dosages of dopamine or other oxidants, making DISC1 protein an oxidation "sensor" (Atkin et al., 2012; Trossbach et al., 2016).

In the tgDISC1 rat, an about 11-fold overexpression, leading to DISC1 misassembly, changed proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment is observed. Furthermore, dysregulation of DISC1 potentially modulates pathways including actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling associated with the synaptic pathologies. DISC1-regulated proteins are also highly associated with neurodevelopmental disorders, autistic disorder, schizophrenia spectrum, anxiety disorders, substance-related disorders and intellectual disability (**Figure 5**).

Previously known DISC1-protein interactors have been reported to modulate synaptic processes. The current study revealed that DISC1 regulates an array of synaptic proteins and processes that complements previous protein interaction results (Supplementary Figure S7). Proteins that were previously reported to interact with DISC1 (Millar et al., 2003; Camargo et al., 2007) were also modified in the current study in the tgDISC1 rat. These include microtubule proteins pericentrin (PCNT), GRIP1 associated protein 1 (GRIPAP1), microtubule associated protein 1A (MAP1A), nudE neurodevelopment protein 1 (NDEL1) and microtubule-actin crosslinking factor 1 (MACF1) that are involved in neuronal cytoskeleton organization and membrane transport processes.

Dysregulation of DISC1 was reported to modulate glutamatergic and dopaminergic systems as previously reviewed (Hayashi-Takagi et al., 2010; Ramsey et al., 2011; Dahoun et al., 2017). Own results herein show that NMDAR1 is increased in the striatum of the tgDISC1 rat. A relationship between NMDAR1 and DISC1 has been shown, as knockdown and antagonists of NMDAR1 reduced numbers of synapses and synaptic DISC1 mainly in the striatum (Ramsey et al., 2011). Further, the DISC1 interactor GRIPAP1 is increased in the tgDISC1 rat. GRIPAP1 controls the AMPA receptors/GRIP-complex transport to the synapse by NMDA receptor activation (Ye et al., 2000).

As shown by MS, dopamine transporter levels were highly increased in the tgDISC1 rats, consistent with own previous studies by immunoblotting (Trossbach et al., 2016). Whereas levels of dopamine receptors 1 and 2 were not significantly altered, pathway enrichment analyses (**Figure 3C**) suggest that proteins (e.g., ADCY3, GNAS) from the dopamine-DARPP32 feedback of the cAMP signaling canonical pathway, may be involved in modulation of the known dopaminergic deficits in tgDISC1. Adenylate cyclase ADCY3 as a downstream effector of dopaminergic pathways catalyzes the formation of cAMP in response to G-protein signaling.

TABLE 1 | Disease-protein association of the DISC1 regulated proteins.

Disease name	*P*-value	Proteins
Neurodevelopmental disorders	1.02E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Mental disorders	5.11E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GC, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, KLHL5, LINGO2, MAGI2, RGS12, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Autistic disorder	3.40E-05	ASIC2, CADM1, DISC1, GJA1, KCNMA1, RIMS1, ROBO2, TCN2
Schizophrenia spectrum and other psychotic disorders	4.10E-04	DISC1, GC, GRIN1, MAGI2, RGS12, SLC6A3
Anxiety disorders	2.53E-02	MAGI2, SLC6A3
Cocaine-related disorders	1.74E-02	GRIN1, KLHL5, SLC6A3
Intellectual disability	2.52E-02	ANK3, DISC1, GNAS, GRIN1, KCNA2, SLC4A4
Psychotic disorders	1.13E-02	GRIN1, SLC6A3
Schizophrenia	1.62E-03	DISC1, GC, MAGI2, RGS12, SLC6A3
Substance-related disorders	3.98E-02	GNAS, GRIN1, KLHL5, LINGO2, SLC6A3

Gene-disease associations on the DISC1 regulated proteins were implemented in the Comparative Toxicogenomics Database, CTD. Fifty-four DISC1-regulated proteins are associated with mental disorders and/or nervous system disease disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) are illustrated.

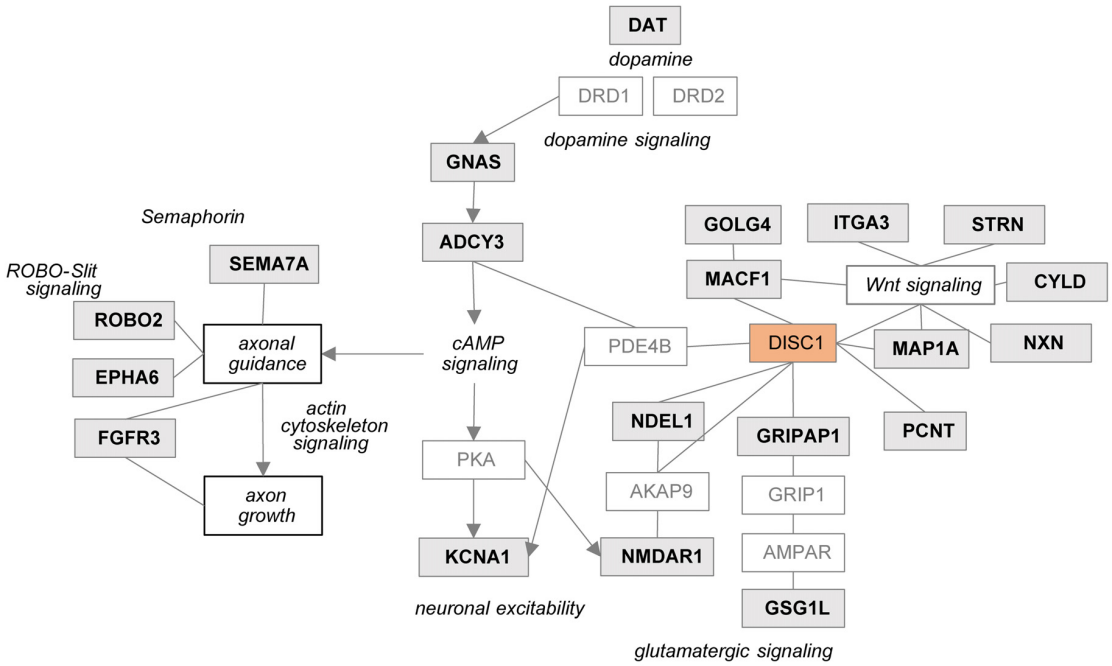

FIGURE 5 | Potential relationship between the DISC1 regulated proteins and synaptic processes. Proteins regulated in tgDISC1 rats from this study are marked in gray boxes.

The protein level changes of this enzyme along with the corresponding G-protein GNAS observed herein supports previous studies proposing dysregulation of cAMP signaling by DISC1 (Millar et al., 2005; Kvajo et al., 2011; Crabtree et al., 2017).

In a mouse Disc1 mutant model, functional reduction of Kv1.1/KCNA1 was proposed to contribute to alterations in neuronal excitability and short-term plasticity. Reduction of this channel was accompanied by reduced phosphodiesterase 4 activity and elevated cAMP levels in the PFC of *Disc1* mutant mice (Crabtree et al., 2017). Interestingly, in our DISC1 overexpressing transgenic model, we found an increase of this and several proteins in the voltage-gated potassium channel complex suggesting potential dyregulation of electrophysiological synaptic functions (Supplementary Figure S8).

Current data also revealed that proteins associated with axonal guidance pathways were altered by DISC1 overexpression: the axonal guidance receptors semaphorin 7A (SEMA7A), EPH receptor A6 (EPHA6), roundabout receptor 2 (ROBO2), fibroblast growth factor receptor 3 (FGFR3) and integrin subunit alpha 3/very late activation protein 3 receptor, alpha-3 subunit (ITGA3) were shown to be modulated by DISC1 (**Figure 3B**). The leading edge of the axons contains receptors that sense guidance cues and aid in the navigation and migration of axons. The attraction or repulsion of cues promotes or decreases active actin polymerization, resulting in axonal extension or retraction by triggering the actin cytoskeleton signaling and Rho-GTPase pathways, as also proposed in the current pathway enrichment analysis (reviewed in Dent et al., 2011; Spillane and Gallo, 2014; Van Battum et al., 2015). The receptor SEMA7A stimulates axonal growth through integrins and MAPK signaling (Pasterkamp et al., 2003). The roundabout receptor 2, ROBO2 is the main receptor from the Slit-Robo pathway, that is involved in axon guidance and which is also associated with DISC1-interacting proteins SRGAP2 and 3 (Camargo et al., 2007). The Ephrin receptor signaling pathway, predicted to be regulated by DISC1, is critical for embryonic development and known as a mediator of axon guidance (Kvajo et al., 2011).

In perspective, alterations of these developmental pathways and processes could explain the subtle neurodevelopmental phenotypes in the tgDISC1, where the substantia nigra (SN) contains fewer dopaminergic neurons (DA), fewer projections into dorsal striatum, and a shift in the parvalbumin-positive interneurons (Hamburg et al., 2016). DA homeostasis deficiency and the proposed disturbed dopaminergic signaling could explain the observed decrease of DA neurons in the SN. The disturbed axonal guidance signaling could lead to the reduction of the projections into the dorsal striatum and the shift of the parvalbumin-positive interneurons. As protein profiles were obtained from adult tgDISC1 rats, it would be interesting to follow up by studying the profiles in the developing brain to reveal the etiopathology effects of DISC1 which exceeds the scope of this study.

Bioinformatics analyses from the current study predicted that the following biological processes were activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation

(**Figure 4**). These results may be relevant for interpretation of previous as well as for the design of future studies on DISC1.

CONCLUSION

Our results suggest that overexpression and/or aberrant DISC1 proteostasis can lead to profound changes in protein networks relevant for mental disorders or endophenotypes and may signify a role for the DISC1 protein alone—in the absence of mutations—in behavioral and neural processes and disorders. DISC1 expression levels likely have to be controlled in a narrow expression window in order to execute adaptive behavior. These findings make the DISC1 protein and its posttranslational modifications a molecular convergence point or sensor for environmental interactions such as oxidative stress. The findings also strongly support the earlier literature indicating involvement of the dopaminergic systems, particularly in the dorsal striatum in functional properties of the DISC1 protein.

AUTHOR CONTRIBUTIONS

FJS, SVT, CK, JPH, MASS and GL conceived and designed the experiments. FJS, A-LW, BF and MK performed experiments. FJS and RS collected data and processed them. FJS, CK, JPH, MASS and GL interpreted the results. FJS, CK, JPH, MASS and GL wrote the article. CK, JPH, MASS and GL revised the intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00026/full#supplementary-material

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REVIEW
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The Emerging Roles for Telomerase in the Central Nervous System

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Telomerase, a specialized ribonucleoprotein enzyme complex, maintains telomere length at the 3′ end of chromosomes, and functions importantly in stem cells, cancer and aging. Telomerase exists in neural stem cells (NSCs) and neural progenitor cells (NPCs), at a high level in the developing and adult brains of humans and rodents. Increasing studies have demonstrated that telomerase in NSCs/NPCs plays important roles in cell proliferation, neuronal differentiation, neuronal survival and neuritogenesis. In addition, recent works have shown that telomerase reverse transcriptase (TERT) can protect newborn neurons from apoptosis and excitotoxicity. However, to date, the link between telomerase and diseases in the central nervous system (CNS) is not well reviewed. Here, we analyze the evidence and summarize the important roles of telomerase in the CNS. Understanding the roles of telomerase in the nervous system is not only important to gain further insight into the process of the neural cell life cycle but would also provide novel therapeutic applications in CNS diseases such as neurodegenerative condition, mood disorders, aging and other ailments.

Keywords: telomerase, central nervous system, proliferation, differentiation, apoptosis

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INTRODUCTION

Telomeres are simple repeat sequences at the physical 3′ end of chromosomes (TTAGGG for human and mouse; Greider, 1996). Since 1989 when it was discovered that the maintenance of telomeric length was mediated by telomerase (Blackburn et al., 1989; Greider and Blackburn, 1989), a large number of studies have focused on the function of telomerase in stem cells. The structure of telomerase was revealed as a specialized ribonucleoprotein complex, consisting of a protein component telomerase reverse transcriptase (TERT) that serves as catalytic subunit (Counter et al., 1997; Harrington et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997; Greenberg et al., 1998), and an essential telomerase RNA component (also known as TERC or RT). The TERC serves as a template for the elongation of a telomere catalyzed by TERT (Blackburn, 2001).

Neural stem cells (NSCs), like other types of stem cells, are self-renewing and multipotent (Gage, 2000). In the adult rodent brain, NSCs are mainly present in two specific regions: the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. In the SGZ, new dentate granule cells are produced from NSCs daily. In the SVZ, new neurons are also born every day and migrate through the rostral migratory stream (RMS) to the olfactory bulb to become interneurons (Gage, 2000; Goh et al., 2003; Alvarez-Buylla and Lim, 2004; Ma et al., 2005; Zhao et al., 2008; Ming and Song, 2011). TERT is present at high levels in NSCs and NPCs in the developing brain (Klapper et al., 2001; Cai et al., 2002) and adult (Caporaso et al., 2003), but then declines rapidly when stem/progenitor cells differentiate or die. Moreover, telomerase has

an influence on several steps of the cell life cycle including proliferation, differentiation, survival, development and apoptosis (Mattson et al., 2008; Ferrón et al., 2009). Studies also show that telomerase has been closely associated with NSCs-/NPCs-related diseases in the central nervous system (CNS) including brain tumorigenesis (Kheirollahi et al., 2013), ischemia (Zhao et al., 2010; Li et al., 2011), neurodegenerative illness (Franco et al., 2006), affective disorders (Lee et al., 2010; Zhou et al., 2011) and schizophrenia (Kao et al., 2008; Porton et al., 2008). Here we will review published literature focusing on the relationship between telomerase, NSCs and other nerve cells in the CNS, as well as cellular proliferation, differentiation, survival, development, apoptosis and their applications and emerging relevance to CNS diseases.

STRUCTURE AND FUNCTION

The telomerase is composed of TERT and TERC in all species (Feng et al., 1995; Harrington et al., 1997). TERC acts as a template for the addition of TTAGGG at the end of telomeric DNA in humans (Feng et al., 1995; Harrington et al., 1997). Telomerase expression and activity is repressed in normal human somatic cells in adulthood (Meyerson et al., 1997; Masutomi et al., 2003). In contrast, it actives only in a small subset of adult cells, including stem cells and progenitor cells in renewal tissues, germline cells, mesenchymal stem cells and activated lymphocytes (Broccoli et al., 1995; Chiu et al., 1996; Wright et al., 1996; Martens et al., 2002; Serakinci et al., 2008). Accumulating evidence indicates that telomere shortening represents a marker and a mechanism of biological aging (Aubert and Lansdorp, 2008). Telomerase is implicated in this process as it contributes to DNA damage accumulation in ASCs (Epel, 2009; Wang et al., 2009; Sahin and Depinho, 2010). Moreover, mutational analysis and knockdown experiments showed that telomerase deficiency led to telomere loss and uncapping, causing progressive atrophy of renewal tissues, gradual depletion of stem cells and eventual failure of organ systems (Jaskelioff et al., 2011; Bär and Blasco, 2016). Above all, telomerase may play a critical role in cellular and organismal aging, and could be a potential target for anti-aging therapy (Bernardes de Jesus et al., 2012; Shay, 2016).

After numerous cycles of cell division, stem/progenitor cells in the adult body lacking telomerase stop proliferating and enter into a state of growth arrest called replicative senescence (Newbold, 1997). However, elevated telomerase expression is almost universal in human tumors which make it a hallmark of cancer cells (Kim et al., 1994). As telomerase counteracts telomere shortening during rounds of cellular proliferation by synthesizing new repeats (TTAGGG) for maintaining the telomeric length at the chromosomal termini, cancer cells are able to maintain their telomere to proliferate continuously without limits, making telomerase a promising target for cancer treatment (Collins and Mitchell, 2002). Accordingly, several telomerase-based strategies have been developed by scientists for cancer therapy and many are in advanced clinical trials (Kim et al., 1994; Vonderheide, 2002; Shay and Keith, 2008; Liu et al., 2010).

SPECIES DIFFERENCE

Although the telomere is composed of TTAGGG repeats in all vertebrates, the length differs significantly in different species (Calado and Dumitriu, 2013). On average, the telomere in humans is about 5–15 kb long, while much longer telomere (50–100 kb) is detected in laboratorial mice (Calado and Dumitriu, 2013). Consistently, in marked contrast to humans, mice have higher levels of telomerase expression and activity (Prowse and Greider, 1995; Burger et al., 1997; Wright and Shay, 2000). The expression of telomerase declines dramatically upon differentiation of stem cells in humans; therefore, it is difficult to detect telomerase in somatic human cells. However, robust levels of telomerase activity are detected in a wide range of somatic tissues and cells in mice (Horikawa et al., 2005).

Horikawa et al. (2005) found that the activity of the mouse TERT promoter is 5.4–16 folds higher than that of the human TERT promoter, in which a non-conserved GC-Box functions critically. Studies investigating site-directed mutations revealed that a GC-box (CCCCGCCC) located at −31 to −24 and a putative E2F site (GCGCG) located at −13 to −9 in the human TERT promoter contribute to the repression of the activity of the human TERT promoter (Horikawa et al., 2005). Nonetheless, the molecular mechanisms of these differences remain largely obscure and need more research focus. The species difference of telomerase implies that it requires a humanized mouse model for deeply studying the role of telomerase in aging and cancer and more attention should be paid when studying the function of telomerase in the brain using regular laboratorial mice.

EXPRESSION OF TELOMERASE IN THE CNS

Telomerase highly exists in the brain at embryonic stages and declines gradually after birth except in adult stem cells (ASCs; Wright et al., 1996; Greenberg et al., 1998; Martín-Rivera et al., 1998; Armstrong et al., 2005). Particularly, TERT expression and activity are confirmed in adult brain tissues including the hippocampus, olfactory bulb and SVZ, possibly due to the rich of NPCs in these places (Martín-Rivera et al., 1998; Caporaso et al., 2003; Zhou et al., 2011). Interestingly, ectopic telomerase in neurons and glial cells are also detected under special conditions (Iannilli et al., 2013). Elucidating the distribution of telomerase is important for understanding its specialized functions in the CNS.

Expression of Telomerase in NSCs

During embryonic development, most of the cells in different tissues display telomerase expression and activities both in humans and rodents (Wright et al., 1996; Greenberg et al., 1998; Armstrong et al., 2005). High levels of telomerase activity have been observed throughout embryonic brain development (Fu et al., 2000). The activity level reaches a peak point at embryonic day 13 and then declines notably from embryonic day 13–18. The activity remains at a low level until postnatal day 3 when it decreases remarkably (Klapper et al., 2001; Mattson and Klapper, 2001). This expression pattern indicates that telomerase is present in the brain during embryonic development, which is

supported by its elevated expression in embryonic neuronal stem or progenitor cells (Mattson and Klapper, 2001). Additionally, NSCs lose telomerase activity upon differentiating into astrocytes or neurons (Kruk et al., 1996; Miura et al., 2001; Caporaso et al., 2003; Cheng et al., 2007). It remains unclear how exactly telomerase activity decreases in differentiating cells. Sporadic research suggests that histone deacetylation and DNA methylation is involved in the silencing of the TERT gene, correlated with the decreased level of telomerase activity in differentiating cells (Lopatina et al., 2003; Hiyama and Hiyama, 2007; Würth et al., 2014).

Adult NSCs (ANSCs) populations are maintained during the adult lifetime in the SVZ and the SGZ in the lateral ventricle and the hippocampal DG (Gage, 2000; Arnold and Hagg, 2012). It is shown that telomerase activity contributes to the viability and self-renewal potential of ASCs including ANSCs (Ostenfeld et al., 2000; Allsopp et al., 2003; Liu et al., 2004; Choi et al., 2008). Indeed, telomerase activities and expression can be detected in the olfactory bulbs, SVZ and hippocampus (Caporaso et al., 2003).

Although the TERT level is significantly lower in the human brain compared to the mouse brain, it is still detectable (Horikawa et al., 2005). Remarkably, increased expression of human TERT is observed with activity in human neural progenitor cells (NPCs; Ostenfeld et al., 2000; Bai et al., 2004). Several human cells such as the teratocarcinoma NTera2 and human neuroblastoma (SK-N-SH) cell lines are generally used for investigating neuronal function (Jain et al., 2007). Abundant telomerase activity is enriched and inhibits neuronal differentiation in these cell lines (Jain et al., 2007; Richardson et al., 2007). Together, these studies display evidence of telomerase existence in both embryonic and adult NSCs.

Ectopic Existence of Telomerase in Neurons and Glial Cells

Although it is believed that telomerase activity is restricted to areas containing stem cells in the brain (Caporaso et al., 2003), ectopic expression of the TERT protein has been shown in post-mitotic neurons without proliferating abilities (Iannilli et al., 2013). In contrast to the claim of Kang et al. (2004), that TERT is not detectable in the adult mouse brain using fluorescent *in situ* hybridization histochemistry, Spilsbury et al. (2015) presented evidence that TERT was expressed in cultured mouse neurons and microglia *in vitro*, which is consistent with a study by Fu et al. (2000). More interestingly, it is found that TERT was detected in the cytoplasm of mature human hippocampal neurons *in vivo* (Spilsbury et al., 2015). Additionally, TERT presents in activated microglia but is absent from astrocytes (Spilsbury et al., 2015). Various insults including ischemia, amyloid peptide administration, and glutamate or NMDA-induced excitotoxicity, substantially induce the expression of TERT in rodent neurons (Fu et al., 2000; Klapper et al., 2001; Zhu et al., 2001; Kang et al., 2004; Lee et al., 2010). Although telomerase levels are low in mature neurons, telomere repeat-binding factor 2 (TRF2) expression is high. Relative deficiency of TERT in new mature neurons during brain development may partially determine their vulnerability to DNA damage (Cheng et al., 2007). In addition, TERT is expressed in microglial cells in the hilus of hippocampus after administration of kainic acid in adult mice (Fu et al., 2002). The evidence of the existence of telomerase in neurons and glial cells implies a potential novel function in these cells, which warrants further investigation.

ROLES FOR TELOMERASE IN BRAIN DEVELOPMENT

Besides the roles of TERT in embryonic stem cells (ESCs), post-transcriptional regulation of TERT is implicated in the survival, self-renewal and differentiation of ASCs (Mattson et al., 2001; Marión and Blasco, 2010; Maeda et al., 2011; Cheng G. et al., 2013; Radan et al., 2014). This function is mediated by telomeric length stability or extra-telomeric telomerase isoforms (Radan et al., 2014; Zeng et al., 2014). In particular, telomerase deficiency impairs normal brain function in mice (Lee et al., 2010; Zhou et al., 2016, 2017). In the brain, telomerase in ASCs plays a critical role in the proliferation of NSCs, neuronal differentiation and development, and neuronal survival, which are involved in CNS diseases (Mattson and Klapper, 2001).

Roles for Telomerase in Proliferation of NSCs

Telomerase is critical for stem cell proliferation. Using 3′-azido-2′,3′–dideoxythymidine (AZT), a type of telomerase activity inhibitor, Haïk et al. (2000) showed that telomerase activity was required for brain organogenesis. Additionally, cell proliferation of NPCs in the SGZ and olfactory bulb is severely decreased in the forebrain of TERC-knockout mice (Ferrón et al., 2009). We have also showed that AZT disrupted neurogenesis in the SGZ of the hippocampal DG both *in vivo* and *in vitro* (Zhou et al., 2011). In contrast, overexpression of telomerase by recombinant adenoviral vector expressing mouse TERT (Ad-mTERT-GFP) stimulates the proliferation of NSC both *in vitro* and *in vivo* (Zhou et al., 2011; Liu et al., 2012). Transduction with human TERT gene also results in increased proliferation in mouse NSCs (Smith et al., 2003).

Roles for Telomerase in Neuronal Differentiation

NSCs possess the capability to self-renew and differentiate into mature nerve cells including neurons, astrocytes and oligodendrocytes (Miura et al., 2001; Ming and Song, 2011; Würth et al., 2014). The activity of telomerase rapidly decreases when NSCs stop dividing and differentiate into nerve cells (Kruk et al., 1996; Klapper et al., 2001). Therefore, the potential relationship between the decrease in telomerase activity and neuronal differentiation was examined. Indeed, overexpression of telomerase can inhibit neuronal differentiation in NPCs (Richardson et al., 2007). Inhibition of the telomerase activity by treatment of cells with telomerase antisense accelerates differentiation, suggesting that telomerase activity may contribute to the blockade of the onset of cell differentiation (Kondo et al., 1998). Moreover, overexpressing TERT in

neuroepithelial precursors caused continuous cell division, but led to disaggregation and cell death, showing that TERT itself is not sufficient to cause termination of differentiation of neural precursors *in vitro* (Richardson et al., 2007). The telomere length regulated by telomerase activity may mediate the control of cell differentiation (Sharpless and DePinho, 2004).

However, a markedly different role for telomerase was reported in NCS differentiation. Schwob et al. (2008) demonstrated that overexpressing TERT in primary ESCs produced markers of neuronal precursors and mature neurons, with a heightened efficiency of neuroectodermal differentiation. It is also reported that TERT promotes neuronal survival and differentiation via reducing excitotoxicity in the CNS (Fu et al., 2002; Kang et al., 2004). Thus, telomerase activity and TERT expression may have different functions in regulation of cellular differentiation. A sharp reduction of telomerase activity during the development of the brain may be a useful signal for cells to begin the process of exiting the cell cycle, thereby differentiating into nerve cells including neurons and glial cells (Mattson et al., 2001). Studies have shown that decreasing telomerase activity was correlated with the differentiation of neural cell lines (Fu et al., 1999) including primary neurons (Fu et al., 2000), supporting such a mechanism. Telomerase-deficient mice reveal impaired neuronal differentiation, which is caused by the expression of RhoA effectors, Rock1 and Rock2, in parallel with the Notch pathway dependent on the modulation of p53 expression, supporting an opposing role of TERT in cell differentiation based on activity (Ferrón et al., 2009). In addition, abnormal telomerase expression significantly inhibited neuronal differentiation of NT2 cells, a model of human NPCs (Mattson et al., 2001). It is also unclear why only a subset of cells in neuronal-inducing conditions are able to attain or sustain terminal differentiation. Research investigating telomerase throughout human neuronal cell differentiation is needed to further answer these questions (Richardson et al., 2007). The different roles of telomerase expression and activity may be involved.

Although the roles of telomerase in neuronal differentiation are unsettled, it is certain that telomerase has important roles in the transition between pluripotent stem cells and committed neuronal cell fate in both NSC and ES cells (Schwob et al., 2008). Therefore, telomerase is a potential target for manipulating NSCs/NPCs, increasing the possibility for autologous cell replacement therapy for CNS illnesses including neurodegenerative diseases, psychiatric disorders, brain ischemia, aging and traumatic injury (Mattson et al., 2001; Sanai et al., 2005). More studies are necessary to determine the exact function of telomeres in neuronal differentiation.

Roles for Telomerase in Neuronal Development

Brain-derived neurotrophic factor (BDNF) is an important molecule for neuronal development. It is found that telomerase is a key mediator of cell survival induced by BDNF in developing neurons (Fu et al., 2002). The high expression of TERT in neurons throughout embryonic and early postnatal development support an important role of telomerase in neuronal development (Mattson et al., 2001). Age-induced impairment of neurogenesis and neuritogenesis are correlated with the telomere length shortening in adult NPCs in the SVZ (Ferrón et al., 2009). The neurons matured from TERC-deficient NSCs fail to acquire a fully mature neuritic arbor (Ferrón et al., 2009). Our recent study found that TERT gene deletion caused a disruption in neuronal development, which was reversed by TERT reactivation (Zhou et al., 2017). The role of telomerase dysfunction in neuronal development may be involved in CNS diseases such as anxiety and memory deficiencies (Lee et al., 2010; Zhou et al., 2017).

Roles for Telomerase in Neuronal Survival

Studies have provided evidence that telomerase may be involved in the regulation of the survival of cells including developing neurons (Fu et al., 2000; Klapper et al., 2001; Lu et al., 2001). Using antisense technology, Fu et al. (2000) demonstrated that suppression of TERT in cultured embryonic neurons induced apoptosis. The mechanisms by which TERT and/or telomerase are involved in survival-promoting activity may involve an interaction with neurotropic factors such as fiber growth factor (FGF), which can induce TERT expression (Haïk et al., 2000). Aside from FGF, Akt kinase, another neurotropic factor, affects TERT function. Phosphorylated TERT with enhanced enzymatic activity induced by Akt account for the neuronal survival-promoting actions of Akt (Mattson et al., 2001). Interestingly, temporary ectopic expression of TERT in neurons following brain ischemia protects hypoxic neurons from excitotoxicity, promoting neuronal survival (Kang et al., 2004; Li et al., 2011; Qu et al., 2011). In addition, TERT also mediates the neuronal survival-promoting actions of brain-derived neurotropic factor (BDNF), counteracting the adverse function of amyloid precursor protein in cultured hippocampal neurons during development (Zhu et al., 2001; Fu et al., 2002). Together, TERT activation is a common pathway for neurotropic factors including FGF, Akt, and BDNF, the well-established mediators of neuronal survival. Hence, the TERT subunit of telomerase in the embryonic brain may safeguard neuronal development, and the ectopic expression of TERT may act on post-mitotic cells to protect neurons from impairment.

Programmed cell death (apoptosis) is an important biological process (Shlezinger et al., 2017). Embryonic NSCs (ENSCs) play a critical role in the complex processes of the CNS formation during embryonic development and apoptosis of ENSCs contribute crucially to the appropriate formation of various biological structures and function of the brain (Gökhan and Mehler, 2001). The establishment of TERC knockout embryos led to a failure of closing the neural tube, which is crucially associated with telomere shortening (Herrera et al., 1999, 2000). TERC knockout mice also display a phenotype of enhanced apoptosis (Phelan et al., 1997). Consistently, reduction in TERT expression in ENSCs *in vitro* forces neurons to undergo apoptosis (Fu et al., 2000). These findings advocate both TERT and TERC as cell survival-promoting factors in neurons.

Most somatic cells enter a non-dividing state called cellular senescence after undergoing cell division (Wright and Shay, 1992). Cell cycle arrest frequently precedes

activation of the molecular cascades of apoptosis, causing morphological changes and death in cells including neurons (Krantic et al., 2005). Interestingly, it is reported that telomerase activity cannot be detected and TERT expression is suppressed during growth arrest and cellular senescence (Wright et al., 1996). Once telomere length activates its checkpoint, cellular senescence is triggered, cell division is suspended and the cell eventually dies (Gilley et al., 2008). Since the well-established function of telomerase is maintaining the telomere length, it is reasonable to consider the involvement of telomerase in an anti-apoptotic role through prevention of DNA damage in a telomere-dependent manner (Rhyu, 1995; Liu, 1999). After differentiation from NSCs/NPCs, the somatic cells have extremely low levels of telomerase, leaving telomeres significantly damaged and cells vulnerable to stress, inducing apoptosis (Kondo et al., 1998). More direct evidence is shown in experiments suppressing TERT expression. In developing neurons, antisense-mediated silencing of TERT gene expression causes them to undergo apoptosis more frequently (Fu et al., 2000). In contrast, overexpression of TERT in human cells prevents cellular senescence and extends the lifespan (Bodnar et al., 1998; Yang et al., 1999). More importantly TERT can protect cultured neurons from apoptosis in experimental cell models relevant to ischemia and Alzheimer's disease (AD; Zhu et al., 1999; Fu et al., 2000). It has been demonstrated that telomerase is associated with DNA repair and promotes cell survival (Peterson et al., 2001). One possible mechanism underling the role of TERT in apoptosis of NSCs is that TERT may suppress DNA damage and the activation of the associated pathways, which aids in the stabilization of chromosome ends (Holt et al., 1999). Collectively, both TERC and TERT may account for the action of telomerase in the modulation of apoptosis of NSCs at different stages, contributing to the balance of brain function.

TELOMERASE AND DISEASES IN THE CENTRAL NERVOUS SYSTEM

Since telomerase appears to have a significant role in the different stages of development of NSCs and NPCs, it may be involved in a variety of CNS diseases. Besides its well-studied role in brain cancer (Kheirollahi et al., 2013), telomerase also contributes to CNS impairment, including ischemic brain injury (Zhao et al., 2010; Li et al., 2011), neurodegenerative disease (Franco et al., 2006), mood disorders (Lee et al., 2010; Zhou et al., 2011) and schizophrenia (Kao et al., 2008; Porton et al., 2008).

Brain Tumors

More than 85% of the tumor cells show telomerase activation for preventing progressive shortening of the telomere as excessive divisions (Harley, 1991). Tumors that originate in the brain are known as primary brain tumors, including astrocytomas, oligodendrogliomas and ependymomas. High telomerase activity was observed in astrocytoma including glioblastoma (GBM, grade IV astrocytoma), the most common type of malignant primary tumors in adults (Cheng L. et al., 2013). Hakin-Smith et al. (2003) found that alternative-lengthening-of-telomere is a prognostic indicator for patients with GBM (Lötsch et al., 2013). Consistently, Tchirkov et al. (2003) reported hTERT mRNA levels may represent a prognostic and diagnostic indicator for GBM patients. Mechanistically, two somatic mutations, C228T (−124 bp) and C250T (−146 bp) located upstream of the ATG start site confer enhanced TERT promoter activity in the GBM (Mosrati et al., 2015). These TERT promoter mutations are associated with shorter overall survival (Mosrati et al., 2015). In addition, the SNP of rs1006969, in the promoter regions, and rs2736100, in the intron 2, were reported to be associated with an increased risk of developing GBM (Mosrati et al., 2015). Interestingly, TERT promoter mutations led to a significant increase in TERT mRNA and enhanced activity of telomerase in tumors. Based on these findings, Marión and Blasco (2010) used a telomerase antagonist, imetelstat, to target glioblastoma tumor-initiating cells efficiently for decreasing proliferation and tumor growth (Ferrandon et al., 2015). However, using telomerase-based drugs for cancer treatment should be conducted with caution, as these therapies may have adverse effects normal tissues.

Following the findings of elevated levels of telomerase in tumors, new anticancer methods targeting telomerase have been highly anticipated. In 1995, the first attempt to use an antisense vector against TERC was reported (Feng et al., 1995). To date, a number of different approaches including antisense, natural compounds, hormones, vaccines, and small molecules have been developed to inhibit telomerase activity in cancer cells (Saretzki, 2003; Shay and Wright, 2006). However, no company has declared success in developing compounds for cancer treatment. Several factors account for this lack of progress. It takes a long time for telomerase inhibition to be clinically effective, which may cause toxicity in normal proliferative cells (Harley, 2008). Another factor is the pharmaceutical industry arguing that telomerase is not a practical drug target (Man et al., 2016). Many efforts are being made to continue investigating novel methods, including oncolytic viral strategies and immunotherapy, to target telomerase (Olaussen et al., 2006). Despite significant progress, issues must be addressed before applying telomerase-based therapies for treating cancer including brain tumors.

Aging Brain

Telomeres play a central role in aging. Shortening of telomeres has been linked to the mechanisms responsible for the aging of cells (López-Otín et al., 2013). Telomerase, preventing the telomere from being too short, thus acts as an anti-aging enzyme, proposing a "telomere theory of aging", a prominent concept in research (Jaskelioff et al., 2011). Since the secret of "the end replication problem" was uncovered owing to the finding of telomerase, its role in cellular aging was predicted and revealed (Greider and Blackburn, 2004). TERT gene knockout studies provide direct evidence that TERT loss provoked tissue degeneration including progressive atrophy of tissues, depletion of stem cells, failure of organ systems and impairment of tissue

response to injuries throughout the whole body including the brain (Jaskelioff et al., 2011). Strikingly, 4 weeks of reactivating telomerase reversed the aging process in the brain, including NSCs proliferation and differentiation, brain size and olfactory function (Jaskelioff et al., 2011). Overexpression of TERT in NSCs or neurons is beneficial for the adult brain by increasing resistance to neurodegenerative changes with aging (Mattson et al., 2001). Clinical evidence also suggests a correlation between telomerase activity in human leukocytes and the volume of the hippocampus in early stages of aging (Jacobs et al., 2014). As noted, the aging brain is associated with extensive accumulation of DNA damage. Telomerase gene therapy in adult and old mice has been shown to delay aging and increase longevity without causing cancer via DNA damage repair (Bernardes de Jesus et al., 2012). Thus, telomerase could be a serious intervention to inhibit degeneration in the aging brain.

AD, with two discrete pathologies including Amyloid-β (Aβ) and tau (p-tau) aggregation, is a common neurodegenerative disorder in elderly patients, and has aging characteristics featured with cell senescence and oxidative stress (Smith et al., 1995; Spilsbury et al., 2015). It is shown that the length of neuronal telomeres are remarkably shorter in hippocampal neurons in patients with AD (Franco et al., 2006). TERT exhibits neuronal protective properties against tau pathology in experimental models of AD (Kota et al., 2015; Spilsbury et al., 2015). Additionally, ROS generation and oxidative damage in neurons, the mediators of pathological tau, are relieved in TERT knockout mice (Spilsbury et al., 2015). In accordance with this evidence, Aβ oligomers-induced cytotoxicity is shown to be potentially mediated by telomerase activity inhibition (Wang et al., 2015). However, TERC knockout mice with AD present with telomere shortening which slows down the progression of Aβ pathology (Rolyan et al., 2011). The inconsistency of telomere in AD pathology may be due to different processes, provoking microglial activation with extreme telomere shortening (Rolyan et al., 2011). Therefore, telomerase may play different roles in the tau and amyloid pathology via multiple mechanisms. Furthermore, leukocyte telomere length is altered in other neurodegenerative disorders including Huntington's disease and dementia, indicating that change in telomere length is a shared characteristic of neurodegenerative disorders (Kota et al., 2015). Thus, the modification of telomerase together with telomere could be a marker of aging-related conditions (López-Otín et al., 2013).

Parkinson's Disease

Parkinson's disease (PD) is an aging-associated long-term degenerative disorder (Singleton and Hardy, 2016). Contrary to short telomeres observed in the aging brain, the relationship between telomere and PD remains unclear. An analysis of 131 PD patients and 115 healthy controls performed by Eerola et al. (2010) found no difference in telomere length between PD patients and healthy controls. Consistently, a case-control study from Wang et al. (2008) reported that shorter telomeres are not associated with a higher risk of PD. Moreover, a large nested case-control study also found that telomere shortening was associated with reduced PD risk (Schürks et al., 2014). To date there is no evidence showing abnormalities of telomerase or telomere in the CNS tissues of PD patients.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is another type of progressive neurodegenerative disease, characterized by the death and dysfunction of nerve cells in the CNS (Boillée et al., 2006; Eitan et al., 2012). Motor neurons in the spinal cord, the cerebral cortex, and brain stem gradually break down and die, causing muscle weakness and atrophy. An investigation on the telomere length in blood leukocytes revealed accelerated telomere attrition and lower levels of telomerase in patients with ALS (De Felice et al., 2014). More importantly, human TERT expression in the spinal cord of ALS patients is extremely low compared to healthy controls (De Felice et al., 2014). A controlled and transient increase in telomerase expression and activity in the brain using a telomerase-increasing compound delayed the onset and progression of ALS (Eitan et al., 2012). This compound increased the survival of motor neurons in the spinal cord by 60% (Eitan et al., 2012). Telomerase-related DNA repair and transcription regulation may contribute to the survival of motor neurons in ALS (Singh et al., 2017). Surprisingly, Linkus et al. (2016) found a trend of longer telomeres in microglia from human post-mortem brain tissue with ALS. However, knocking out telomerase in mice accelerated the ALS phenotype (Linkus et al., 2016). The longer telomeres in microglial cells may play a role in microglial proliferation, which contributes to ALS disease progression (Linkus et al., 2016). Therefore, the contribution of telomerase in different neural cells in the development of ALS may have distinct mechanisms.

Brain Ischemia

Normally, TERT expression and telomerase activity are at a very low level and undetectable in post-mitotic cells including neurons in the brain. After ischemic injury, ectopic expression of TERT was detected in neurons (Kang et al., 2004). Transgenic overexpression of TERT showed a significant resistance to injury. Induction of TERT in injured neurons protects against NMDA excitotoxicity, ameliorating ischemic neuronal cell death (Kang et al., 2004). Aside from neurons, astrocytes appear to have a role in TERT-related neuronal protection. Baek et al. (2004) show TERT co-localization with glial fibrillary acidic protein (GFAP), a marker of astrocyte, in the neonatal brain 3 days after stroke. Consistently, it was reported that TERT mRNA and protein were up-regulated in neurons 2 days after hypoxia-ischemia but shifted to astrocytes at day 3 (Qu et al., 2011). The distribution of temporary ectopic expression of TERT supports the concept that both promotion of neuronal survival and attenuation of astrocyte proliferation in the developing brain contribute to a TERT-based neuroprotective mechanism of hypoxia–ischemia (Qu et al., 2011). Additionally, a shift of the cytosolic free Ca^{2+} into the mitochondria is important for TERT to inhibit apoptosis and excitotoxicity, decrease angiogenesis and promote neuronal survival (Li et al., 2011). Reduction of telomerase activity leads to an intensified neuroinflammatory response

and blood-brain barrier disruption after experimental stroke (Zhang et al., 2010). Decreasing ROS generation and increasing mitochondrial membrane potential were also reported as TERT's neuroprotective mechanisms (Li et al., 2013). Better understanding these novel mechanisms may assist in the development of more effective neuroprotective strategies in the treatment of ischemic brain injury.

Mood Disorders

The World Health Organization ranks mood disorders as the leading causes of years (Murray and Lopez, 1996). Brain structural and functional abnormalities mediate the pathophysiology of mood disorders, including major depressive disorder (MDD), bipolar disorder (BD), and anxiety. Increasing studies suggest a strong causal link between impaired neurogenesis and etiology of mood illnesses. Considering the function of telomerase in stem cells, especially ANSCs/ANPCs, it is highly expected that telomerase plays an important role in the modulation of mood disorders (Monroy-Jaramillo et al., 2017). Epidemiologic studies reveal a close association between telomere length and psychiatry illness (Darrow et al., 2016). Dysfunctional telomeres in peripheral leukocytes have been observed in several psychiatric conditions (O'Donovan et al., 2011; Lindqvist et al., 2015). A pilot study found that 16 un-medicated subjects with MDD had increased basal telomerase activity in comparison with healthy controls in males (Wolkowitz et al., 2012) but not females (Simon et al., 2015). Post-mortem research shows a significant reduction in telomere length across brain regions, especially in the hippocampus, of patients with MDD (Mamdani et al., 2016). Repression of telomere-associated genes leads to microglial senescence, a mechanism of neuropsychiatric diseases (Kronenberg et al., 2017).

Life stress is the main environmental factor causing MDD. Accelerated telomere shortening and decreased telomerase activity has also been reported in response to chronic stress (Epel et al., 2004, 2010). Chronic mild stress lowered hippocampal TERT protein levels and telomerase activity was reversed by fluoxetine treatment (Zhou et al., 2011). In addition, a low level of TERT and its activity was detected in the hippocampus in a rat model of MDD (Wei et al., 2015). Both overexpression of TERT and TERT activity inhibition or knockout demonstrated that hippocampal telomerase played an essential role in modulating depressive and aggressive behaviors (Zhou et al., 2011, 2016).

Deficiencies in telomerase can also be associated with other mood disorders. Telomerase expression is correlated with anxiety (Perna et al., 2016). Brain structural and functional changes of aging were more pronounced in subjects with anxiety than controls, including reduced gray matter density, white matter alterations, and impaired functional connectivity of large-scale brain networks. Moreover, molecular correlates of brain aging such as telomere shortening, Aβ accumulations, and oxidative/nitrosative stress, were overrepresented in anxious subjects (Perna et al., 2016). Both rodent and human findings showed an association between anxiety and telomere shortening. TERT-deficient mice displayed significantly higher anxiety-like behaviors (Lee et al., 2010). Consistently, deficiency of telomerase resulted in increased anxiety-like behavior in aged transgenic mice (Lee et al., 2011). In non-psychiatric human brain samples, associations were found between exposure to chronic stress (e.g., childhood adverse experiences/stressful caregiving status) or high phobic anxiety and accelerated telomere shortening, which may be related to dysregulation of inflammatory markers, HPA axis, and autonomic system function (Surtees et al., 2011; Okereke et al., 2012; Wolkowitz et al., 2012; Révész et al., 2014).

Schizophrenia

Generally, schizophrenia is not regarded as an aging-related disorder. However, pathology of aging may be a component of this disorder, since there are similar structural brain abnormalities (Buchsbaum and Hazlett, 1997; DeLisi, 1997; Surtees et al., 2011; Okereke et al., 2012; Wolkowitz et al., 2012; Révész et al., 2014). Research shows the average length of telomeres in peripheral blood lymphocytes from individuals with schizophrenia is markedly shortened (Kao et al., 2008; López-Otín et al., 2013). A recent study also demonstrated shorter telomere length among patients with schizophrenia (Galletly et al., 2017). In line with this finding, a significant decrease was reported in telomerase activity in peripheral blood lymphocytes taken from individuals with schizophrenia (Porton et al., 2008). While these studies provide exciting correlative results, further research is needed to determine the exact role of telomerase in schizophrenia.

Summary

More and more evidence displays a correlation between changes in TERT activity or telomere length and CNS diseases besides brain tumors. Therefore, it is possible that additional applications of TERT manipulation may be useful in the treatment of various CNS disorders. Due to potential adverse effects on normal cells, treatments based on TERT manipulation must be carefully planned and should exclude patients with conditions such as tumorigenesis. Regardless, this is an exciting new avenue for research and translational medicine, as scientists are making breakthroughs in telomerase gene therapy (Bernardes de Jesus et al., 2012). Bernardes de Jesus et al. (2012) show that administration of TERT to aged mice, using an adeno-associated virus, reduced the incidence of glucose intolerance and osteoporosis, and improved the function of the neuromuscular junction. More notably, they found this gene therapy strategy improved the ability of memory formation without increasing tumorigenesis (Bernardes de Jesus et al., 2012). While these new studies are encouraging, there is still much research needed before telomerase therapy can be translated into clinical application for the treatment of CNS disorders.

CONCLUSION AND PERSPECTIVES

In this review article, we have presented the existence of telomerase in the CNS and its roles in the developing and adult brain, including proliferation, differentiation, maturation

and apoptosis of nerve cells, as well as CNS diseases. The mechanisms whereby telomerase acts within these processes are becoming more clear, however its exact role and mechanisms remain unknown (Harley, 1991). Small molecules screened and tested for telomerase inhibition targeting telomerase activity are anticipated for drug discovery of cancers, although they are not yet approved (Harley, 2008). Understanding the precise functions of TERT and other telomere-associated proteins in the CNS may be helpful for recruiting them as novel targets for treatment of brain tumor, neurodegenerative diseases, and mood disorders. Hence, knowing the role of telomerase in the nervous system is not only important to gain further insight into the process of the neural cell cycle, but also provides a novel therapeutic application for the treatment of nervous system diseases. Although failure of developing telomerase therapeutics for clinical use is possible, the manipulation of the telomere/telomerase system is still a promising and novel approach for therapeutic purpose. The knowledge of this system, reviewed here, may be vital in the development of future treatments of neurological dysfunction.

AUTHOR CONTRIBUTIONS

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Development of Structural Covariance From Childhood to Adolescence: A Longitudinal Study in 22q11.2DS

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Background: Schizophrenia is currently considered a neurodevelopmental disorder of connectivity. Still few studies have investigated how brain networks develop in children and adolescents who are at risk for developing psychosis. 22q11.2 Deletion Syndrome (22q11DS) offers a unique opportunity to investigate the pathogenesis of schizophrenia from a neurodevelopmental perspective. Structural covariance (SC) is a powerful approach to explore morphometric relations between brain regions that can furthermore detect biomarkers of psychosis, both in 22q11DS and in the general population.

Methods: Here we implement a state-of-the-art sliding-window approach to characterize maturation of SC network architecture in a large longitudinal cohort of patients with 22q11DS (110 with 221 visits) and healthy controls (117 with 211 visits). We furthermore propose a new clustering-based approach to group regions according to trajectories of structural connectivity maturation. We correlate measures of SC with development of working memory, a core executive function that is highly affected in both idiopathic psychosis and 22q11DS. Finally, in 22q11DS we explore correlations between SC dysconnectivity and severity of internalizing psychopathology.

Results: In HCs network architecture underwent a quadratic developmental trajectory maturing up to mid-adolescence. Late-childhood maturation was particularly evident for fronto-parietal cortices, while Default-Mode-Network-related regions showed a more protracted linear development. Working memory performance was positively correlated with network segregation and fronto-parietal connectivity. In 22q11DS, we demonstrate aberrant maturation of SC with disturbed architecture selectively emerging during adolescence and correlating more severe internalizing psychopathology. Patients also presented a lack of typical network development during late-childhood, that was particularly prominent for frontal connectivity.

Conclusions: Our results suggest that SC maturation may underlie critical cognitive development occurring during late-childhood in healthy controls. Aberrant trajectories of SC maturation may reflect core developmental features of 22q11DS, including disturbed cognitive maturation during childhood and predisposition to internalizing psychopathology and psychosis during adolescence.

Keywords: schizophrenia, graph theory, connectome, synaptic stabilization, cortical development, executive functions, structural covariance

INTRODUCTION

Mounting evidence has suggested that schizophrenia arises from a disorder of brain development (Feinberg, 1982; Weinberger, 1987; Crow et al., 1989; Murray et al., 1991; Insel, 2010; Rapoport et al., 2012). Indeed, psychosis typically emerges when critical brain maturation is still underway, during late-adolescence and early-adulthood. Moreover, recent work has highlighted that cognitive deficits, which represent a core dimension of schizophrenia, can manifest as early as childhood (Kremen et al., 2010; Gur et al., 2014) and help predict subsequent emergence of psychosis (Riecher-Rössler et al., 2009; Seidman et al., 2016). The neurodevelopmental model carries at least two critical implications. Firstly, it predicts that, for therapeutic interventions to be truly effective, they should target neurodevelopmental events that precede clinical manifestations (Marín, 2016; Millan et al., 2016). Secondly, it implies that biomarkers of vulnerability to psychosis can potentially manifest earlier than disease onset, in form of atypical neurodevelopmental trajectories (Weinberger, 1987; Insel, 2010; Rapoport et al., 2012). An improved characterization of such neurodevelopmental biomarkers could prove critical for informing future therapeutic interventions (Millan et al., 2016).

In the field of schizophrenia, converging evidence has pointed to the role of disturbed structural and functional connectivity (Stephan et al., 2009; van den Heuvel and Fornito, 2014) in the pathogenesis of the disease. At the most basic anatomical scale, neuropathological studies have consistently reported synaptic alterations, particularly in prefrontal regions (Garey et al., 1998; Glantz and Lewis, 2000; Rosoklija et al., 2000; Black et al., 2004; Glausier and Lewis, 2013) that are thought to arise from excessive synaptic pruning during adolescence (Feinberg, 1982; Sekar et al., 2016). Neuroimaging allows to investigate brain connectivity *in-vivo* and at a higher anatomical scale. For instance, using diffusion-weighted MRI it is possible to investigate white-matter connectivity non-invasively throughout the entire brain (Beaulieu and Allen, 1994; Basser and Pierpaoli, 1996; Mori and Barker, 1999; Basser et al., 2000; Mori and van Zijl, 2002), while functional MRI and EEG allow to explore patterns of synchronized activity underlying functional communication between brain regions (Vértes and Bullmore, 2015). Connectomics analysis can then characterize non-trivial aspects of network architecture with tools from graph theory (Sporns et al., 2005; Hagmann et al., 2007; Bullmore and Sporns, 2009; Rubinov and Sporns, 2010). Such an approach has demonstrated that healthy structural and functional brain networks find a balance between the local segregation of sub-communities of densely connected regions and the overall integration of multiple sub-networks (Sporns, 2013). This optimal organization, also known as community structure (van den Heuvel et al., 2009; Park and Friston, 2013) has shown to underlie higher cognitive performance in healthy controls (HCs) (Langer et al., 2012; Hilger et al., 2017). In patients with psychosis, on the contrary, this optimal balance is altered, with insufficient architectural integration and excessive segregation (van den Heuvel and Fornito, 2014). Moreover insufficient brain network integration might at least partially account for cognitive symptoms of schizophrenia (Langer et al., 2012; Alloza et al., 2017). So far however, little is known about how network architecture matures in children and adolescents who are at risk for developing psychosis. The characterization of such developmental trajectories could prove informative, particularly in the context of the neurodevelopmental model of schizophrenia (Insel, 2010; Rapoport et al., 2012; Marín, 2016).

22q11.2 Deletion Syndrome (22q11DS) is a powerful model to investigate the pathogenesis of psychosis from a neurodevelopmental perspective (Jonas et al., 2014). Indeed, patients with 22q11DS are at a very high risk for psychosis, with up to 30% of patients developing schizophrenia by adulthood and up to 80% presenting prodromal psychotic symptoms, typically during adolescence (Schneider et al., 2014a; Tang et al., 2014). Furthermore, compared to idiopathic psychosis, patients are typically diagnosed prior to psychiatric manifestations due to a complex somatic phenotype (McDonald-McGinn et al., 2015). 22q11DS offers therefore a unique opportunity to investigate connectivity development in young patients at risk for psychosis. 22q11DS is also characterized by insufficient maturation of executive functions, starting during childhood (Maeder et al., 2016) and recapitulating deficits in executive functions observed in idiopathic psychosis (Seidman et al., 2016). Moreover cognitive decline during childhood can predict subsequent emergence of psychosis in 22q11DS (Vorstman et al., 2015). However the relationship between brain network development and cognitive dysmaturation has not yet been investigated in 22q11DS.

Structural covariance (SC) is a powerful morphometric approach to investigate brain connectivity (Alexander-Bloch et al., 2013). This technique measures how the morphology of different brain regions is correlated across populations, based on the observation that regions connected, either functionally or by white-matter tracts, also tend to co-vary in their morphology (Alexander-Bloch et al., 2013). Several mechanisms are thought

to contribute to this phenomenon (Alexander-Bloch et al., 2013), including the mutually trophic effect of axonal connections (Burgoyne et al., 1993; Gong et al., 2012), coordinated activity induced plasticity (Draganski et al., 2004; Driemeyer et al., 2008; Dehaene et al., 2010) or common genetic influences (Pezawas et al., 2005; Schmitt et al., 2008, 2009, 2010). The emergence of SC networks has moreover shown to be functionally relevant, given that the optimal organization of SC network architecture was associated with higher cognitive performance in healthy and clinical pediatric populations (Bonilha et al., 2014; Khundrakpam et al., 2016). Importantly, networks reconstructed using SC are altered in patients suffering from psychosis, with insufficient architectural integration and excessive segregation (Bassett et al., 2008; Zhang et al., 2012). Similar architectural disturbances of SC networks have been recently replicated in 22q11DS, with specific alterations affecting patients with psychotic symptoms (Sandini et al., 2017). However, so far, little is known about how SC network architecture matures in children and adolescents with 22q11DS and how this relates to cognitive maturation.

Methods to investigate the development of SC have to date mostly consisted of comparisons between age bins (Zielinski et al., 2010; Khundrakpam et al., 2013; Nie et al., 2013). Indeed, SC inherently relies on a group of subjects and thus cannot be retrieved at an individual level (Alexander-Bloch et al., 2013). Age-bin comparisons have contributed significantly to understanding the development of SC in HCs, highlighting for example differential maturation across cortical regions (Zielinski et al., 2010) and significant late-childhood architectural reorganization (Khundrakpam et al., 2013). This technique however presents several methodological limitations. Firstly, age-bin definition is arbitrary, potentially leading to inconsistencies across studies (Richmond et al., 2016). Secondly, temporal resolution is generally insufficient to truly characterize developmental trajectories. In an effort to overcome these limitations recent work has implemented a sliding-window approach, originally developed to investigate dynamics of functional connectivity, to study the development of structural covariance (Váša et al., 2018). The higher temporal resolution offered by such approach demonstrated non-linear maturation of SC networks during adolescence (Váša et al., 2018). This study was however constrained by its cross-sectional nature and in that it did not cover the childhood age range. Indeed particularly late childhood was previously shown to be a critical period for the maturation of both SCNs (Khundrakpam et al., 2013) and cognitive performance (Chelune and Baer, 1986; Anderson, 2002; Crone et al., 2006). Moreover no link with behavioral development was made.

Here we employ a sliding-window technique to investigate structural covariance network (SCN) development in a large longitudinal cohort of children and adolescents with 22q11DS and HCs. We propose a novel approach to cluster regions according to developmental trajectories of structural connectivity strength. Furthermore we correlate maturation of SCNs with development of working memory (WM), a core executive function that is highly affected in idiopathic psychosis. Finally in 22q11DS we explore correlations between SC dysconnectivity and severity of internalizing symptoms. Indeed high internalizing psychopathology represents a hallmark of the psychiatric phenotype of 22q11DS (Shashi et al., 2012; Klaassen et al., 2013, 2015) and higher internalizing symptoms such as anxiety represent a risk factor for psychosis in 22q11DS (Gothelf et al., 2007, 2013).

We hypothesized that in HCs SCNs would undergo non-linear maturation with critical reorganization during late childhood and progressive fine-tuning during adolescence, recapitulating previous findings in general population (Khundrakpam et al., 2013; Váša et al., 2018). We hypothesized that network architecture and particularly fronto-parietal connectivity maturation would be associated with WM performance. In patients we expected to observe aberrant developmental trajectories of particularly frontal connectivity given previous reports of frontal dysmaturation in 22q11DS (Schaer et al., 2009) and idiopathic psychosis (Wood et al., 2008). We expected that previously reported disturbed network architecture would emerge during adolescence, since adolescence is a critical period of vulnerability to psychosis in the general population (Insel, 2010) and in 22q11DS (Schneider et al., 2014a,b). We hypothesize that SC dysconnectivity would be associated with higher internalizing psychopathology.

METHODS

Participants

All participants with 22q11DS were recruited at the Geneva School of Medicine in the context of a prospective longitudinal study [details about recruitment can be found in (Schaer et al., 2009; Maeder et al., 2016)].

For the present work our cohort consisted of 110 patients with 22q11DS, followed up for an average of 2 time-points (varying from 1 to 4) amounting to 221 visits. Prior to selecting our cohort structural brain scans were visually inspected to screen for the presence of gross morphological abnormalities, leading to the exclusion of two patients with 22q11DS presenting with polymicrogyria. Additionally, we recruited 117 (M/F = 58/59) HCs that were followed for an average of 1.8 time-points (varying from 1 to 4), amounting to 211 visits. Groups did not differ in terms of mean age ($p = 0.1$), time between visits, ($p = 0.35$), gender ($p = 0.63$) or handedness ($p = 0.61$). Only full-scale IQ was significantly lower in patients compared to controls ($p < 0.001$). For demographic details see **Supplementary Table 1** and **Supplementary Figure 1**. Prevalence of main psychiatric and neurological diagnoses are reported in **Supplementary Table 2**. Written informed consent was obtained for all participants, and the study was approved by the Institutional Review Board of the Geneva University School of Medicine.

Image Acquisition and Processing

T1-weighted images were acquired with a three-dimensional volumetric pulse sequence with a Philips 1.5T Intera scanner (sequence parameters: TR = 35 ms, TE = 6 ms, flip angle = 45°, NEX = 1, matrix size = 256 × 192, field of view = 24 cm^2, slice thickness = 1.5 mm, 124 slices) and Siemens Trio or Prisma 3T scanners (sequence parameters for 3T scanners: TR = 2,500 ms,

TE = 3 ms, flip angle=8°, acquisition matrix = 256 × 256, field of view = 22 cm, slice thickness = 1.1 mm, and 192 slices). A test-retest approach has previously demonstrated high consistency for morphological measures across scanners (Mutlu et al., 2013). Furthermore scanner type did not differ significantly across populations ($p = 0.14$ see demographic table) and any potential effects of scanner were rigorously accounted for during statistical analysis.

Images were imported in FreeSurfer software package (http://surfer.nmr.mgh.harvard.edu/fswiki) for a precise and semi-automatic reconstruction of the internal and external cortical surfaces (Fischl and Dale, 2000). The mean cortical thickness of 148 brain regions was then computed for each scan using the Destrieux parcellation, implemented in FreeSurfer (Destrieux et al., 2010).

Definition of Age-Bins Through Sliding-Window Approach

Visits were ordered according to age separately for HCs and patients. Subsequently, a window of 35 visits was progressively slid across the two cohorts, starting for the 35 visits of the youngest subjects, and proceeding with one visit at a time (See **Figure 1**, Step 1). Definition of window-width is an inherently arbitrary step of sliding-window approaches and implies a trade of between higher statistical power and lower temporal resolution with increasing window-width (Preti et al., 2016). We opted for a window-width of 35 subjects given that 30 data-points are generally considered sufficient for the estimation of reliable correlations (Hogg et al., 2007). Moreover a window-width of 35 subjects was associated with a mean age-range in each window of 2.86 ± 0.72 years in 22q11DS and 2.83 ± 0.72 in HCs that was only slightly inferior to the mean time between longitudinal visits of 3.8 and 3.6 years in 22q11DS and HCs respectively. This guaranteed that subjects were generally not included twice, at different time-points in a single window. Age-windows that included two repeated visits of the same subject were excluded (37 and 43 age-window in 22q11DS and HCs respectively). This yielded a total of 148 and 132 partially overlapping age-windows, of progressively increasing mean age, in 22q11DS and HCs, respectively. In the subsequent steps, SC was estimated in every age-window.

Structural Covariance Estimation

Before computing SC we accounted for the effects of nuisance variables. Specifically, we controlled for the effects of scanner and gender in two steps: at the level of the entire cohort, and then again in each individual age-window. In each window, we additionally controlled for the effects of age and overall cortical thickness as commonly described in SC literature (Alexander-Bloch et al., 2013).

Subsequently, we computed SCNs using Pearson correlations of cortical thickness across subjects between each couple of brain regions, yielding a symmetrical 148 × 148 covariance matrix for each age-window.

We computed several measures to characterize SCNs after thresholding to consider only positive correlations (**Figure 1**, Step 3a). Mean R Coefficient across all connections was computed as an index of mean connectivity strength (MCS). MCS was also computed for each local region. We employed graph theory to quantify features of network architecture using functions implemented in the Brain Connectivity Toolbox for MATLAB (The MathWorks, Inc., Natick, MA; http://www.brain-connectivity-toolbox.net/). As graph-theoretical measures are influenced by overall network connectivity, we normalized for this by dividing each correlation for the mean of all R-coefficients (Rubinov and Sporns, 2010; van Wijk et al., 2010).

We quantified Mean Clustering Coefficient (MCC) as an architectural measure of local segregation. MCC measures the proportion of neighbors of a node that are also neighbors to each other (Rubinov and Sporns, 2010) and quantifies how efficiently information is transferred within segregated clusters of regions. We also quantified Network Efficiency (NE). NE gauges how efficiently connections are distributed to limit the distance separating each couple of regions of a network (Rubinov and Sporns, 2010).

Extraction of SC Developmental Trajectory and Analysis

We firstly tested the age-relationship of SC measures in both populations separately. Specifically, models of increasing order (from constant to cubic age-relationship) were fit to each measure of SC and a Bayesian information criterion (BIC) based approach was used to select the optimal model order (Mutlu et al., 2013; **Figure 1**, Step 4). Subsequently visits included in each age-window were randomly resampled for 900 leave-one-out-substitution Bootstrap Samples (BSs) (**Figure 1**, Step 2). Covariance matrices were re-computed at each BS, yielding a distribution of SC measures in each age-window. Model fitting was then repeated at each bootstrap iteration and correlation with age was considered significant if a model of non-zero order could be fit to at least 95% of the BSs. For each population we additionally computed confidence intervals (CIs) of curve parameters (such as CI of ages of peak maturation).

Subsequently we employed K-means clustering to identify clusters of regions showing common maturation (**Figure 1**, Step 3b.2).Indeed K-means clustering allows to group together variables (i.e., brain regions) that are similar throughout multiple dimensions (i.e., connectivity at different ages) (Bair, 2013). See **Figure 1**, Step 3b.2 for a graphical representation of K-means clustering approach. Here, the algorithm yielded clusters of regions whose connectivity strength was similar throughout multiple age-windows, indicating a common developmental trajectory. We tested several cluster solutions (from $k = 2$ to $k = 7$) and employed a silhouette approach to identify the optimal number of clusters (Rousseeuw, 1987). We subsequently averaged connectivity strength across regions within each cluster and employed bootstrapping to define CIs at each age-window. We then fit models of increasing order to the bootstrapped data to test and characterize developmental trajectories of each cluster.

Lastly we tested for between-group differences in developmental trajectories of SC measures. Specifically group differences were defined when either models of different order were fit to the two populations, or when the CI of

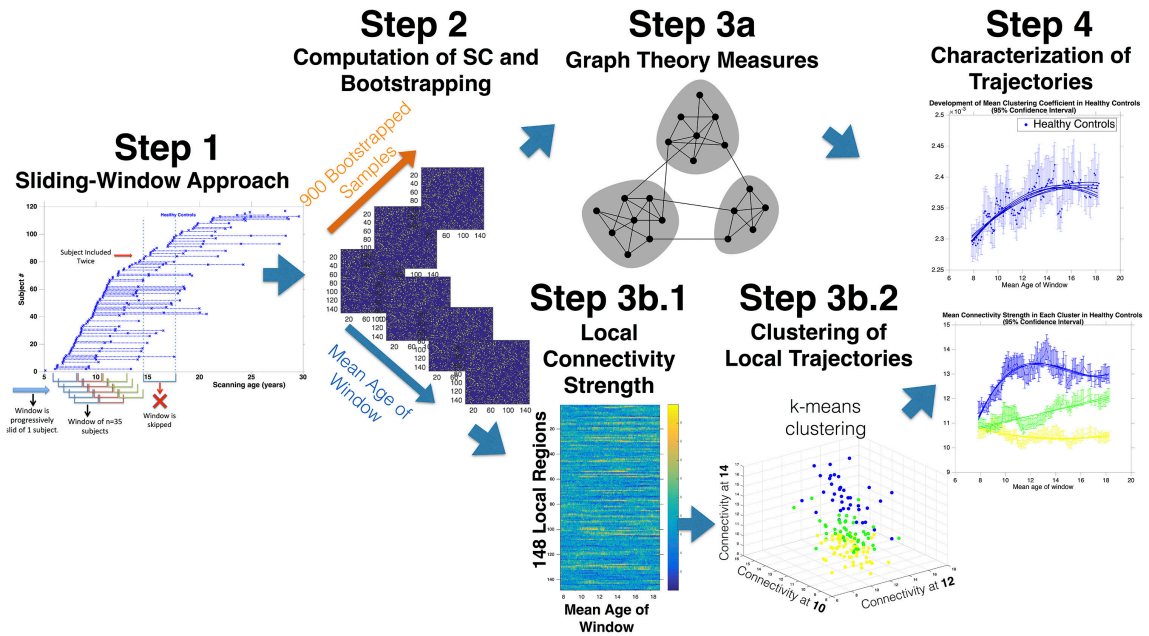

FIGURE 1 | Sliding-Window Methodological Protocol: **Step 1:** Scans are ordered according to age. Subsequently a window of 35 subjects is progressively slid across the cohort starting for the 35 youngest scans at advancing by of one scan at a time. The procedure yields partially overlapping age-windows of progressively increasing mean age. **Step 2**: Covariance matrices are computed in each partially overlapping age-window. Subsequently scans included in each age-window are resampled for 900 leave-one-out substitution bootstrapped samples (BSs). **Step 3a**: The global architecture of covariance matrices computed in each age-window and each BS are characterized with Graph Theory measures, obtaining a distribution of each measure at each age-window. **Step 3b.1**: Local connectivity strength is computed for each region and each age-window yielding a matrix of regions by age. **Step 3b.2:** K-means clustering is then employed to identify clusters of regions showing common maturation. Connectivity strength is averaged within each cluster at each age-window. **Step 4**: Models of increasing order are fit to measures of SC and BIC are employed to select model order. The process is repeated for 900 BSs to define confidence intervals of developmental trajectories.

peak-maturation age did not overlap. To test for quantitative differences in SC measures we identified the 120 couples of windows that were closest in terms of mean-age. We then computed p-values for each measure as the proportion of overlap in bootstrapping-derived distributions, between the 2 populations.

Correlation With Trajectories of Working-Memory

Working-Memory (WM) performance was measured using the Wechsler Digit Span subtest, backward part, considering raw-scores (Wechsler, 1991, 1997). In this task, participants were asked to repeat backward a gradually increasing set of numbers. WM was tested at each scanning session and WM scores were available for 206/211 visits in HCs and 216/221 visits in 22q11DS.

To characterize developmental trajectories of WM we firstly employed mixed model linear regression at the level of individual subjects in the two populations. Detailed description of the specific algorithm employed is available in previous work (Kremen et al., 2010). Briefly models of increasing order (from constant to cubic) were fit to WM scores and a BIC based approach was used to select optimal model order. Hence a likelihood ratio test was employed to test differences in both curve shape, also known as an interactions effect, and in curve intercept, also known as group effect. To qualitatively characterize differences in curve shape, we furthermore plotted the derivatives of mean developmental curves that express the rate of WM maturation as a function of age in the two populations.

To allow a direct comparison with SC, mean WM scores were furthermore computed in each window using the previously described sliding window approach. Mean WM was then correlated with measures of SC computed in each window using Pearson's correlation. Finally to define CIs, correlations between WM and SC were recomputed for 900 BSs. Correlations were considered significant if the null hypothesis could be rejected at $p < 0.05$ in at least 95% BSs. Moreover to exclude that age was not exclusively responsible for correlations between WM performance and SC, we repeated correlations after accounting for the effect of age.

Correlation With Severity of Internalizing Psychopathology in 22q11DS

Severity of internalizing psychopathology was measured with the Child Behavior Checklist (CBCL) (Achenbach, 1991), filled out by parents of patients with 22q11DS younger than 18 years of age. Patients with 22q11DS older than 18 filled out the Adult Behavior Checklist (ABCL) (Achenbach and Rescorla, 2003). To account for systematic inconsistencies across the two instruments values obtained from CBCL and ABCL were separately z-scored prior to being merged. Subsequently the same statistical procedure

adopted for WM scores was employed to describe developmental trajectories of internalizing psychopathology, using both mixed models linear regression and the sliding-window approach. Finally, as described for WM, internalizing symptom scores computed from the sliding window approach were correlated with measures of SC for each of 900 BSs, before and after accounting for the effect of age. Correlations were considered significant if the null hypothesis could be rejected at $p < 0.05$ in at least 95% BSs.

RESULTS

Developmental Trajectories of Mean Connectivity Strength

Mean network connectivity strength in HCs showed a significant quadratic developmental curve with peak maturation occurring at [15.1–16.5] Years of Age (YoA). In 22q11DS, **MCS** showed an altered cubic development with a first negative peak at [10.9–11.2] YoA, followed by a second positive peak at [17.7–17.9] YoA. Overlapping developmental curves between patients and controls revealed a significantly higher mean connectivity in 22q11DS for 29 age-windows, mostly (23/29) located between 14.8 and 18.1 YoA (See **Figure 2**).

Developmental Trajectories of Network Architecture

MCC in HCs showed a quadratic trajectory of development peaking at [15.2–17.3] YoA. In 22q11DS, MCC showed a deviant cubic development with a first negative peak at [10.35–10.78] YoA, followed by a positive peak at [17.5–17.71] YoA. Direct comparison of two curves revealed a significantly higher MCC in

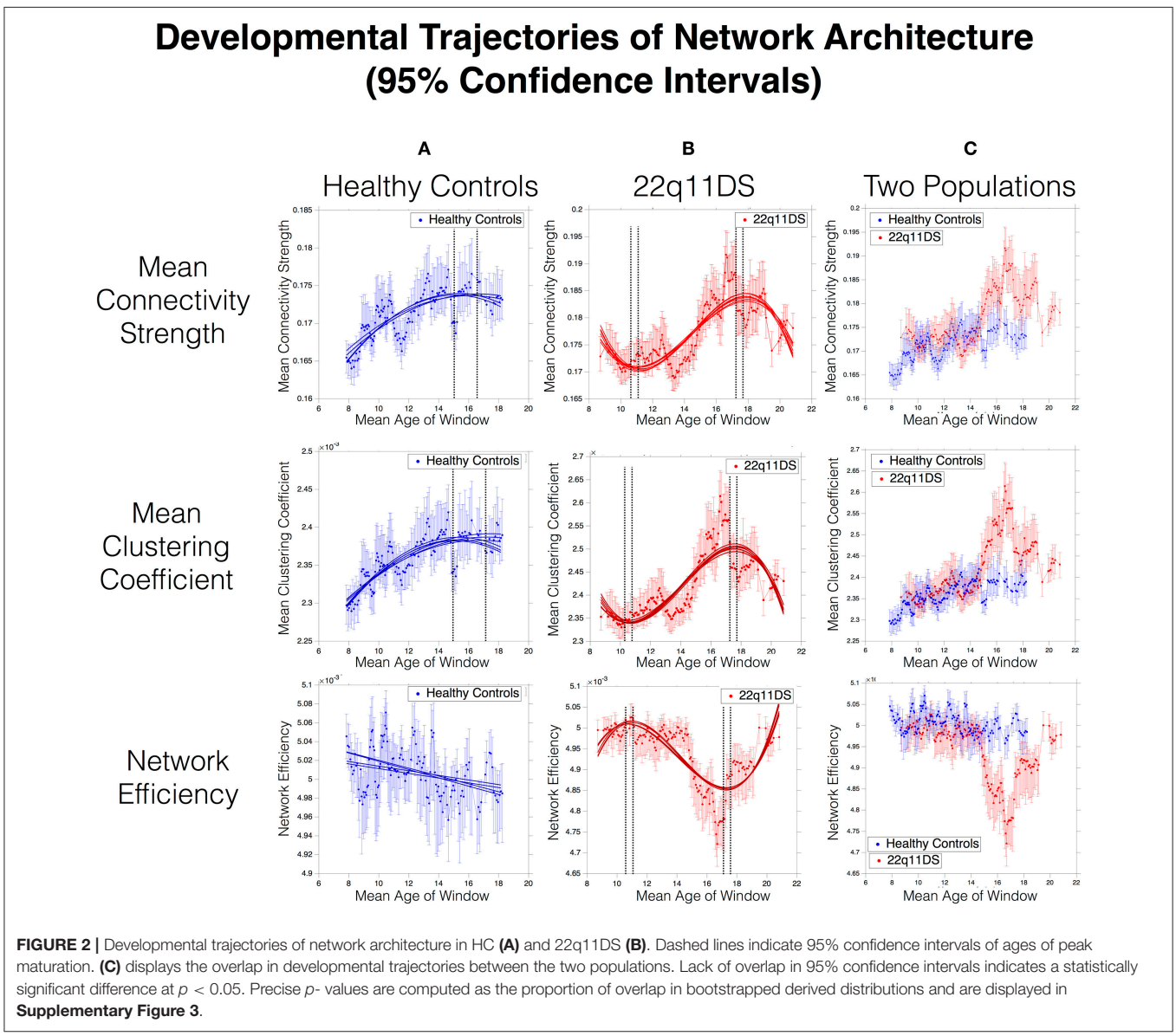

FIGURE 2 | Developmental trajectories of network architecture in HC **(A)** and 22q11DS **(B)**. Dashed lines indicate 95% confidence intervals of ages of peak maturation. **(C)** displays the overlap in developmental trajectories between the two populations. Lack of overlap in 95% confidence intervals indicates a statistically significant difference at $p < 0.05$. Precise p- values are computed as the proportion of overlap in bootstrapped derived distributions and are displayed in **Supplementary Figure 3**.

22q11DS for 22 age-windows spanning between 14.8 and 18.15 YoA (See **Figure 1**).

In HCs, NE showed a linear decrease throughout the age range. In 22q11DS, maturation of NE was altered and was best captured by a cubic trajectory with a first negative peak at [10.69–10.99] YoA, followed by a positive peak at [17.25–17.42] YoA. Overlapping the two curves revealed that NE was significantly lower in 22q11DS ($p < 0.05$) for 30 age-windows mostly (25 out of 30) located between 14.9 and 18 YoA (See **Figure 1**).

Clustering of Regions According to Developmental Trajectories of Connectivity Strength

The silhouette approach identified $K = 2$ and $K = 3$ as the two best cluster solutions for both populations. Here we describe results for the 3-cluster solution, that best characterized differences between populations (See **Figure 3**). Results for the 2-cluster solution are reported in **Supplementary Figure 2**.

In HCs, a first dominant cluster presented a cubic developmental trajectory, with a first positive peak at [12.34–12.73] YoA, followed by a negative peak at [17.12–18.25]. This first cluster encompassed mostly bilateral fronto-parietal regions including bilateral middle and superior frontal gyri and pre-central gyrus, inferior and superior parietal gyrus, the precuneus, cunes, and superior occipital gyrus.

A second cluster of regions showed a more retarded and protracted linear maturation and included the bilateral middle and inferior temporal gyri, fusiform cortices, anterior cingulate cortex (ACC), orbito-frontal cortex (OFC), sub-parietal sulcus, and left inferior parietal lobule.

A third cluster of regions, that was characterized by the weakest connectivity strength, encompassed bilateral insular cortices, parahippocampal giri, posterior cingulate gyri and pericallous gyrus and presented a subtle quadratic development with a negative peaking at [13.98–14.83] YoA.

In 22q11DS, the 3-cluster solution revealed a strikingly different developmental pattern.

A first cluster showed a cubic development with a first positive peak at [13.63–14.28] YoA, followed by a second negative peak at [17.41–18.26] YoA. This trajectory is similar to the one observed in HCs. However, this first cluster included exclusively parietal and occipital regions such as bilateral middle and superior occipital gyri, occipital poles, cuneus, inferior and superior parietal gyri and post-central gyri.

Frontal regions, on the other hand, were grouped with a second cluster that showed a more postponed and protracted linear development. This second cluster encompassed the bilateral middle and superior frontal gyri, precentral gyrus, along with several regions that showed a comparable developmental trajectory in HCs, such as the middle and inferior temporal gyri, fusiform cortex, OFC, parietal lobule and right ACC.

Lastly, a third cluster of regions was characterized by weaker connectivity strength along with a subtle quadratic development throughout the age range. This last cluster encompassed the insular cortex, inferior frontal gyrus, gyrus rectus, parahippocampal gyrus, left anterior and posterior cingulate cortex and left superior temporal gyrus.

Developmental Trajectories of Working Memory Performance

Overall WM performance was significantly lower in 22q11DS compared to HCs, as estimated by a significant group effect ($p < 0.0001$). WM underwent a quadratic developmental trajectory in both populations with the strongest increase occurring in late childhood and early adolescence (See **Figure 4**). However the shape of the trajectory was significantly different in 22q11DS (p of interaction = 0.01). Inspection of the derivatives of mean developmental trajectories revealed that rate of WM development was significantly reduced in 22q11DS particularly during late childhood and early adolescence. By late-adolescence/early adulthood rate of WM development was similar across the two populations. A similar picture was depicted when estimating developmental trajectories with the sliding-widow approach (See **Figure 4**).

Correlation of Working Memory and Network Architecture

In HCs before accounting for the effect of age, WM performance was positively correlated with **MCC** ($r = [0.73/0.62]$ $p < 0.001$) and **MCS** ($r = [0.73/0.62]$, $p < 0.001$) whereas it was negatively correlated with **NE** ($r = [-0.43/-0.27]$, $p = [<0.0001/0.001]$). After accounting for the effect of age, WM remained positively correlated with **MCC** ($r = [0.43/0.23]$, $p = [<0.0001/0.006]$) and **MCS** ($r = [0.42/0.25]$, $p = [<0.0001/0.003]$) whereas correlation with **NE** became positive and non-significant ($r = [0.33/0.12]$, $p = [0.0001/0.135]$) (See **Figure 5**).

In 22q11DS **MCC** ($r = [0.64/0.56]$ $p < 0.0001$) and **MCS** ($r = [0.62/0.53]$ $p < 0.0001$) were positively correlated while NE was negatively correlated ($r = [-0.5/-0.4]$ $p < 0.0001$) with WM before accounting for the effect of age. However after accounting for the effect of age WM was not significantly correlated with either **MCC** ($r = [-0.08/0.08]$ $p = [0.28/0.97]$), **MCS** ($r = [-0.16/0.01]$ $p = [0.04/0.94]$) or NE ($r = [0.14/-0.01]$ $p = [0.07/0.97]$) in 22q11DS.

Correlation of Working Memory and Local Connectivity Strength

As pertains to local connectivity strength in HCs, connectivity of the (blue) fronto-parietal cluster ($R = [0.64/0.57]$ $p < 0.0001$) and the (green) ACC-OFC cluster ($R = [0.8/0.74]$ $p < 0.0001$) were positively correlated with WM whereas connectivity of the yellow cluster was negatively correlated with WM ($R = [-0.39/-0.25]$ $p = [<0.0001/0.0027]$) (See **Figure 6**). However after accounting for the effect of age only the blue fronto-parietal cluster remained positively correlated with WM ($R = [0.73/0.63]$ $p < 0.0001$) while no correlation was observed for the green cluster ($R = [0.11/-0.06]$ $p = [0.98/0.1858]$)

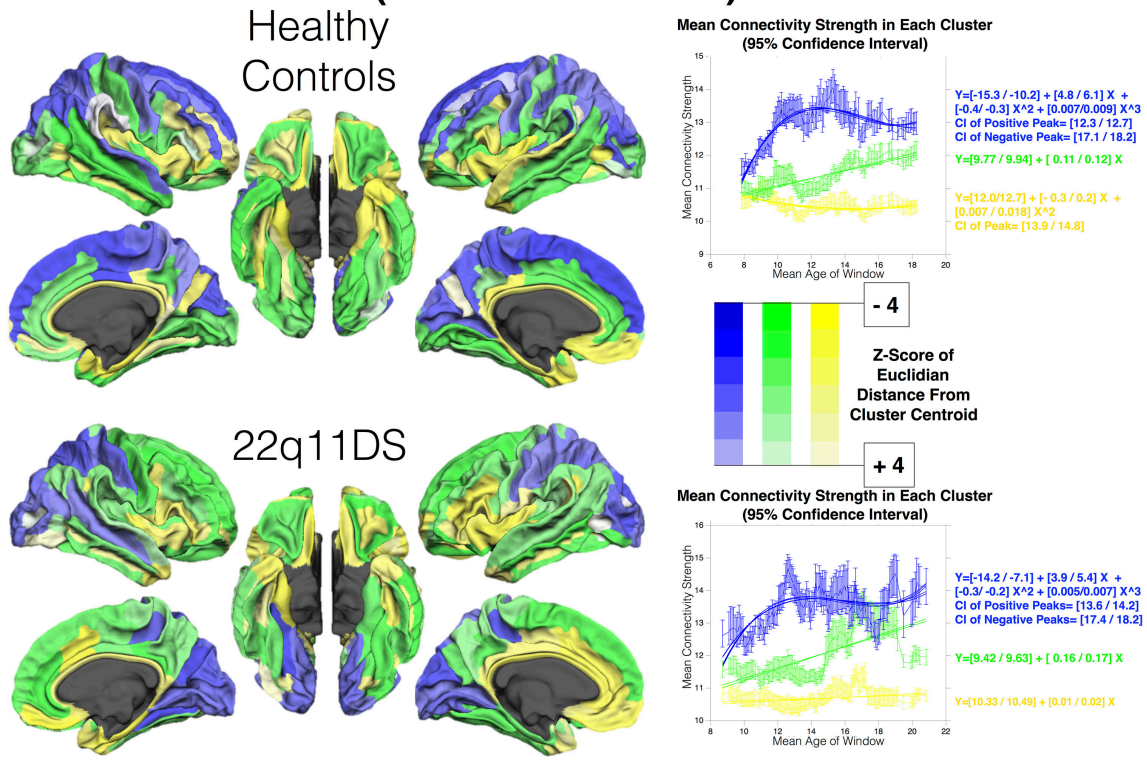

FIGURE 3 | Clustering of regions according to developmental trajectories of connectivity strength in HCs and patients. Color-coding (green, blue, and yellow) indicates correspondence between cluster and developmental trajectory. Regions are shaded according to Z-score of mean Euclidian distance from cluster centroid computed over 900 bootstrapped samples, which is indicative of how closely maturation of each region is reflected in that of the corresponding cluster.

and a negative correlation was observed for the yellow cluster ($R = [-0.47/-0.31]$ $p = [<0.0001/0.00)$.

In 22q11DS connectivity of the blue ($R = [0.55/0.44]$ $p < 0.0001$) and the green ($R = [0.65/0.58]$ $p < 0.0001$) clusters were positively correlated with WM whereas no significant correlation was observed for the yellow cluster ($R = [0.09/0.22]$ $p = [0.22/0.005]$). After accounting for the effect of age only the blue parito-occipital cluster remained positively correlated with WM ($R = [0.48/0.33]$ $p < 0.0001$) whereas no significant correlation was observed for the green cluster ($R = [-0.19/-0.04]$ $p = [0.01/0.55]$) and a negative correlation was observed for the yellow cluster ($R = [-0.41/0.25]$ $p = [<0.001/0.002]$).

Correlation of SC and Severity of Internalizing Symptoms in 22q11DS

According to mixed linear regression severity of internalizing symptoms remained stable with age in 22q11DS. However the sliding window approach revealed a more complex pattern with reduced symptom severity during childhood and a transient increase in internalizing symptom severity during mid to late adolescence (See **Figure 7**, Column **A**).

Before accounting for the effect of age severity of internalizing symptoms was negatively correlated with **MCC** ($r = [-0.28/-0.18]$, $p = [0.0004/0.02]$) whereas no significant correlation was observed for NE ($r = [0.18/0.08]$, $p = [0.02/0.3]$) or for **MCS** ($r = [-0.24/-0.13]$, $p = [0.001/0.09]$). However after accounting for the effect of age severity of internalizing symptoms was positively correlated with both **MCC** ($r = [0.34/0.21]$, $p = [<0.0001/0.007]$) and **MCS** ($r = [0.41/0.28]$, $p = [<0.0001/0.004]$) and negatively correlated with NE ($r = [-0.31–/0.20]$, $p = [<0.0001/0.01]$) (See **Figure 7**, Column **B,C**).

As pertains to local connectivity strength, before accounting for the effect of age, internalizing symptom severity was negatively correlated with connectivity of both the blue parieto-occipital cluster ($r = [-0.58/-0.48]$, $p < 0.0001$) and green cluster ($r = [-0.25/-0.17]$, $p = [0.001/0.03]$) whereas no significant correlation was observed for the yellow cluster ($r = [0.18/0.05]$, $p = [0.02/0.47]$). However after accounting for the effect of age internalizing symptom severity remained negatively correlated with connectivity of the blue parieto-occipital cluster ($r = [-0.48/-0.35]$, $p<0.0001$) but was positively correlated with connectivity of both the green ($r = [0.46/0.37]$, $p < 0.0001$) and yellow ($r = [0.45/0.31]$, $p < 0.0001$) clusters (See **Figure 7**, Column **D**).

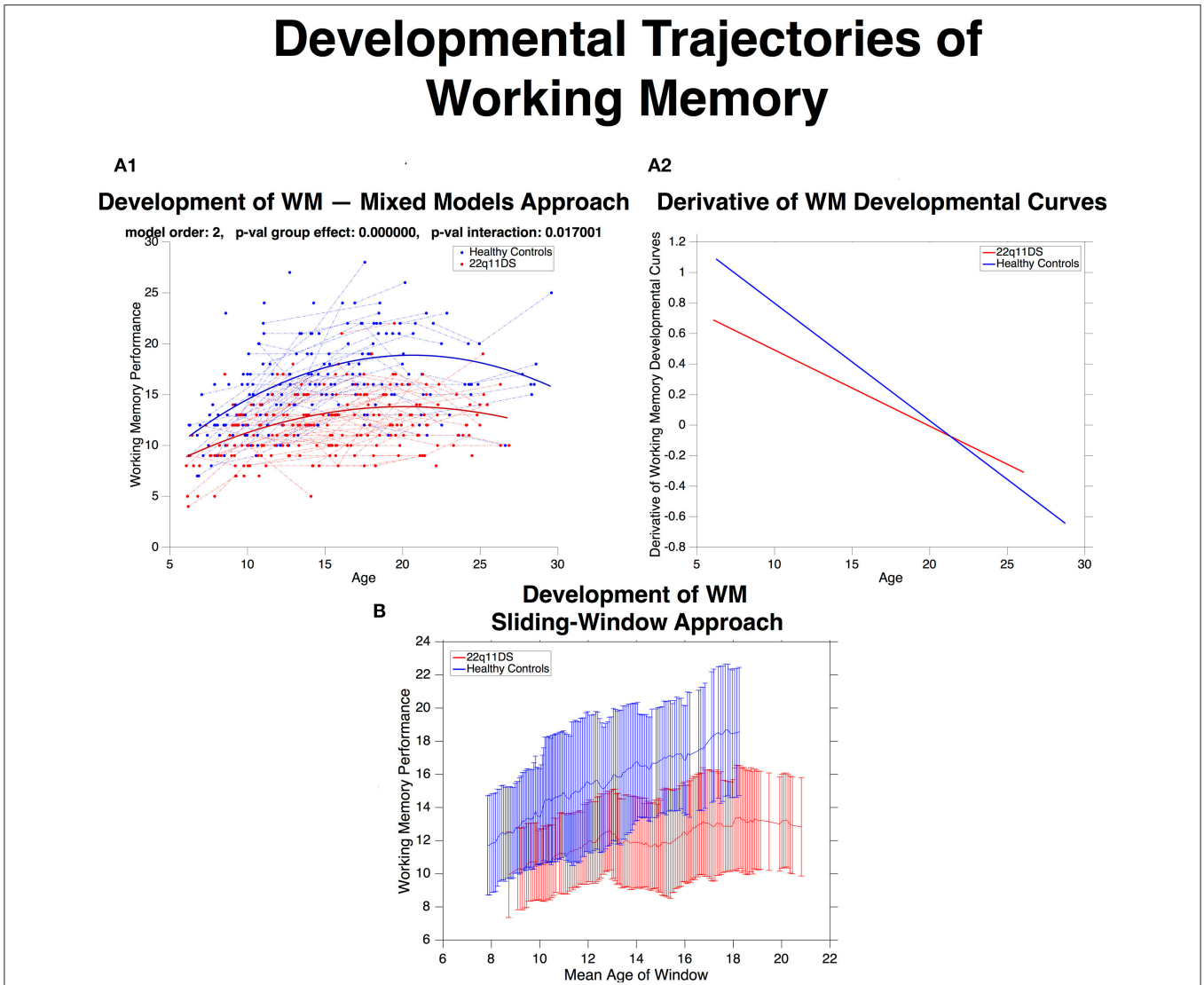

FIGURE 4 | (A.1) Developmental trajectories of working memory (WM) described using mixed-model linear regression for HCs in blue and 22q11DS in red. WM is on average lower in 22q11DS (p-val group effect < 0.0001) and undergoes aberrant development with age (p-val interaction $= 0.01$). **(A.2)** Derivatives of WM developmental curves, express mean rate of WM maturation as a function of age for HCs in blue and 22q11DS in red. Strongest differences in rate of WM development are observable at the youngest ages, during late-childhood and early adolescence while by late adolescence rate WM maturation is similar between the two populations. **(B)** Developmental trajectories WM are estimated using the same sliding-window approach used to compute structural covariance. Error-bars (HCs in blue and 22q11DS in red) indicate mean ± standard deviation of WM scores in each window.

DISCUSSION

In this work, we implement a state-of-the are sliding window approach to investigate developmental trajectories of SC networks in a large cohort of patients with 22q11DS and HCs.

We will first discuss findings in HCs, in relation to typical trajectories of cognitive maturation and offering hypotheses on the neurobiological processes underlying SC maturation. We will then discuss how this development deviates in patients with 22q11DS. We advance that disturbed SC maturation may contribute to core developmental features of the syndrome, including disturbed cognitive maturation during childhood and internalizing psychopathology and psychosis predisposition during adolescence.

Maturation of Structural Covariance Network Architecture in Healthy Controls

Our results in HCs point to late-childhood and early-adolescence as critical periods for the maturation of SCNs. Indeed, until early-adolescence, networks underwent a significant increase in mean correlation strength along with a prominent increase of MCC, indicative of a more segregated and less random organization. Architectural maturation was also reflected by a decrease in NE, that was however more gradual throughout

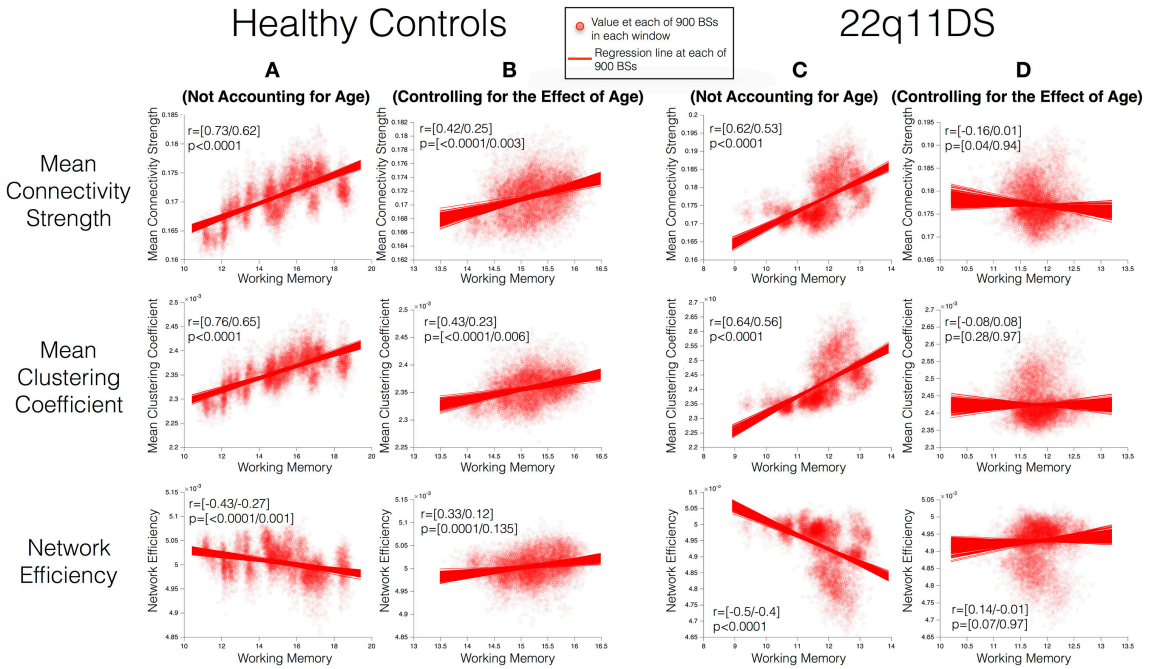

FIGURE 5 | Correlation between structural covariance network architecture and working memory. Method: Mean WM scores and measures of SC network architecture are computed in each window and correlated using Pearson's correlation. The process is repeated for 900 BSs in each window to define confidence intervals of correlations. Correlations are furthermore repeated after controlling for the effect of age. **(A)** Correlations in HCs before controlling for age. **(B)** Correlations in HCs after controlling for age. **(C)** Correlations in 22q11DS before controlling for age. **(D)** Correlations in 22q11DS after controlling for age.

the examined age range. Late-childhood re-organization of SCN architecture was previously reported in a large cross-sectional cohort (Khundrakpam et al., 2013). Our results firstly replicate this finding in a longitudinal sample, furthermore allowing a more precise characterization of developmental trajectories.

From the perspective of cortical development, the period of late-childhood has until recently been relatively overlooked, mainly due to histological studies reporting little changes in neuronal or synaptic organization during this time period (Huttenlocher, 1979; Rakic et al., 1986; Huttenlocher and Dabholkar, 1997; Petanjek et al., 2011). Indeed synaptogenesis is completed by early-childhood while most synaptic pruning occurs after the onset of adolescence (Huttenlocher, 1979; Rakic et al., 1986; Huttenlocher and Dabholkar, 1997; Petanjek et al., 2011). However, late-childhood is a period of important maturation for multiple cognitive domains, including verbal and non-verbal intelligence, attentional performance and executive functions (Chelune and Baer, 1986; Anderson, 2002; Crone et al., 2006). A possible interpretation for this discrepancy is that late-childhood may be a critical period for synaptic fine-tuning (Changeux and Danchin, 1976). During synaptic fine-tuning, functionally relevant synapses are stabilized in order to be protected from subsequent pruning during adolescence. Interestingly, it was proposed that late-childhood maturation of SCNs could capture this process of synaptic fine-tuning (Khundrakpam et al., 2013), which is critical for cognitive maturation.

An alternative interpretation is that the reorganization of SCNs is reflecting white matter maturation. Indeed, developmental trajectories of white matter from childhood to adulthood are best described by quadratic curves, with most of the development occurring precociously, during childhood and early adolescence (Lebel and Beaulieu, 2011; Lebel et al., 2012). Furthermore SC has shown considerable overlap with white matter connectivity (Sui et al., 2014).

Importantly SC network architecture was correlated with WM performance suggesting a potential functional relevance of SC maturation. A more segregated and organized SC network architecture has been previously associated with higher cognitive performance in childhood and adolescence (Khundrakpam et al., 2016). When considering that in HCs network segregation significantly increases during childhood and early adolescence, correlation between MCC and WM suggests that maturation of network architecture could contribute to cognitive development during this critical developmental period. On the other hand NE underwent a linear decrease with age, but showed a non-significant positive correlation with WM when the effect age was accounted for. Recent findings pointed to a positive correlation

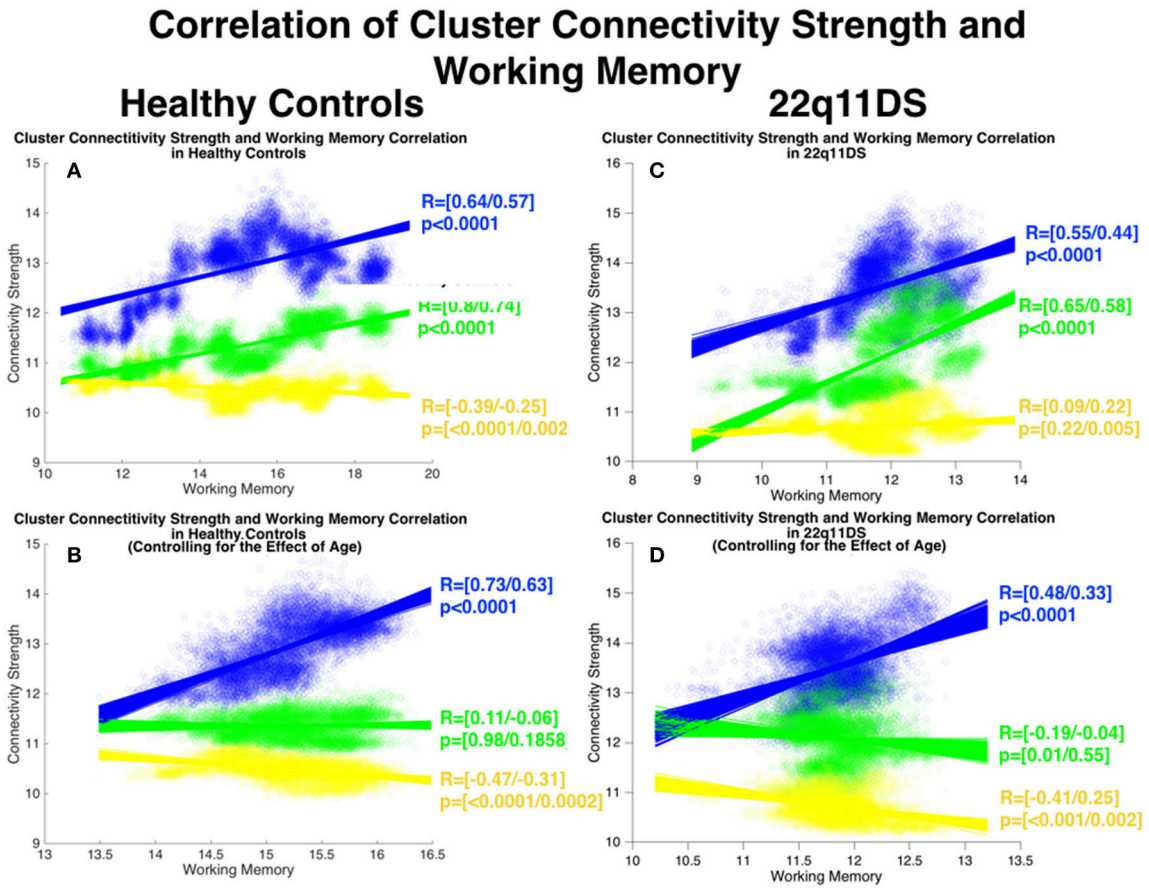

FIGURE 6 | Correlation between structural covariance local connectivity strength and working memory. Method: Mean connectivity strength in each cluster and mean WM scores are computed in each window and are correlated using Pearson's correlation. The process is repeated for 900 BSs in each window to define confidence intervals of correlations. Correlations are furthermore repeated after controlling for the effect of age. **(A)** Correlations in HCs before controlling for age. **(B)** Correlations in HCs after controlling for age. **(C)** Correlations in 22q11DS before controlling for age. **(D)** Correlations in 22q11DS after controlling for age.

between cognitive performance and SC NE (Khundrakpam et al., 2016). Our results, albeit at trend level, tend to confirm that higher SC NE is associated with better WM irrespective of age. However, when considering developmental trajectories, our results suggest that maturation of NE is not strongly implicated in development of WM, at least in the examined age range.

Deviant Maturation of Structural Covariance Network Architecture in 22q11DS

Trajectories of SC network architecture were altered in 22q11DS with a lack of development during childhood, followed by a prominent reorganization during adolescence. Network development was not only postponed, but also aberrant with patients presenting increased connectivity strength coupled with excessive segregation and insufficient integration, for several age-windows, during mid-to-late adolescence.

In a previous cross-sectional investigation of SC in 22q11DS, we reported increased correlation strength, coupled with decreased architectural integration and increased segregation, that selectively affected patients presenting prodromal psychotic symptoms both compared to HCs and non-psychotic patients (Sandini et al., 2017). Several studies investigating SC in patients suffering from psychotic symptoms have also reported increased correlation strength (Wible et al., 1995, 2001; Buchanan et al., 2004; Mitelman et al., 2005a,b, 2006; Modinos et al., 2009; Zugman et al., 2015), along with increased architectural segregation and decreased integration (Bassett et al., 2008; Zhang et al., 2012). Increased segregation and decreased integration are furthermore consistent with reports of white-matter dysconnectivity in idiopathic psychosis (van den Heuvel and Fornito, 2014) and in 22q11DS (Ottet et al., 2013b; Váša et al., 2016) as well as with findings of increasingly segregated functional networks in 22q11DS (Scariati et al., 2016b).

Here disturbed network architecture was correlated with higher internalizing symptoms after accounting for the effect of age. High internalizing psychopathology, including anxiety, depression, social withdraw, and thought disorders represents a hallmark of psychiatric phenotype of 22q11DS (Shashi et al., 2012; Klaassen et al., 2013, 2015). Moreover internalizing symptoms such as anxiety, exert a prominent negative impact

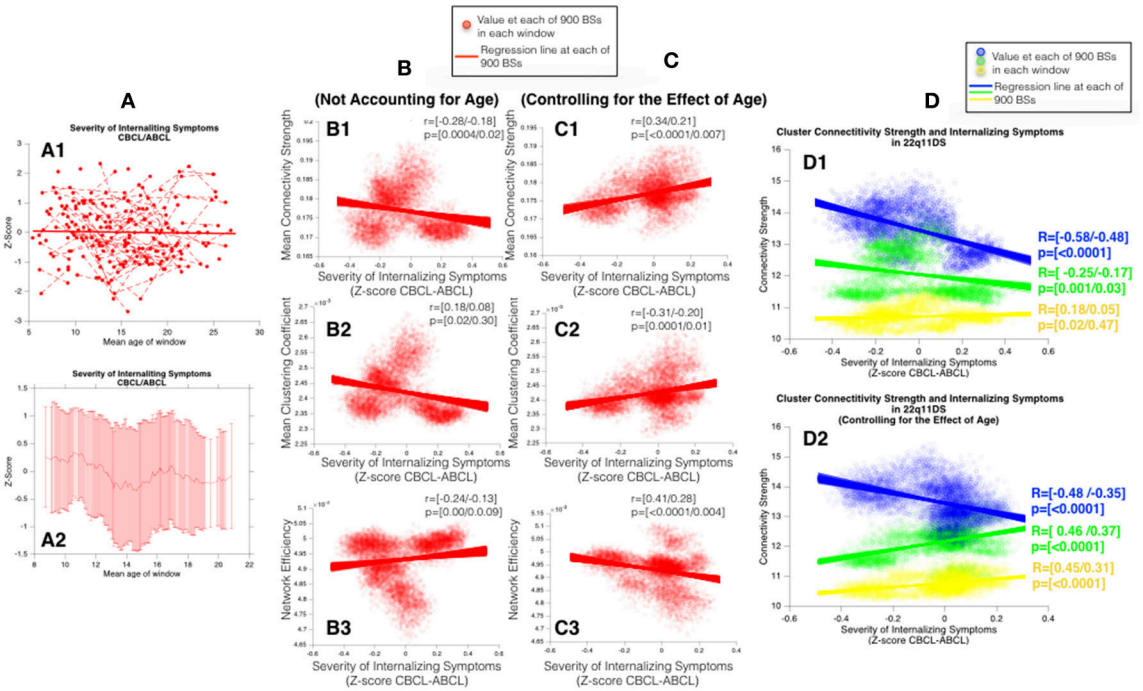

FIGURE 7 | Correlation of structural covariance and internalizing psychopathology in 22q11DS. **(A)** Developmental trajectories of internalizing symptoms as quantified from the CBCL/ABCL subscale. Measures obtained from the two instruments are separately z-scored prior to being merged. **(A1)** Mixed models linear regression approach **(A2)** Sliding Window Approach. **(B)** Correlation of internalizing psychopathology and network architecture before accounting for the effect of age **(B1)** MCS, **(B2)** MCC, **(B3)** MCS. **(C)** Correlation of internalizing psychopathology and network architecture after accounting for the effect of age **(C1)** MCS, **(C2)** MCC, **(C3)** MCS. **(D)** Correlation of structural covariance local connectivity strength and internalizing psychopathology **(D1)** Before accounting for the effect of age **(D2)** After accounting for the effect of age.

on overall functioning (Angkustsiri et al., 2012) and represent a strong risk factor for the subsequent development of psychosis in 22q11DS (Gothelf et al., 2007, 2013). Internalizing symptoms have furthermore been shown to increase in prevalence during adolescence in 22q11DS (Duijff et al., 2013). Our finding suggest that the development of disturbed network architecture, with insufficient integration and excessive segregation, could contribute to the emergence of internalizing symptoms and potentially to increased vulnerability to psychosis during adolescence in 22q11DS. Indeed disturbed architecture could lead to an insufficient integration of signals originating from functionally specialized sub-networks, which could in turn predispose to the emergence of the syndrome's psychiatric phenotype (Sandini et al., 2017).

A second observation is that the aberrant development of network architecture during adolescence is preceded by a lack of typical maturation during late-childhood. As discussed in the previous section, late-childhood is the critical period for the maturation of SCNs in HCs (Khundrakpam et al., 2013) and development of particularly MCC could contribute to improved WM in this period. The lack of architectural development during childhood in 22q11DS could therefore contribute to the blunted WM maturation observed in this population. However measures of SC network architecture were not significantly correlated with WM performance in 22q11DS, after accounting for the effect of age.

From the perspective of the underlying neurobiology, if SCNs maturation were indeed capturing a process of synaptic stabilization (Changeux and Danchin, 1976), insufficient late-childhood network maturation in 22q11DS could reflect impairments in this process. Interestingly, synaptic instability has been reported in LGDel± mouse models of 22q11DS (Moutin et al., 2016). A similar deficit of synaptic stabilization might therefore also be affecting patients with 22q11DS, and manifest with a lack of network reorganization observed during late-childhood. Synaptic instability could then potentially predispose to aberrant SC development and vulnerability to internalizing psychopathology and psychosis during adolescence. Indeed, recent neuro-pathological evidence has suggested that synaptic deficits, that are highly replicable in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000; Rosoklija et al., 2000; Black et al., 2004; Glausier and Lewis, 2013), might be linked to insufficient synaptic stabilization (MacDonald et al., 2017).

Moreover disturbed SC maturation during late-childhood could also be reflective of deficient white-matter maturation, that might contribute to typical development of SC in HCs. Although several cross-sectional investigations have consistently reported white matter dysconnectivity in 22q11DS (Ottet et al., 2013a,b; Scariati et al., 2016a; Váša et al., 2016) so far no longitudinal studies have been conducted. Our results could suggest that developmental trajectories of white-matter connectivity start to diverge during late-childhood potentially inducing vulnerability to the emergence of psychosis during adolescence.

Developmental Trajectories of Local Connectivity Strength in Healthy Controls

In HCs, a first set of regions displayed a precocious cubic maturation, with increase in connectivity up to late-childhood, followed by a more subtle pruning during adolescence and a stabilization by early adulthood. Recent work, employing a similar sliding window approach, reported comparable trajectories for SC connectivity maturation for regions corresponding to this first cluster, albeit limited to the age range between late adolescence to early adulthood. From a functional perspective this first cluster was mainly composed of frontal-parietal regions, strongly resembling the Central Executive Network (CEN) (Seeley et al., 2007; Menon, 2011). The CEN is critical for goal-directed cognitive processes also known as executive functions (EFs) (Wager and Smith, 2003; Fan et al., 2005; Müller and Knight, 2006; Markett et al., 2014; McKenna et al., 2017). EFs, and particularly (WM), undergo dramatic improvements during late-childhood as described both in our cohort (See **Figure 4**) and previous literature (Chelune and Baer, 1986; Crone et al., 2006; Tamnes et al., 2013; Ullman et al., 2014). Moreover morphological and connectivity maturation of the CEN have been specifically correlated with the development of WM (Crone et al., 2006; Tamnes et al., 2013; Ullman et al., 2014). In accord with these findings, we observe a significant positive correlation between SC connectivity of the CEN and WM performance, even when accounting for the effect of age. When considering developmental trajectories, our findings suggest that SC maturation of the CEN might contribute to the critical **WM** improvements observed during late-childhood in HCs.

A second bilateral group of regions showed a more postponed and protracted linear maturation. Connectivity of this cluster was not significantly correlated with WM performance after accounting for the effect of age. However, ACC and OFC, included in this cluster, are critical for cognitive processes underlying decision-making (Wallis and Kennerley, 2011; Khani et al., 2015) that continue to mature into early-adulthood (Blakemore and Robbins, 2012). Furthermore the inferior temporal cortices together with the ACC, sub-parietal sulcus, and inferior parietal lobule are key nodes of the default-mode-network (DMN) (Lee et al., 2013). The DMN is involved in self-referential cognitive processes (Raichle, 2015) that also continue to mature throughout adolescence (Dumontheil et al., 2010). Moreover white-matter tracts connecting most of these regions such as the cingulate bundle, or the uncinate fasciculus, continue to mature until early-adulthood (Lebel and Beaulieu, 2011), potentially explaining the delayed development of SC.

On the other hand the Salience Network, involved in the attributing subjective salience to internal and external events and classically encompassing the dorsal anterior cingulate cortex (dACC) and anterior insula (AI)(Uddin, 2015), did not appear to display a coherent developmental trajectory in HCs. Indeed while the dACC displayed a linear increase in connectivity strength, together with other DMN related regions, the AI presented a different and more subtle negative quadratic development.

Deviant Developmental Trajectories of Local Connectivity Strength in 22q11DS

Developmental trajectories of local connectivity strength were altered in 22q11DS. Indeed, compared to controls, the cluster showing a more precocious maturation included exclusively bilateral parietal and occipital regions. Frontal regions, on the contrary, were mostly included in a second cluster showing no maturation during childhood followed by increased connectivity strength during mid-to-late adolescence.

Frontal cortical dysmaturation was previously reported by our group in 22q11DS (Schaer et al., 2009). Indeed individuals with 22q11DS were found to undergo a lack of typical cortical maturation during childhood, leading to excessive cortical thickness, which was followed by accelerated cortical thinning during adolescence (Schaer et al., 2009). This pattern was particularly striking at the level of the pre-frontal cortex (Schaer et al., 2009). Our findings are therefore consistent with notion of frontal dysmaturation in 22q11DS, but expand it to consider also frontal connectivity.

As discussed in the previous section, morphological and connectivity maturation of the CEN sustain improvements in EFs and **WM**, which are particularly important during late-childhood (Crone et al., 2006; Tamnes et al., 2013; Ullman et al., 2014). Disturbed frontal connectivity maturation might therefore contribute to retarded maturation of EFs and **WM**, occurring during late-childhood in 22q11DS (Maeder et al., 2016).

Moreover in 22q11DS WM was positively correlated selectively with parieto-occipital connectivity, after accounting for the effect of age. This could suggest that in 22q11DS, parieto-occipital connectivity maturation might sustain development of **WM** during childhood, and at least partially compensate for blunted frontal maturation. Indeed two studies suggested that children and adolescents with 22q11DS might rely on more parietal-dependent cognitive strategies, with a conserved parietal activation compared to reduced frontal activation during a similar n-back non-spatial WM task (Kates et al., 2007). Moreover adolescents with 22q11DS were found to present an excessive parietal activation during an arithmetical-reasoning task compared to healthy controls (Eliez et al., 2001). However conflicting evidence has also been reported with children and adolescents presenting reduced parietal activation during a visuo-spatial WM task (Azuma et al., 2009).

A further consideration is that the prominent increase of frontal connectivity during mid to late adolescence, coincides with the emergence of disturbed network

architecture. Connectivity the green fronto-temporal cluster was also positively correlated with severity of internalizing psychopathology. The increase in frontal-temporal connectivity could therefore contribute to the development of architectural disturbances, and corresponding emergence of internalizing psychopathology and vulnerability to psychosis during adolescence. Indeed several regions displaying a postponed maturation, such as the right superior frontal gyrus and right ACC were previously found to presented aberrant connectivity, selectively in patients with 22q11DS with psychotic symptoms (Sandini et al., 2017).

Moreover severity of internalizing symptoms was also positively correlated with connectivity of the yellow cluster encompassing bilateral dACC, AI and fronto-opercular cortex, that represent key regions of the Salience Network (SN) (Uddin, 2015). Disturbances in the process of salience attribution, critically governed by the SN, have been highly implicated in the pathogenesis of psychosis (Kapur, 2003). Moreover recent evidence suggests that increased connectivity of the SN might also be correlated with higher internalizing psychopathology in the peripubertal age-range (Ordaz et al., 2017). Our findings would suggest that higher connectivity of the SN might indeed contribute to increased vulnerability to internalizing psychopathology in 22q11DS, possibly as a consequence of aberrant salience attribution.

Lastly it could be tentatively hypothesized that the lack of frontal maturation during late-childhood might predispose to the subsequent aberrant frontal maturation and emergence of internalizing psychopathology and psychosis vulnerability during adolescence. Indirect support comes from the observation that a decline of frontally mediated cognitive performance, occurring as early as childhood (Kremen et al., 2010; Gur et al., 2014), is a well-documented predictor of psychosis both in 22q11DS (Vorstman et al., 2015) and in the general populations (Riecher-Rössler et al., 2009; Seidman et al., 2016).

CONCLUSIONS

Our findings highlight critical late-childhood maturation of SCNs in HCs that could be instrumental to the significant cognitive maturation occurring during this developmental phase.

In 22q11DS, we observe disturbed development of SCNs with aberrant architecture emerging during adolescence and being preceded by a lack of typical late-childhood maturation. Disturbed development was furthermore particularly striking for frontal lobe connectivity. These results are the first to demonstrate aberrant longitudinal connectivity maturation in 22q11DS, reinforcing the hypothesis that psychosis originates from a neurodevelopmental disorder of connectivity.

LIMITATIONS

The present work comes with one main limitation. Indeed, SC is an inherently population based measure that cannot be computed at the individual level. This consideration strongly limits direct correlations with individual cognitive and clinical variables. More advanced analysis will be required to confirm the functional relevance of our findings at level of individual subjects.

ETHICS STATEMENT

In agreement with Frontiers in Neuroscience guidelines, all participants have given their informed consent, in accordance with protocols approved by the Institutional Review Board of the University of Geneva Medical School.

AUTHOR CONTRIBUTIONS

CS: Performed conceived the study, performed statistical analysis and redacted a first draft of the manuscript; DZ and MarS: Contributed in development of methodological pipeline, in interpretation of results and in drafting the manuscript; ES: Contributed in conceiving the study and in drafting the manuscript; MP and MauS: Contributed in drafting the manuscript and in interpretation of results; DV: Co-supervised the study, contributed in development of methodological pipeline, in interpretation of results and in drafting the manuscript; SE: Co-supervised the study, obtained funding, contributed in development of methodological pipeline, in interpretation of results and in drafting the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00327/full#supplementary-material

Supplementary Table 1 | Demographic characteristics of sample.

Supplementary Table 2 | Prevalence of main psychiatric and neurological diagnoses in 22q11DS sample.

Supplementary Figure 1 | Age distribution of sample.

Supplementary Figure 2 | Developmental trajectories of local connectivity strength (two cluster solution).

Supplementary Figure 3 | *P*-values of differences in network architecture between 22q11DS and Healthy Controls.

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REVIEW
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Common Defects of Spine Dynamics and Circuit Function in Neurodevelopmental Disorders: A Systematic Review of Findings From *in Vivo* Optical Imaging of Mouse Models

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In vivo optical imaging is a powerful tool for revealing brain structure and function at both the circuit and cellular levels. Here, we provide a systematic review of findings obtained from *in vivo* imaging studies of mouse models of neurodevelopmental disorders, including the monogenic disorders fragile X syndrome, Rett syndrome, and Angelman syndrome, which are caused by genetic abnormalities of *FMR1, MECP2,* and *UBE3A*, as well as disorders caused by copy number variations (15q11-13 duplication and 22q11.2 deletion) and BTBR mice as an inbred strain model of autism spectrum disorder (ASD). Most studies visualize the structural and functional responsiveness of cerebral cortical neurons to sensory stimuli and the developmental and experience-dependent changes in these responses as a model of brain functions affected by these disorders. The optical imaging techniques include two-photon microscopy of fluorescently labeled dendritic spines or neurons loaded with fluorescent calcium indicators and macroscopic imaging of cortical activity using calcium indicators, voltage-sensitive dyes or intrinsic optical signals. Studies have revealed alterations in the density, stability, and turnover of dendritic spines, aberrant cortical sensory responses, impaired inhibitory function, and concomitant failure of circuit maturation as common causes for neurological deficits. Mechanistic hypotheses derived from *in vivo* imaging also provide new directions for therapeutic interventions. For instance, it was recently demonstrated that early postnatal administration of a selective serotonin reuptake inhibitor (SSRI) restores impaired cortical inhibitory function and ameliorates the aberrant social behaviors in a mouse model of ASD. We discuss the potential use of SSRIs for treating ASDs in light of these findings.

Keywords: two-photon imaging, calcium imaging, autism spectrum disorders (ASDs), dendritic spines, excitatory-inhibitory balance, serotonin

INTRODUCTION

Neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASDs) and other genetic syndromes, are an etiologically heterogeneous group of neuropsychiatric conditions that manifest very early in life due to the rapid development of brain circuitry during this period (Rutter et al., 2008). While demonstrating many specific symptoms, these disorders often share common deficits such as intellectual disability, epilepsy, sleep disturbances, and abnormal sensory processing. Despite these pronounced deficits, gross brain anatomy often appears largely normal, suggesting that the abnormalities result from relatively subtle changes in connectivity and communication among neurons. The pathogenesis of these disorders thus should be sought at the level of neural circuits and, more specifically, in how neural circuits are initially constructed during development, subsequently refined by experience, and operate when they subserve various cognitive and motor functions. Proper formation, stabilization, function, and remodeling of synapses, each of which is achieved by complex molecular machineries, are essential to ensure the function of these processes.

Neurodevelopmental disorders are caused by various genetic abnormalities and non-genetic factors, such as exposure to toxins and pathogens. To facilitate research, many mouse models that recapitulate the genetic abnormalities known to cause relevant disorders in humans have been established. These genetic mouse models often display phenotypes similar to the symptoms found in individuals with the relevant disorders. Discovering potential targets for effective therapies requires a thorough understanding of these disorders at the molecular, cellular, and neuronal network levels. However, methodologies used to examine the structure and function of the human brain, such as magnetic resonance imaging (MRI), electroencephalography (EEG), and sensory-evoked potentials, do not yet have sufficient spatiotemporal resolution or specificity to distinguish the underlying abnormalities at the cellular level. Alternatively, *in vivo* optical imaging using various microscopic techniques combined with different labeling methods in mice can visualize circuit structure and function in living animals, with cellular and subcellular resolution. This technology is thus well suited for studies that aim to reveal basic pathogenic mechanisms and guide clinical research. In fact, these techniques have been applied successfully to investigate structural and functional abnormalities in several mouse models of NDDs. In this review, we discuss the findings obtained by these studies and explore their implications for a better understanding of the corresponding human disorders.

METHODOLOGIES FOR *IN VIVO* OPTICAL IMAGING

Human postmortem studies have revealed changes in the density and morphology of dendritic spines, while electrophysiological, psychophysical and neuroimaging analyses indicate abnormal sensory processing and dysfunctional activity in a variety of otherwise etiologically distinct NDDs. However, postmortem studies are limited by uncontrolled tissue changes after death and cannot elucidate changes in spine dynamics, while the aforementioned diagnostic techniques lack the resolution to reveal changes at the single-cell and microcircuit levels. Thus, many *in vivo* optical imaging studies of mouse models have examined the structural dynamics of dendritic spines on cortical neurons and the responsiveness of cortical neuronal populations to sensory stimuli. These experiments are often conducted by acquiring images of the same set of fluorescently labeled spines over time, using two-photon microscopy. Alternatively, circuit function can be revealed by imaging changes in signals of fluorescent indicators of neural activity (e.g., calcium- or voltage-sensitive indicators) or intrinsic optical signals from brain tissues during sensory stimulation (**Figure 1**).

Dendritic spines are tiny (∼1 μm) protrusions on dendrites on which synapses form, primarily excitatory glutamatergic synapses. Historically, the density and shape of spines were examined by Golgi's impregnation method, which can reveal the detailed morphology of a subset of neurons in fixed brain tissues. However, these images provide only a "snapshot" of highly dynamic spines, so the information provided by these images may be misleading. For example, normal spine density may be a consequence of abnormally enhanced (or reduced) spine formation and concomitantly increased (or decreased) elimination occurring at balanced rates. Thus, *in vivo* time-lapse imaging is a powerful method to determine whether model mice exhibit abnormalities in spine density, shape, and turnover by tracking the fate of individual spines over time. The spines to be imaged *in vivo* are usually labeled with fluorescent marker proteins expressed by means of transfection using viral vectors or *in utero* electroporation (Cruz-Martín et al., 2010; Isshiki et al., 2014) or by crossing the model mouse with a transgenic reporter mouse line (Pan et al., 2010; Landi et al., 2011; Padmashri et al., 2013; Reiner and Dunaevsky, 2015; Kim et al., 2016; Hodges et al., 2017). Labeled spines can then be imaged by two-photon excitation laser scanning fluorescence microscopy, which can efficiently excite fluorescent molecules within otherwise opaque brain tissues via highly penetrating near-infrared pulsed laser light (Denk and Svoboda, 1997). However, the best resolution is still usually obtained from the superficial spines located in layer (L) 1, within ∼100 μm of the cortical surface, which are nonetheless on apical dendrites of pyramidal neurons in L2–5. Imaged spines are classified into different morphological categories, such as thin (immature), stubby, and mushroom (mature), and their appearance, persistence, and disappearance are analyzed in multiple images acquired at intervals of hours, days, and even weeks. In young adolescent mice, over the course of 2 weeks, approximately 10% of spines imaged on L5 pyramidal neurons are eliminated, while 5-8% of them are formed (Zuo et al., 2005). These rates become smaller in adult mice, showing that 3-5% of imaged spines are eliminated or newly formed. Images of spine dynamics have been acquired from multiple cortical areas, including somatosensory (Cruz-Martín et al., 2010; Pan et al., 2010; Landi et al., 2011;

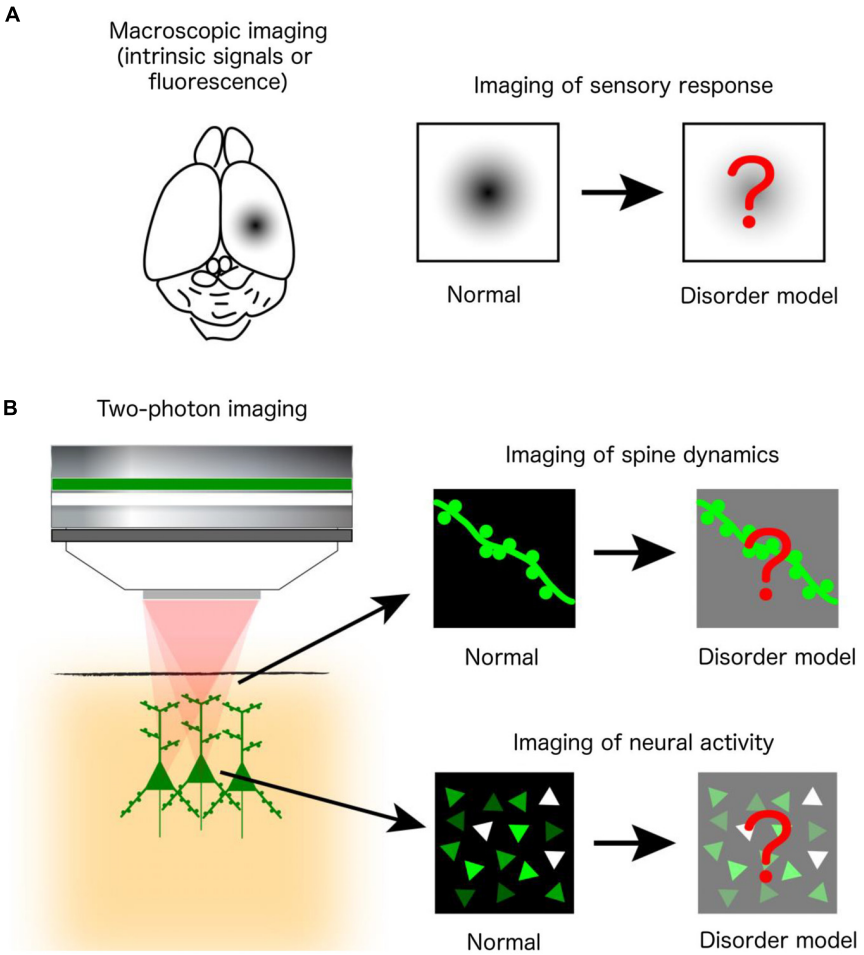

FIGURE 1 | *In vivo* optical imaging in mouse models of NDDs. **(A)** Macroscopic imaging of cortical activity visualizes intrinsic optical signals from brain tissues or the fluorescence of exogenously introduced calcium or voltage indicators, all of which reflect localized brain activity. Studies are designed to identify abnormalities of cortical sensory responses in mouse models of NDDs compared to normal mice. **(B)** Two-photon microscopy at subcellular and cellular resolution images the morphological dynamics of dendritic spines labeled by fluorescent proteins (upper right) or the activity of a population of neurons labeled by fluorescent calcium indicators (lower right).

Isshiki et al., 2014), visual (Kim et al., 2016), motor (Padmashri et al., 2013; Reiner and Dunaevsky, 2015; Hodges et al., 2017) and frontal (Isshiki et al., 2014) cortices, during area-specific postnatal developmental periods. Spine dynamics in the cortex are known to be influenced by sensory experience and learning through the effects of neuronal activity on molecular machinery in spines, and this reorganization of synaptic connections is thought to underlie adaptive changes in circuit functions (Holtmaat and Svoboda, 2009). The effects of sensory input can be examined through various experimental manipulations, such as whisker trimming to alter the degree and topology of somatosensory input (Pan et al., 2010; Isshiki et al., 2014), visual deprivation by eyelid suture or rearing animals in darkness (Kim et al., 2016), or training mice to acquire new motor skills (Padmashri et al., 2013; Reiner and Dunaevsky, 2015; Hodges et al., 2017).

There are also multiple techniques to image neural circuit activity in the living mouse brain. Intrinsic-signal optical imaging visualizes the area and intensity of cortical activity by extracting optical signals intrinsic to brain tissues, such as subtle changes in red light reflectance, that are correlated with local brain activity (Kalatsky and Stryker, 2003; Tropea et al., 2009; Sato and Stryker, 2010; Arnett et al., 2014; Castro et al., 2014; Gogolla et al., 2014; Banerjee et al., 2016). This technique enables the measurement of sensory-evoked activity in neuronal populations, particularly from superficial cortical layers, without the application of exogenous indicator molecules. However, the temporal resolution of this modality is limited to the order of seconds. In contrast, fast spatiotemporal dynamics of cortical electrical activity can be measured at millisecond resolution with voltage-sensitive dyes (VSDs) that change fluorescence in a membrane potential-dependent manner (Zhang et al., 2014; Connor et al., 2016; Lee L.J. et al., 2017). Alternatively, changes in intracellular calcium ion concentrations associated with neuronal activity can be measured with high spatiotemporal resolution using synthetic

calcium-sensitive dyes or genetically encoded calcium indicators (GECIs) introduced into the living brain (Nakai et al., 2001; Looger and Griesbeck, 2012). To label neuronal populations, cell-permeable analogs of synthetic dyes are microinjected locally into the target brain area (Gonçalves et al., 2013; Banerjee et al., 2016). Alternatively, GECIs are expressed by viral vectors (He et al., 2017; Zaremba et al., 2017) or by crossing disorder model mice with transgenic mice expressing the GECI (Nakai et al., 2017). These GECIs are often under the control of promoters specific to certain cell types. *In vivo* calcium imaging can be performed using two-photon microscopy to achieve cellular resolution (Gonçalves et al., 2013; Banerjee et al., 2016; He et al., 2017; Zaremba et al., 2017) or by single-photon excitation macroscopy using charge-coupled device or complementary metal-oxide-semiconductor cameras for wide-field imaging (Nakai et al., 2017). In sum, these techniques allow for a variety of experimental measurements, ranging from imaging spontaneous network activity (Gonçalves et al., 2013; Connor et al., 2016) to sensory encoding (Arnett et al., 2014; Zhang et al., 2014; Banerjee et al., 2016; He et al., 2017; Lee L.J. et al., 2017; Nakai et al., 2017) and neuroplastic changes elicited by altered experience (Tropea et al., 2009; Sato and Stryker, 2010; Castro et al., 2014; Banerjee et al., 2016; Zaremba et al., 2017).

Although it is beyond the scope of this review to discuss all currently available imaging methodologies, *in vivo* brain imaging in mice includes not only optical but also other techniques such as MRI. In particular, resting-state functional MRI (rsfMRI) of the mouse brain is an emerging technique that enables mapping of both local and long-range functional connectivity between distinct brain areas by visualizing anatomical patterns of the low-frequency oscillatory blood-oxygen-level dependent (BOLD) signals associated with spontaneous correlated activity (Gozzi and Schwarz, 2016; Liska and Gozzi, 2016). Although the spatial resolution of this technique is not as high as the above-mentioned optical imaging and its application so far is primarily limited to mapping of intrinsic functional brain networks in anesthetized or sedated animals, it has a great advantage over the optical methods in that it can examine the functional connectivity at the whole-brain scale, including deep subcortical areas that are difficult to access optically. This technique should complement optical methods in future studies such that functional connectivity defects identified by rsfMRI are subsequently examined more closely via two-photon microscopy. A few initial studies using rsfMRI have revealed impaired functional connectivity in mouse models of ASD (Haberl et al., 2015; Sforazzini et al., 2016; Liska et al., 2018).

In the following sections, we review studies on mouse models of NDDs using these *in vivo* optical imaging techniques. Our comprehensive literature searches that covered 35 NDDs (Bishop, 2010) were conducted according to the PRISMA guidelines for systematic reviews (Moher et al., 2009) and identified a total of 22 *in vivo* optical imaging studies in mouse models of fragile X syndrome (FXS), Rett syndrome (RTT), Angelman syndrome (AS), ASDs (including 15q duplication syndrome) and 22q11.2 deletion syndrome (22q11.2DS). The studies selected are summarized in **Table 1**, and the details of the searches are described in the appended footnote.

IN VIVO OPTICAL IMAGING OF FXS MODEL MICE

Fragile X syndrome is the most common inherited mental impairment and also the most common known single genetic cause of ASD, with a total frequency of approximately 1 in 4,000 males and 1 in 8,000 females (Riley et al., 2017). Individuals with FXS exhibit a broad range of symptoms, including intellectual disabilities, autism, macroorchidism, seizures, sensory hypersensitivity, and facial abnormalities such as a long face and large ears (Garber et al., 2008). In almost all cases, FXS is caused by a CGG repeat expansion in the 5′ untranslated region of the fragile X mental retardation 1 gene (*FMR1*) and hypermethylation of its promoter region on the X chromosome, which results in *FMR1* transcriptional silencing and absence of the fragile X mental retardation protein (FMRP) encoded by this gene (Santoro et al., 2012).

Fragile X mental retardation protein is a polyribosome-associated RNA-binding protein localized in the soma and nucleus as well as the dendrites and spines of neurons (Feng et al., 1997). FMRP is also expressed in astrocytes of the developing brain (Pacey and Doering, 2007). It regulates the trafficking and local translation of mRNAs for numerous genes important for synaptic growth, function, and plasticity (Bagni and Greenough, 2005; Darnell and Klann, 2013; Contractor et al., 2015). *Fmr1* knockout (KO) mice, including the often-used male hemizygous *Fmr1*-null ($Fmr1^{-/y}$) mice, lack FMRP expression and recapitulate many behavioral features observed in FXS (Bernardet and Crusio, 2006). Golgi impregnation of cerebral cortical autopsy materials from individuals with FXS has revealed higher spine density and greater numbers of long, thin, immature-looking spines on L3 and L5 pyramidal neurons of parieto-occipital neocortex (Rudelli et al., 1985; Hinton et al., 1991) and on L5 pyramidal neurons of temporal and visual cortices (Irwin et al., 2001). Adult *Fmr1* KO mice exhibited similar morphological defects of spines on visual cortical L5 pyramidal neurons when examined by Golgi staining of fixed tissues (Comery et al., 1997). These spine abnormalities appear to be age-dependent because the aberrantly high spine density and greater spine lengths observed on green fluorescent protein (GFP)-transfected L5 pyramidal neurons of somatosensory barrel cortex during the early postnatal period (1 week of age; Nimchinsky et al., 2001) and on Golgi-stained L5 pyramidal neurons during adulthood (postnatal day 73-75 of age (P73-75); Galvez and Greenough, 2005) were less apparent in juvenile mice at approximately 4 weeks of age (Nimchinsky et al., 2001; Galvez and Greenough, 2005).

Fmr1 KO mice are among the most extensively studied NDD models, and cortical defects are relatively well described at both the molecular and circuit levels (Contractor et al., 2015). *In vivo* two-photon time-lapse imaging of *Fmr1* KO mice

TABLE 1 | *In vivo* optical imaging studies of mouse models of NDDs.

Imaging modality	Disorder	Mouse model	Labeling method	Imaging method	Imaged area	Age	Findings	Reference
Spine	FXS	*Fmr1* KO	*In utero* electroporation	Two-photon	Somatosensory cortex, L2/3 neurons	P7-24	Normal spine density and length. Delayed downregulation of spine turnover and transition from immature to mature spines at P10-12.	Cruz-Martín et al., 2010
	FXS	*Fmr1*$^{-/y}$	Tg mice (YFP-H)	Two-photon	Somatosensory cortex, L5 neurons	3 w.o. -adult	Normal spine density. Enhanced formation and elimination of spines. Increased fraction of transient (immature) spines. Reduced sensitivity of spine formation and elimination to altered sensory experience.	Pan et al., 2010
	FXS	*Fmr1*$^{-/y}$	Tg mice (YFP-H)	Two-photon	Motor cortex, L5 neurons	5 w.o.	Impaired motor learning. Normal density of total spines and filopodia. Enhanced baseline spine formation and elimination. Lack of training-induced increases in spine density and formation.	Padmashri et al., 2013
	FXS	*Fmr1*$^{-/y}$	Tg mice (YFP-H)	Two-photon	Motor cortex, L5 neurons	P35-42	Lack of clustering but normal stabilization of new spines formed after motor skill training.	Reiner and Dunaevsky, 2015
	FXS	Astrocyte-specific *Fmr1*$^{-/y}$	Tg mice (YFP-H)	Two-photon	Motor cortex, L5 neurons	4 w.o. -adult	Normal spine density, normal motor learning, and enhanced basal spine formation in young mice. Increased density of total and thin (immature) spines, impaired motor learning, and lack of enhanced spine formation and elimination during training in adult mice.	Hodges et al., 2017
	FXS	*Fmr1*$^{-/y}$	Tg mice (GFP-M)	Two-photon	Visual cortex, L5 neurons	2-6 m.o.	Enhanced gain and loss of spines. No increased spine turnover was observed in the enriched environment. Rescue of enhanced spine turnover by MMP-9 inhibition.	Nagaoka et al., 2016
	RTT	*Mecp2*$^{-/y}$	Tg mice (GFP-M)	Two-photon	Somatosensory cortex, L5 neurons	P25-40	Reduced spine and filopodia density and reduced short-term changes in spine length and head volume at P25–26. Rescue of short-term spine dynamics defects but not reduced spine density by IGF-1. Reduced spine density and normal short-term spine dynamics at P40.	Landi et al., 2011
	AS	*Ube3a*$^{m-/p+}$	Tg mice (GFP-O)	Two-photon	Visual cortex, L5 neurons	P14-38	Decreased spine density. Normal spine formation and increased spine elimination. Increased fraction of thin spines.	Kim et al., 2016
	ASD	15q dup (also Neuroligin-3 R451C)	*In utero* electroporation	Two-photon	Somatosensory cortex and anterior frontal cortex, L2/3 neurons	2-8 w.o.	Normal spine density. Enhanced gain and loss of PSD-95 containing spines. Reduced sensitivity of spine formation to altered sensory experience.	Isshiki et al., 2014
Activity	FXS	*Fmr1* KO	Synthetic calcium indicator injection	Calcium imaging (two-photon)	Somatosensory cortex, L2/3 neurons (anesthetized and awake)	P9-40	Higher synchrony of spontaneous ensemble activity. Higher proportion of neurons participating in the synchrony. Higher synchrony during sleep.	Gonçalves et al., 2013

(Continued)

TABLE 1 | Continued

Imaging modality	Disorder	Mouse model	Labeling method	Imaging method	Imaged area	Age	Findings	Reference
							Lack of synchrony modulation by anesthesia.	
	FXS	*Fmr1*$^{-/y}$	No labeling	Intrinsic-signal optical imaging	Somatosensory cortex (anesthetized)	12-14 w.o.	Increased area of response to tactile stimulation.	Arnett et al., 2014
	FXS	*Fmr1*$^{-/y}$	Synthetic VSD application	VSD imaging	A large cortical area including somatosensory and motor cortices (anesthetized)	10-16 w.o.	Accelerated spread of tactile-evoked cortical activity.	Zhang et al., 2014
	FXS	*Fmr1* KO	AAV vector-mediated GCaMP6s expression	Calcium imaging (two-photon)	Somatosensory cortex, L2/3 neurons (awake)	P14-adult	Increased avoidance behavior to tactile stimulation and reduced fraction of cells responding to tactile stimulation in young mice. Impaired neuronal adaptation to repetitive tactile stimulation in young and adult mice.	He et al., 2017
	RTT	*Mecp2*$^{-/+}$	No labeling	Intrinsic-signal optical imaging	Visual cortex (anesthetized)	P28-60	Abnormally prolonged plasticity at P60 in response to altered visual experience and rescue by a tripeptide form of IGF-1 or full-length IGF-1	Tropea et al., 2009; Castro et al., 2014
	RTT	*Mecp2*$^{-/y}$ and PV^{+}- or SOM^{+}-interneuron specific *Mecp2*$^{-/y}$	Synthetic calcium indicator injection and no labeling	Calcium imaging (two-photon) and intrinsic-signal optical imaging	Visual cortex (anesthetized and awake), L2/3 neurons	P28-60	Reduced response rate, reliability, selectivity and signal-to-noise ratio of pyramidal neurons to visual stimuli in *Mecp2*$^{-/y}$ mice. Recapitulation of visual response defects in PV^{+}- but not SOM^{+}-specific *Mecp2*$^{-/y}$ mice. Improvement of visual response defects by IGF-1. Abnormally prolonged plasticity of PV^{+}-specific *Mecp2*$^{-/y}$ mice at P60.	Banerjee et al., 2016
	RTT	*Mecp2*$^{-/y}$	Synthetic VSD application	VSD imaging	Somatosensory cortex (anesthetized)	1-2 m.o.	Weaker and more diffuse tactile-evoked responses.	Lee L.J. et al., 2017
	AS	*Ube3a*$^{m-/p+}$	No labeling	Intrinsic-signal optical imaging	Visual cortex (binocular zone) (anesthetized)	P21-37	Lack of rapid plasticity in response to altered visual experience in P25-28 mice. Abnormally immature form of plasticity in P33-37 mice.	Sato and Stryker, 2010
	ASD	15q dup	Tg mice (GLT-1-G-CaMP7)	Calcium imaging (macro-scopic)	Somatosensory cortex (anesthetized)	7-8 w.o.	Reduced magnitude, slower decay and broader area of response to tactile stimulation.	Nakai et al., 2017
	ASD	BTBR T+tf/J (also *Shank3* KO and *Mecp2* KO)	No labeling	Intrinsic-signal optical imaging	Insular cortex (anesthetized)	P16-adult	Impaired multisensory integration and its maturation. Increased area of auditory response. Rescue of impaired integration in the adult by enhanced inhibition early in life.	Gogolla et al., 2014
	ASD	*Mdga2*$^{+/-}$	Synthetic VSD application	VSD imaging	Nearly entire dorsal cortex of one hemisphere (anesthetized)	Adult (6-10 w.o.)	Enhanced spontaneous cortical activity in motor and retrosplenial cortices. Functional hyperconnectivity in lateral cortical areas.	Connor et al., 2016

(Continued)

TABLE 1 | Continued

Imaging modality	Disorder	Mouse model	Labeling method	Imaging method	Imaged area	Age	Findings	Reference
	22q11.2DS	*Df(16)A*$^{+/-}$	AAV vector-mediated GCaMP6f expression	Calcium imaging (two-photon)	Hippocampal CA1 neurons (awake)	8-12 w.o.	Impaired goal-oriented learning. Reduced place cell map stability. Absence of goal-directed reorganization of place cell maps.	Zaremba et al., 2017

FXS, fragile X syndrome; RTT, Rett syndrome; AS, Angelman syndrome; ASD, autism spectrum disorder; 22q11.2DS, 22q11.2 deletion syndrome; Fmr1$^{-/y}$*, male hemizygous Fmr1-null mice; Mecp2*$^{-/y}$*, male hemizygous Mecp2-null mice; Mecp2*$^{-/+}$*, female heterozygous Mecp2-null mice; Ube3a*$^{m-/p+}$*, maternal Ube3a-deficient mice; 15q dup, paternal 15q duplication mice; Mdga2*$^{+/-}$*, heterozygous Mdga2-null mice; Df(16)A*$^{+/-}$*, mice carrying a hemizygous deletion of the syntenic region of mouse chromosome 16; w.o., weeks old; m.o., months old. We comprehensively searched the PubMed database of the U.S. National Library of Medicine (https://www.ncbi.nlm.nih.gov/pubmed/) as of early April 2018 using the keywords "imaging" and "mice" in combination with each of 35 NDDs that were identified from Rutter's Textbook of Child and Adolescent Psychiatry, 5th Edition by Bishop (Rutter et al., 2008; see Table 1 of Bishop, 2010). The number of studies retrieved for each NDD ranged from 0 to 178. We further selected from these studies the ones that conducted cellular, subcellular or macroscopic-resolution optical imaging of brain function or structure in living animals, after which 8, 3, 2, 2 and 1 remained for mouse models of FXS, RTT, AS, ASD and 22q11.2DS. Finally, we added two FXS studies (Padmashri et al., 2013; Nagaoka et al., 2016), two RTT studies (Tropea et al., 2009; Castro et al., 2014) and two ASD studies (Gogolla et al., 2014; Nakai et al., 2017), which are relevant but did not appear in the initial database searches, probably because some keywords were not included in the searchable part of the literature.*

revealed elevated spine turnover (i.e., greater spine formation and elimination) along apical dendritic tufts of L2/3 (Cruz-Martín et al., 2010) and L5 (Pan et al., 2010) pyramidal neurons in the barrel cortex and on L5 pyramidal neurons in the motor cortex (Padmashri et al., 2013) at different ages. Although the overall spine density in these studies appeared surprisingly normal, larger populations of short-lived small spines were observed in *Fmr1* KO mice (Cruz-Martín et al., 2010; Pan et al., 2010). Another study using astrocyte-specific *Fmr1* KO mice reported normal spine density but increased spine formation in young mice as well as higher densities of total and thin spines on the apical dendritic tufts of L5 motor cortex pyramidal neurons in adult mice (Hodges et al., 2017), demonstrating a significant contribution of astrocytic FMRP to the neuronal wiring defects in global *Fmr1* KO mice. Further, these mice demonstrate reduced sensitivity of spine turnover to altered sensory experience (Pan et al., 2010) and motor learning (Padmashri et al., 2013; Hodges et al., 2017). For instance, trimming all whiskers reduced the spine elimination rate of L5 pyramidal neurons in the contralateral barrel cortex in wild-type mice but not in *Fmr1* KO mice, while trimming alternate whiskers in a chessboard-like pattern increased the spine formation rate in wild-type mice but not in *Fmr1* KO mice (Pan et al., 2010). Moreover, training wild-type mice on a forearm-reaching task increased total spine numbers on L5 pyramidal neurons of the motor cortex contralateral to the trained arm through an increase in spine formation rate. Conversely, in *Fmr1* KO mice, motor-skill learning was impaired, and spine number and formation rate were not altered (Padmashri et al., 2013). A follow-up study by the same group reported that the new spines formed after motor skill training in *Fmr1* KO mice lacked clustering on dendrites but exhibited a degree of stabilization similar to that in wild-type mice (Reiner and Dunaevsky, 2015). Impaired motor-skill learning and lack of training-induced increases in spine formation and elimination rates were also observed in adult astrocyte-specific *Fmr1* KO mice trained on a similar task, although learning appeared normal when these mice were trained during adolescence (Hodges et al., 2017).

A recent study reproduced the elevated basal spine turnover and the lack of experience-dependent enhancement in L5 visual cortical pyramidal neurons of *Fmr1* KO mice (Nagaoka et al., 2016) and further reported that the abnormal baseline spine turnover can be rescued by pharmacological inhibition of matrix metalloproteinase-9 (MMP-9). The transport and translation of mRNAs encoding MMP-9 are regulated by FMRP at synapses (Janusz et al., 2013), and pharmacological inhibition and genetic deletion of MMP-9 rescued the dendritic spine and behavioral abnormalities in *Fmr1* KO mice (Bilousova et al., 2009; Sidhu et al., 2014). Moreover, a clinical trial of the antibiotic MMP-9 inhibitor minocycline demonstrated some global benefits to children and adolescents with FXS (Leigh et al., 2013). Together, the findings from these *in vivo* imaging studies demonstrate that the absence of FMRP reduces baseline synaptic stability and impairs experience-dependent and learning-induced spine remodeling in the cortex. These defects support the idea that developing synaptic circuits may not be properly shaped by sensory stimuli and learning in FXS.

Individuals with FXS and *Fmr1* KO mice are also known to display features of hyperexcitability at both neurological and behavioral levels (Bernardet and Crusio, 2006; Garber et al., 2008; Contractor et al., 2015). Functional imaging studies of *Fmr1* KO mice have shed light on potential neural circuit bases for such symptoms. In one study, two-photon calcium imaging of somatosensory cortical networks in early postnatal *Fmr1* KO mice demonstrated greater and more widespread synchrony of spontaneous ensemble activity during both wakefulness and sleep and a delay in the developmental decorrelation of this synchrony (Gonçalves et al., 2013). In adult *Fmr1* KO somatosensory cortex, tactile stimulation evoked larger response regions than in wild-type mice, as revealed by intrinsic-signal optical imaging (Arnett et al., 2014), and accelerated spread of cortical activity, as evidenced by VSD imaging (Zhang et al., 2014). Subsequent electrophysiological recordings revealed that this hyperexcitability can be partly attributed to defects in neuronal dendritic h-channels and BK_{Ca} channels (Zhang et al., 2014). Collectively, these findings suggest that similar circuit and molecular defects in the human brain may underlie FXS symptoms associated with neuronal hyperexcitability, such

as hyperarousal, high susceptibility to seizures, and sensory hypersensitivity. A recent two-photon calcium imaging study of somatosensory cortex in head-fixed behaving *Fmr1* KO mice reported that increased avoidance to tactile stimulation, analogous to the FXS symptom known as tactile defensiveness, was associated with a reduced fraction of cells responding to the stimuli in young mice and with impaired neuronal adaptation to repetitive tactile stimulation in young and adult mice (He et al., 2017).

IN VIVO OPTICAL IMAGING OF RTT MODEL MICE

Rett syndrome is a severe postnatal neurological disorder named after the Austrian pediatrician Andreas Rett, who first described this condition in 1966 (Hagberg et al., 1983). RTT is caused by loss-of-function mutations of the methyl-CpG binding protein 2 gene (*MECP2*), which is located on the X chromosome and encodes a chromatin protein that is involved in epigenetic transcriptional regulation of many genes (Chahrour and Zoghbi, 2007; Lyst and Bird, 2015). MECP2 is expressed broadly throughout the body but most abundantly in mature neurons. RTT is an X-linked dominant disorder and almost exclusively affects females because males hemizygous for *MECP2*-null mutations usually display severe early postnatal encephalopathy and do not survive infancy. RTT affects approximately 1 in 10,000–15,000 girls (Hagberg, 1985), and the vast majority of cases are sporadic. Typical RTT is characterized by apparently normal development during the first 6–18 months of life, followed by a period of regression and then recovery or stabilization of various symptoms, including loss of acquired purposeful hand skills, loss of acquired spoken language, gait abnormalities, and stereotypic hand movements such as wringing, clapping, mouthing and washing (Neul et al., 2010). Individuals with RTT often display a variety of other symptoms, including slowing of the rate of head growth, breathing abnormalities, impaired sleep patterns, and seizures. Some individuals eventually diagnosed with RTT are diagnosed initially with autism, and autistic features such as social withdrawal are more common in less severely affected individuals or those with milder, atypical variants of RTT in which speech is preserved (Neul, 2012). *Mecp2*-null mutant mice, as a mouse model of RTT, largely recapitulate the phenotypes and gender differences in severity observed in individuals with RTT (Lombardi et al., 2015). Female mice heterozygous for a *Mecp2*-null allele ($Mecp2^{-/+}$) display a delayed onset of neurological and behavioral deficits at approximately 4 months of age or older, whereas male mice hemizygous for the null allele ($Mecp2^{-/y}$) are more severely affected, with more rapid regression at approximately 3–4 weeks of age and approximately 50% dying by 8–10 weeks of age.

Studies on postmortem samples from RTT individuals and fixed brains of RTT model mice reported lower spine density of pyramidal neurons in the cortex and hippocampus (Belichenko et al., 1994; Chapleau et al., 2009; Xu et al., 2014). An *in vivo* time-lapse two-photon imaging study revealed defects in short-term spine dynamics in $Mecp2^{-/y}$ mice (Landi et al., 2011). Densities of spines and filopodia were reduced, and changes in spine length and head volume measured at 5 min intervals for 1 h were smaller in the apical dendrites of L5 pyramidal neurons in somatosensory cortex at P25–26, when the neurological deficits in these mice begin to emerge. In contrast, the short-term spine motility of $Mecp2^{-/y}$ mice was not different from wild-type mice at P40 or older, when the connectivity within the primary somatosensory cortex was considered mature, although the reduced spine density persisted. A subcutaneous injection of insulin-like growth factor-1 (IGF-1) 24 h prior to the imaging rescued the defects in short-term spine dynamics without ameliorating the reduced spine density. These results indicate that the deficits in structural plasticity of dendritic spines are present at the onset of neurological abnormalities and suggest that pharmacological treatment with IGF-1 during a certain time window in postnatal development may be beneficial for treating RTT.

Although MECP2 is expressed in both principal neurons and interneurons, the excitatory and inhibitory synaptic pathophysiology and circuit dysfunction resulting from loss of MECP2 *in vivo* are poorly understood. Two-photon calcium imaging of the visual cortex of $Mecp2^{-/y}$ mice has revealed that reduced visually driven excitatory and inhibitory conductances of pyramidal neurons lead to circuit-wide reductions in response reliability, selectivity and signal-to-noise ratios in these cells under both anesthetized and awake conditions (Banerjee et al., 2016). Such cortical processing deficits were recapitulated by *Mecp2* deletion specific to parvalbumin-expressing (PV^+) interneurons but not somatostatin-expressing (SOM^+) interneurons and were ameliorated by 2 weeks of daily systemic injections of IGF-1. Moreover, intrinsic-signal optical imaging revealed a role of MECP2 in PV^+ interneurons in cortical plasticity. Ocular dominance (OD) plasticity, the change in responsiveness of the binocular visual cortex to the eyes after brief monocular deprivation of vision, is a form of visual cortical plasticity whose sensitivity is greatest at approximately 4 weeks of age in mice (Sato and Stryker, 2008). Female mice with heterozygous PV^+ interneuron-specific *Mecp2* deletion exhibit abnormally prolonged adult OD plasticity, which is a hallmark of reduced firing of PV^+ interneurons (Kuhlman et al., 2013). This is similar to the previous findings by the same group that female symptomatic $Mecp2^{-/+}$ mice exhibited prolonged adult OD plasticity at P60, and this abnormal plasticity was corrected by daily systemic injections of the active tripeptide fragment of IGF-1 or of full-length IGF-1 (Tropea et al., 2009; Castro et al., 2014). Banerjee et al. (2016) further demonstrated that $Mecp2^{-/y}$ mice exhibit reduced expression of the cation-chloride cotransporter KCC2 and altered GABA reversal potential in pyramidal neurons. Moreover, IGF-1 treatment of these mice restored KCC2 expression in addition to PV^+ interneuron and pyramidal neuron responses, providing a mechanistic basis for the action of IGF-1. Together, these results demonstrate that loss of MECP2 in the brain alters both excitation and inhibition via multiple mechanisms, and MECP2 deletion in a

specific cell type critically contributes to circuit-wide deficits in RTT.

IGF-1 is a growth factor important for brain development and activates multiple intracellular signaling pathways, such as the phosphoinositide 3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAPK) pathways. IGF-1 mRNA expression is decreased in the cerebellum of *Mecp2*$^{-/y}$ mice (Mellios et al., 2014), and IGF-1 protein levels are reduced in the serum of *Mecp2*$^{-/y}$ and *Mecp2*$^{-/+}$ mice (Castro et al., 2014; Mellios et al., 2014). Systemically administered IGF-1 or its tripeptide fragment crosses the blood-brain barrier and improves a wide range of behavioral and cellular phenotypes in *Mecp2*$^{-/y}$ and *Mecp2*$^{-/+}$ mice (Tropea et al., 2009; Castro et al., 2014). IGF-1 treatment also increases the number of glutamatergic synapses in neurons derived from induced pluripotent stem cells from individuals with RTT (Marchetto et al., 2010). An open-label phase I clinical trial of recombinant human IGF-1 (mecasermin) in girls with RTT indicated good tolerance and improvement of certain breathing and behavioral abnormalities (Khwaja et al., 2014).

A recent study using VSD for imaging of the primary somatosensory cortex of *Mecp2*$^{-/y}$ mice revealed weaker and more diffuse whisker-evoked responses at 1–2 months of age (Lee L.J. et al., 2017). This functional deficit was accompanied by reduced complexity of thalamocortical axon terminals at P7 as well as reduced dendritic complexity and spine density of L4 spiny stellate neurons at P30–45, as examined in fixed sections. These findings suggest that similar functional and anatomical defects may underlie sensorimotor behavioral abnormalities in RTT, such as hand-to-mouth stereotypies.

IN VIVO OPTICAL IMAGING OF AS MODEL MICE

Angelman syndrome is a relatively rare NDD, afflicting only 1 in 10,000-40,000 people (Clayton-Smith and Laan, 2003), and was first documented by the British pediatrician Harry Angelman (Angelman, 1965). Individuals with AS display a wide variety of symptoms, including severe developmental delay, speech impairment, ataxia, seizures and abnormal EEG, and behavioral uniqueness, such as frequent laughter and hand flapping (Williams et al., 2006). This disorder is caused by genetic abnormalities affecting the maternal expression of the ubiquitin E3 ligase gene *UBE3A*, resulting in marked loss of function in the brain because the expression of this gene on the paternal chromosome is silenced by genomic imprinting (Sato, 2017). Consistent with a causal role for AS, maternal *Ube3a*-deficient (*Ube3a*$^{m-/p+}$) mice exhibit behavioral deficits analogous to those of human AS. UBE3A reportedly ubiquitinates several substrate proteins (Sell and Margolis, 2015), but none of them has been directly linked to symptoms observed in individuals with AS.

Although AS is reported to have a high comorbidity with ASD, this must be interpreted with caution because fulfillment of diagnostic criteria for ASD by children with AS could be better explained by severe developmental delay and language impairment in AS rather than by the specific deficits in social and communicative skills typically seen in ASD (Trillingsgaard and Østergaard, 2004; Hogart et al., 2010). Instead, accumulating evidence suggests that while loss of UBE3A in the brain leads to AS, abnormally elevated expression levels or activity of UBE3A may contribute to the pathogenesis of ASD. Maternal duplication or triplication of the chromosomal region 15q11–13, which encompasses *UBE3A*, is one of the most frequent genetic causes of ASD (Takumi and Tamada, 2018; see also the next section). Mice carrying a triple dose of *Ube3a* display an ASD-like phenotype (Smith et al., 2011; Krishnan et al., 2017). In addition, an ASD-linked mutation of *UBE3A* leads to enhanced UBE3A activity through disruption of protein kinase A-mediated phosphorylation control (Yi et al., 2015). These findings suggest a dual role of UBE3A in the pathogenesis of AS and ASD in a gene dosage- and ubiquitin ligase activity-dependent manner.

A postmortem histological study of individuals with AS demonstrated reduced dendritic spine density on L3 and L5 pyramidal neurons of the visual cortex (Jay et al., 1991). AS model mice exhibited reduced dendritic spine density not only on basal dendrites of L5 pyramidal neurons in binocular visual cortex (Sato and Stryker, 2010) but also on L2/3 pyramidal neurons in visual cortex (Yashiro et al., 2009) and on secondary apical dendrites of L3–5 cortical pyramidal neurons (Dindot et al., 2008). To elucidate the mechanisms behind this reduced spine density, GFP-labeled spines on L5 pyramidal neurons in the visual cortex of AS model mice were imaged by *in vivo* time-lapse two-photon microscopy (Kim et al., 2016). This study found that in juveniles, spine formation was normal but elimination was enhanced. Moreover, when AS mice were raised in darkness, spine density and turnover were indistinguishable from those in wild-type mice. Thus, the absence of UBE3A function appears to impair experience-driven spine maintenance, which may explain the decreased excitatory synaptic connectivity in this AS mouse model (Wallace et al., 2012).

To elucidate specific functions of UBE3A in brain development and maturation, intrinsic-signal optical imaging was used to examine experience-dependent plasticity of the visual cortex in juvenile and mature AS model mice (Sato and Stryker, 2010). OD plasticity was impaired in juvenile AS mice (P25-28) compared to age-matched wild-type mice. Moreover, while monocular visual deprivation in mature AS mice (P33-37) elicited plasticity of a magnitude similar to that in wild-type mice, the nature of the plasticity was reminiscent of that during the juvenile period (i.e., weakening of the deprived-eye response). In contrast, mature wild-type mice exhibited strengthening of the non-deprived-eye response. Thus, this functional imaging study reveals that UBE3A is indispensable for adaptability and maturation of neuronal circuits in the cortex.

IN VIVO OPTICAL IMAGING OF ASD MODEL MICE

Autism spectrum disorders are etiologically heterogeneous early-onset neuropsychiatric disorders but nonetheless typically exhibit three core symptoms: social deficits, language impairment, and restricted and repetitive patterns of behavior. In most cases,

ASD is accompanied by various co-morbid symptoms, such as seizures, sleep disorders, hyperactivity, and anxiety. Abnormal responses to sensory stimuli are also common in ASD. ASD is approximately four times more frequent in males, with a reported frequency of 1 in 42 compared to only 1 in 189 females (Christensen et al., 2016). Although the causes are unknown for the majority of cases, a genetic contribution to ASD susceptibility is strongly supported by family and twin studies. While many single genes have been associated with ASD, symptom heterogeneity suggests multiple genetic abnormalities. Indeed, copy number variants (CNVs) ranging from kilobases to megabases (Mb) and produced by deletion or duplication of chromosomal fragments have recently been implicated in a variety of disorders, including ASD (Takumi and Tamada, 2018). Recent studies report that 10-20% of ASD cases can be ascribed to CNVs, whereas only 5-10% of cases may be due to coding-sequence mutations in genes expressed in the brain (Huguet et al., 2013). Among several CNVs associated with ASD, the 15q11-13 duplication, which causes an NDD often called 15q duplication syndrome, is one of the most frequent, found in 0.25% of ASD cases (Pinto et al., 2014). Several CNV mouse models have been generated by chromosome engineering (Takumi and Tamada, 2018), and collectively, these models are thought to better reflect the genetic and phenotypic heterogeneity of ASD than single-gene KO mouse models. For instance, mice that mimic the paternally inherited 15q11-13 duplication by 6.3-Mb duplication of the syntenic region of mouse chromosome 7 (15q dup mice) display ASD-like behavioral phenotypes, including impaired social interaction, abnormal ultrasonic vocalization, and behavioral inflexibility (Nakatani et al., 2009).

Postmortem studies of individuals with non-syndromic ASD have so far mostly focused on global changes in neuronal cytoarchitecture and number, while relatively few have examined spine abnormalities (Martínez-Cerdeño, 2017; Varghese et al., 2017). An early qualitative study documented reduced spine density on apical dendrites of cortical pyramidal neurons (Williams et al., 1980). A more recent quantitative Golgi-impregnation study on the superficial and deep cortical layers of the frontal, temporal, and parietal lobes found greater spine densities, primarily on L2 pyramidal neurons of each cortical area and L5 pyramidal neurons of the temporal lobe, in individuals with ASD (but without FXS) compared to age-matched controls (Hutsler and Zhang, 2010). In younger ASD cases, increased spine density was also reported on basal dendrites of L5 pyramidal neurons of the temporal lobe (Tang et al., 2014) and on dendrites of principal neurons in the lateral nucleus of the amygdala (Weir et al., 2018).

The spine dynamics of 15q dup mice were examined by *in vivo* two-photon time-lapse imaging (Isshiki et al., 2014). This study specifically labeled subsets of excitatory and inhibitory postsynaptic spines on L2/3 cortical pyramidal neurons by expressing the GFP-tagged postsynaptic marker proteins PSD-95-GFP and gephyrin-GFP, respectively, while dendrites and all spines were filled with the red fluorescent protein DsRed2. These 15q dup mice showed normal spine density but enhanced turnover rates of PSD-95-GFP-labeled spines in somatosensory and anterior frontal cortices. In contrast, gephyrin-GFP-labeled spines were unaffected. Furthermore, altered sensory experience did not alter the elevated basal spine formation rate in the somatosensory cortex of 15q dup mice. Chessboard-like whisker trimming, which increased the formation rate but not the elimination rate of gephyrin-GFP-negative spines in wild-type mice, did not affect the formation rate in 15q dup mice, as it was already comparable to the enhanced spine formation of wild-type mice after whisker trimming. These results indicate that the basal spine turnover rate is constantly high even in the absence of sensory alterations and suggest that the neurons in 15q dup mice lack the capacity to remodel neuronal connectivity in response to new sensory experience. Similar overall spine defects were also observed in another ASD mouse model, neuroligin-3 R451C point mutant mice. These results suggest that ASD is associated with selective impairments of reorganization of PSD-95-containing excitatory synapses receiving intracortical afferents.

Interestingly, the dynamics of gephyrin-GFP clusters on spines remained unchanged, but those on dendritic shafts showed enhanced dynamics in 15q dup mice. Dynamic inhibitory synapses and remodeled spines are clustered close to each other on dendrites, and the occurrence of clustered changes is influenced by sensory experience (Chen et al., 2012; Wefelmeyer et al., 2016). Further, blockade of $GABA_A$ receptors elicits increased spine elimination (Chen, 2014), suggesting that altered GABA inhibition may underlie the enhanced spine turnover in 15q dup mice.

Given that 15q dup mice show high spine turnover rates regardless of alterations in sensory experience, this may reflect that sensory-evoked activity is abnormal in the cortex of these mice. A macroscopic calcium imaging study reported that 15q dup mice exhibited broader whisker-evoked response areas in the primary somatosensory cortex (Nakai et al., 2017). Further anatomical and electrophysiological analyses revealed fewer inhibitory synapses and concomitant hyperexcitability of pyramidal neurons, suggesting that the impaired sensory tuning is a consequence of reduced cortical inhibition, since inhibition is known to sharpen the whisker-evoked response by suppressing the responses of the surrounding areas (Foeller et al., 2005; Isaacson and Scanziani, 2011). Although the full mechanisms linking the enhanced spine turnover and the altered sensory circuit function remain to be understood, subsequent analyses revealed that serotonin is a key molecule in the pathophysiology of ASD. The 15q dup mice show reduced brain serotonin levels and decreased size, excitatory drive, and glucose metabolism of the dorsal raphe nucleus, which contains a large proportion of serotonin neurons innervating the cortical forebrain (Tamada et al., 2010; Ellegood et al., 2015; Nakai et al., 2017). Consistent with hypofunction of the serotonergic system in ASD, restoration of brain serotonin levels during the early postnatal period by administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine alleviated impaired inhibitory synaptic function at 2-3 weeks of age by reinstating the normal mIPSC frequency without affecting amplitude, implying that restoration of inhibition was mediated by an increased number of inhibitory synapses rather than by enhanced strength of individual synapses (Nakai et al., 2017).

Moreover, fluoxetine improved a subset of social behavioral defects in both young and adult 15q dup mice. While rescue by fluoxetine is encouraging, it remains to be elucidated whether reduced serotonin also contributes to the ethology of other ASD mouse models. In humans, maternal 15q duplication is a more frequent cause of ASD than paternal duplication. However, ASD-like behavior and reduced brain serotonin are observed in paternal but not maternal 15q duplication mice, implying that susceptibility to serotonergic hypofunction is determined by species- and parent-of-origin-specific genetic mechanisms.

The BTBR T+tf/J inbred mouse strain is considered a robust mouse model of idiopathic ASD, displaying impaired social interactions, communication deficits, and repetitive self-grooming compared to the commonly used C57BL/6 inbred strain (McFarlane et al., 2008; Scattoni et al., 2008). The insular cortex serves to combine sensory, emotional, and cognitive inputs from other brain networks, and aberrant insular connectivity and activation have been reported in ASD (Di Martino et al., 2009; Uddin and Menon, 2009; Ebisch et al., 2011). Intrinsic-signal optical imaging of the insular cortex revealed impaired multisensory integration in the BTBR T+tf/J mouse model (Gogolla et al., 2014). Specifically, these mice exhibited exaggerated auditory responses and lacked enhancement of insular responses to concurrent audio-tactile stimuli. The impaired postnatal maturation of integrated responses reflected weakened cortical GABA circuits. Consistent with deficient GABA inhibition in insular circuit dysfunction, transient pharmacological enhancement of inhibition by diazepam early in life rescued these deficits in the adult. Moreover, impaired insular multisensory integration has been observed across different monogenic ASD mouse models, including *Shank3* KO mice and *Mecp2* KO mice. These models also exhibit altered excitatory-inhibitory (E/I) balance, suggesting that optimal E/I balance is indispensable for proper maturation of circuits mediating multisensory integration.

Several genes associated with ASD, such as those encoding neuroligins, neurexins, and Shanks, are involved in the formation and function of synapses (Huguet et al., 2013). *MDGA2* (MAM domain-containing glycosylphosphatidylinositol anchor 2) is a recently identified ASD susceptibility gene that encodes a protein that negatively regulates the synaptogenic activity of neuroligins by suppressing their interaction with neurexins (Bucan et al., 2009; Connor et al., 2016). *Mdga2* haploinsufficient ($Mdga2^{+/-}$) mice exhibit an ASD-like behavioral phenotype and increased excitatory synaptic number and function (Connor et al., 2016). VSD imaging of resting-state cortical activity of $Mdga2^{+/-}$ mice revealed widespread increases in cortical spontaneous activity, notably in the posterior secondary motor cortex, retrosplenial cortex and primary motor cortex for whiskers and forelimbs (Connor et al., 2016). This technique also demonstrated intrahemispheric cortical functional hyperconnectivity, particularly in lateral cortical areas involving secondary somatosensory cortices and primary auditory cortex (Connor et al., 2016). Functional connectivity analysis using VSD imaging could be comparable to the rsfMRI described above, although VSD imaging is an optical method and can only image the superficial cerebral cortex. The midline cortical regions that displayed enhanced activity in $Mdga2^{+/-}$ mice are considered part of a putative mouse equivalent of the so-called default-mode network (Gozzi and Schwarz, 2016), a distributed intrinsic resting-state brain network implicated in various cognitive processes and brain disorders (Raichle, 2015). Indeed, functional brain hyper- or hypoconnectivity has also been observed in fMRI of ASD children (Ecker et al., 2015).

IN VIVO OPTICAL IMAGING OF 22q11.2DS MODEL MICE

22q11.2DS (also known as vero-cardio-facial syndrome or DiGeorge syndrome) is a complex neurogenetic disorder caused by a hemizygous microdeletion of 1.5–3 Mb on chromosome 22 and is one of the most common CNVs with a frequency of approximately 1 in 2,000–4,000 live births (Kobrynski and Sullivan, 2007; Karayiorgou et al., 2010). The clinical features of this disorder include cleft palate, hypocalcemia, cardiac defects, immune dysfunction, short stature and developmental delays. 22q11.2 deletion is associated with an increased risk for neuropsychiatric disorders. Strikingly, up to one-third of individuals with 22q11.2 deletion develop schizophrenia (SZ), and this accounts for 1–2% of sporadic SZ in the general population (Karayiorgou et al., 2010). In addition, 22q11.2 deletion carriers often meet diagnostic criteria for other disorders such as attention-deficit/hyperactivity disorder (ADHD), anxiety disorder, mood disorder and ASD over their lifetimes, although it remains controversial whether neuropsychiatric diagnoses other than SZ represent non-specific deficits in brain development and function or true genetic pleiotropy of this deletion (Jonas et al., 2014). A mouse model carrying a hemizygous 1.3 Mb-deletion of the syntenic region of mouse orthologous chromosome 16 [$Df(16)^{+/-}$] shows reduced dendritic complexity and decreased density of mushroom-shaped spines and excitatory synapses in fixed sections of the hippocampal CA1 area (Mukai et al., 2008). Multiple mouse models of 22q11.2 deletion, including $Df(16)^{+/-}$, exhibit SZ-related behavioral phenotypes, including deficits in sensory-motor gating, working memory, and fear memory (Stark et al., 2008; Drew et al., 2011). In addition, $Df(16)^{+/-}$ mice exhibit impaired social cognition, a symptom commonly observed in SZ and ASD (Piskorowski et al., 2016).

A recent calcium imaging study sought a neural substrate of cognitive deficits by examining hippocampal neural circuit dynamics of awake, behaving $Df(16)^{+/-}$ mice (Zaremba et al., 2017). Hippocampal pyramidal cells are known to exhibit place cell activity; they are active when an animal visits specific locations within an environment. In this study, head-fixed $Df(16)^{+/-}$ mice under a two-photon microscope were trained to find a reward on a sensory cue-rich treadmill belt, and their hippocampal CA1 neurons labeled by adeno-associated virus (AAV) vector-mediated GCaMP6f expression were imaged though a window implanted after surgical removal of the overlying cortex. $Df(16)^{+/-}$ mice showed a significant deficit in goal-oriented learning when the environmental context or the reward location was changed, along with reduced day-to-day stability of place cell maps compared to wild-type mice. In

addition, they lacked reorganization of place cell maps toward the goal location. These results demonstrate that hippocampal neuronal ensemble dynamics that support cognitive flexibility are impaired in a mouse model of 22q11.2 DS.

SEROTONIN-MEDIATED E/I REBALANCING AS A POTENTIAL THERAPEUTIC TARGET FOR ASD

An E/I imbalance as a key factor in ASD etiology was proposed more than a decade ago (Rubenstein and Merzenich, 2003), and since then, substantial evidence has accumulated in support of this hypothesis. Postmortem studies of individuals with ASD demonstrate downregulation of markers for GABA inhibition (Oblak et al., 2009; Blatt and Fatemi, 2011), and many ASD mouse models display altered E/I balance through multiple mechanisms (Lee E. et al., 2017; Bozzi et al., 2018). Moreover, pharmacological compounds that modulate the GABA system have been tested for therapeutic efficacy in mouse models, and clinical trials are currently ongoing (Braat and Kooy, 2015). However, a recent theoretical study proposed that a simple unidimensional E/I imbalance model cannot fully account for the aberrant neural circuit activity in *Fmr1* KO mice (Gonçalves et al., 2013; O'Donnell et al., 2017), implying that restoration of E/I balance by direct modulation of GABA signaling alone may be insufficient for some symptoms or forms of ASD.

The serotonin system plays multiple roles in brain development and function (Lesch and Waider, 2012). Positron emission tomography studies revealed that the serotonin synthesis capacity is reduced in the brains of children with ASD (Chugani et al., 1999; Chandana et al., 2005). Moreover, elevated whole-blood serotonin, which may reflect increased platelet serotonin uptake as shown in mice harboring an ASD-associated gain-of-function mutation in the gene encoding serotonin transporter (SERT Ala56 mice; Veenstra-VanderWeele et al., 2012), is found in more than 25% of affected children, suggesting hyperserotonemia as a biomarker for ASD (Muller et al., 2016). Although a concomitant increase in brain serotonin clearance in SERT Ala56 mice may lead to decreased synaptic serotonin availability and compensatory serotonin receptor hypersensitivity (Veenstra-VanderWeele et al., 2012), it remains to be investigated whether hyperserotonemia is correlated with reduced brain serotonin levels in ASD. SERT Ala56 mice also show behavioral deficits in multisensory processing (Siemann et al., 2017), implying that abnormal serotonin levels may be involved in altered multisensory processing in ASD. Serotonin modulates the strengths of excitatory and/or inhibitory synapses in a serotonin receptor subtype-specific manner (Ciranna, 2006; Lesch and Waider, 2012) and enhances sensory representation and discrimination by adjusting the relative strengths of distinct input pathways (Stutzmann et al., 1998; Kapoor et al., 2016; Tang and Trussell, 2017). Serotonergic modulation has therefore been proposed to be a suitable target for restoring E/I balance and sensory processing that are altered in ASD.

In light of this putative role of serotonin in the pathophysiology of ASD, several clinical studies have investigated the therapeutic effects of SSRIs, particularly on repetitive behaviors, as SSRIs are the established first-line treatment for obsessive–compulsive disorder (OCD) (Kellner, 2010). However, the results thus far are inconsistent, and there is currently no positive consensus on their efficacy according to recent systematic reviews (McPheeters et al., 2011; Doyle and McDougle, 2012; Williams et al., 2013). There have been several published placebo-controlled SSRI studies (McDougle et al., 1996; Hollander et al., 2005, 2012; Sugie et al., 2005; King et al., 2009). Among them, a single-site double-blind placebo-controlled 8-week crossover study of 39 children and adolescents (5-16 years old; 90% ASD and 10% Asperger) found that fluoxetine (2.4-20 mg/d) was superior to placebo in decreasing repetitive behaviors, with no significant difference in occurrence of adverse effects (Hollander et al., 2005). Similarly, a double-blind placebo-controlled 12-week parallel study of 37 adults [18-60 years old; 65% Asperger, 32% ASD and 3% pervasive developmental disorder not otherwise specified (PDD-NOS)] found a significantly greater reduction in repetitive behaviors in the fluoxetine-treated group (10-80 mg/d) than in the placebo-treated group, with only mild and moderate side effects (Hollander et al., 2012). However, preliminary results from a larger, multi-site, double-blind placebo-controlled study of 158 children (5-7 years old) have found that a novel fluoxetine formulation is no more effective than placebo for the treatment of repetitive behaviors (Autism Speaks, 2009). In addition, another multi-site, double-blind placebo-controlled study of the SSRI citalopram (2.5-20 mg/d), including 149 children and adolescence (5-17 years old; including ASD, Asperger and PDD-NOS with unknown percentages), reported no significant difference between citalopram and placebo groups in measures of repetitive behavior (King et al., 2009). Moreover, adverse events, including increased energy, impulsiveness, decreased concentration, hyperactivity, stereotypy, diarrhea, insomnia, and itchy dry skin (pruritus), were significantly more frequent in the citalopram group (King et al., 2009).

These discordant results may be due to the varying degrees of selectivity for the serotonin transporter over other actions and the different pharmacokinetic and pharmacodynamic profiles of SSRIs used. Furthermore, the pharmacological properties of a single SSRI can also change during development. Currently available SSRIs may not be effective for ASD in general because of the extreme heterogeneity of ASD etiology and the diversity of serotonin signaling systems. It is possible, however, that SSRIs may benefit certain forms of ASD. This outcome highlights the necessity of personalized medicine for ASD treatment, a strategy considered routine for cancer treatment. As discussed above, 15q11-13 duplication is a relatively common genetic abnormality in ASD, but the absolute frequency is rather low. In addition, administration of SSRIs to ASD individuals without reduced brain serotonin levels will not only be ineffective but also will likely cause adverse effects. Pre-treatment genotyping and assessment of brain serotonin synthesis capacity are thus recommended to identify cases potentially treatable with SSRIs. Serotonin receptors include several classes and numerous

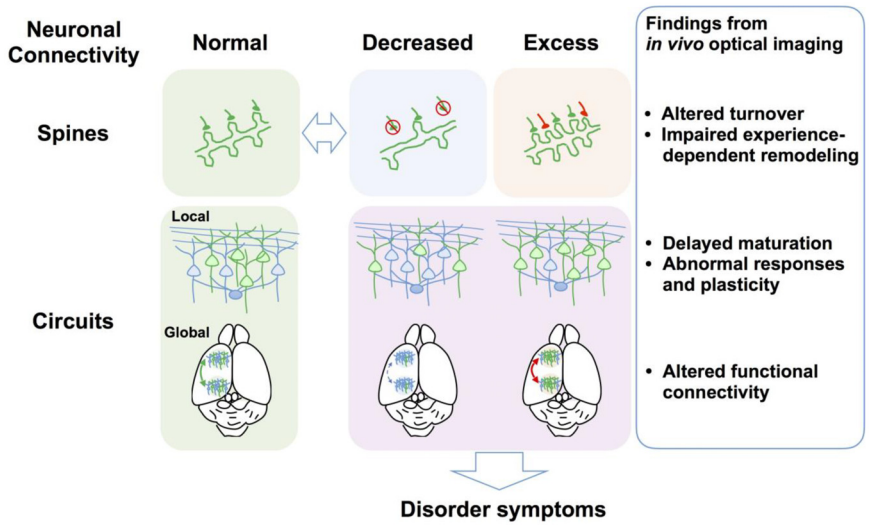

FIGURE 2 | Defects of spine dynamics and circuit function as common pathophysiological underpinnings of NDDs. Multiple NDD model mice and postmortem brains from individuals with NDDs exhibit reduced (e.g., RTT and AS) or increased spine density (e.g., FXS), which is a hallmark of abnormal neuronal connectivity. Evidence from *in vivo* optical imaging of NDD mouse models suggests that altered spine turnover (i.e., formation and elimination) and impaired experience-dependent remodeling are putative common phenotypes across NDDs. At the local circuit level, *in vivo* optical imaging has revealed abnormal sensory responses and plasticity and defects of circuit maturation in common, although causal links between observed aberrant neuronal connectivity and impaired local circuit function at the gross level remain to be elucidated. *In vivo* optical imaging has recently demonstrated altered functional connectivity between different brain areas at the global circuit level. Findings obtained using *in vivo* optical imaging at multiple levels thus greatly advance the understanding of the neural circuit bases for neurological and behavioral symptoms of NDDs.

subtypes, and these subtypes are differentially expressed among neuronal types and brain regions, making it difficult to predict the effects of SSRI-mediated serotonin increases in individuals with ASD. Indeed, while postnatal fluoxetine treatment improved impaired social behavior in 15q dup mice, the same treatment increased anxiety-like behavior in these mice and even impaired reversal spatial learning and exploratory behavior in wild-type mice (Nakai et al., 2017). These observations imply that despite the relatively well-established safety of SSRIs as antidepressants in adults, late-emerging adverse effects of postnatal SSRI treatment require further investigation by longitudinal assessment. Administration of SSRIs to depressed women during pregnancy or after delivery is known to increase serotonin levels in the fetus via the placenta or in newborns via breast milk. Although still controversial, accumulating evidence suggests that such perinatal SSRI exposure is a potential risk for a wide range of symptoms, including ASD, and placental and lactational transfer of SSRIs leads to abnormal behavior and various structural and functional alterations of the brain in rodents (Kinast et al., 2013). Furthermore, SSRIs are less well tolerated in children than adults, and the FDA has not approved SSRIs for OCD in children younger than 6-8 years (Williams et al., 2013). In sum, there are many issues to be resolved before safe and effective pharmacological interventions for restoration of brain serotonin levels in children with ASD are possible. Further elucidation of developmental changes in serotonin subsystems and downstream mechanisms underlying different ASD symptom domains is needed for the development of more-specific pharmacological therapies.

CONCLUSION AND OUTLOOK: LESSONS FROM *IN VIVO* OPTICAL IMAGING

In vivo optical imaging is a powerful technique for investigating brain structure and function in living animals at the circuit, cellular, and synaptic levels, and it will thus continue to be widely applied to new NDD mouse models, since the range of its application has so far been limited to several currently available mouse models among many NDDs. Further, the spatiotemporal resolution and modality of *in vivo* optical imaging are expected to increase with new developments in optics, microscopes, and fluorescent indicators. These findings and implications lead us to the following conclusions.

(1) *In vivo* time-lapse imaging of dendritic spines of NDD mouse models can illuminate dynamic turnover processes that cannot be revealed by examination of fixed brains. In most cases, spine density imaged *in vivo* is consistent with observations in fixed tissues, but it can also differ depending on factors such as age, genetic background, species, cortical area, cortical layer, cell type, labeling technique, and imaging method. Technically, *in vivo* imaging can better visualize dendrites close to the brain surface, such as apical dendritic tufts in L1, so the results accumulated thus far are not necessarily applicable to deeper cortical layers and other brain areas. Studies have also demonstrated that the direction of spine density change differs according to the disorder model, with different models exhibiting

increased (FXS), reduced (RTT and AS), or unchanged (15q dup) spine density. However, altered spine turnover rate and impaired experience-dependent remodeling appear to be common phenotypes across multiple disorder models (**Figure 2**).

(2) *In vivo* functional imaging has revealed that multiple NDD model mice exhibit abnormal cortical sensory responses, such as broader, fast-spreading, or undifferentiated cortical responses, suggesting abnormal cortical representations of external stimuli. Neuronal hyperexcitability and associated behavioral phenotypes such as seizures in some mouse models suggest that altered E/I balance underlies these abnormal sensory responses. *In vivo* calcium imaging can simultaneously record the activity of a large, dense population of neurons. The large datasets obtained with this technique are useful for testing the validity of theoretical models, and such a combined optical and theoretical approach may yield alternative theoretical models regarding the etiology of disorders (O'Donnell et al., 2017).

(3) *In vivo* optical imaging can also guide the development of new pharmacological interventions for NDDs (Tropea et al., 2009; Landi et al., 2011; Castro et al., 2014; Gogolla et al., 2014; Zhang et al., 2014; Banerjee et al., 2016; Nagaoka et al., 2016; Nakai et al., 2017). *In vivo* optical imaging is particularly useful for identifying and validating potential therapeutic targets when combined with anatomical, molecular, electrophysiological, and behavioral analyses. The findings obtained from such studies are expected to provide foundational support for clinical studies on improved therapeutic strategies.

(4) One of the strengths of *in vivo* optical imaging is the ability to visualize specific subtypes of neurons and synapses through differential labeling (Isshiki et al., 2014). However, this strategy has not yet been fully exploited in studies on mouse models of NDDs, so substantial advances in our understanding of cell- and synapse-specific defects are expected in the years to come. This approach is also very important to fill the gap between spine defects and abnormal circuit function because circuit functions emerge from complex neuronal networks that contain different types of synapses, including those made by local excitatory and inhibitory neurons as well as long-range connections from distant areas (**Figure 2**).

(5) Due to optical limitations, most *in vivo* imaging studies have focused on the cerebral cortex. However, subcortical brain areas should also be imaged in future studies because NDDs affect these deep brain regions as well (Zaremba et al., 2017). Recent advances in deep brain imaging techniques, such as targeted cortical excavation and microendoscopy using gradient refractive-index lenses (Ji et al., 2016; Sato et al., 2016, 2017b), may allow such applications. In addition, most experiments discussed here imaged sensory responses under anesthesia. The use of awake head-fixed animals (Banerjee et al., 2016; He et al., 2017; Sato et al., 2017a; Zaremba et al., 2017) and miniature head-mounted fluorescence microscopes attached to freely moving animals (Ghosh et al., 2011; Cai et al., 2016) will enable imaging of neural circuit activity while mice perform cognitive tasks relevant to the disorder of interest.

Pathophysiological changes at the molecular and circuit levels are complex even for monogenic NDDs, so non-syndromic and idiopathic conditions present enormous challenges. However, elucidating such changes is a necessary step toward the development of safe and effective therapies for these lifelong conditions. *In vivo* optical imaging of mouse models of NDDs will continue to contribute to this endeavor by providing evidence for dysfunction in the living brain.

AUTHOR CONTRIBUTIONS

NN and MS wrote the manuscript with input from TT and JN.

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Toxic Effects of Trichloroethylene on Rat Neuroprogenitor Cells

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Trichloroethylene (TCE) is a common volatile organic solvent which is considered as an ubiquitous environmental pollutant. It is claimed to be a developmental neurotoxicant. Our group evaluated previously its impact on three-dimensional neurospheres *in vitro*. The current work aims to investigate the neurotoxic effects of a lower concentration of TCE on the same system. To perform the experiment, neural progenitor cells were obtained from the brains of nine newborn rats. Afterward, these cells were cultured in both growth and differentiation media to get the neurospheres. Cell cultures were divided into two groups: group 1 (control), group 2 (exposed to 0.25 μM TCE). Neurospheres were photographed at different durations and assessment of the morphological changes such as proliferation and differentiation of neurospheres was done. In addition, cell viability, apoptosis, and necrosis were analyzed using flow cytometry to clarify the mechanism of involved cytotoxicity. The results revealed that TCE-treated neurospheres showed significantly decreased proliferation on days 7 and 14. These cells failed to show the neurogenic differentiation seen in the neurospheres of the control group. Furthermore, a highly significant decrease in viability and a significant increase in the number of apoptotic cells were observed in the treated cells in comparison to the control group. The present work confirmed that TCE, at very low doses relevant to daily life exposure in humans, caused neurotoxic effects in 3D neurosphere model through the affection of neural proliferation and differentiation as well as disturbance of cell viability and apoptosis.

Keywords: trichloroethylene, neurotoxicity, neurospheres, *in vitro*, developmental

INTRODUCTION

There is a strong evidence indicating that environmental exposure to various chemicals at critical developmental stages affects the behavioral and neurological development in children (De Felice et al., 2015). It is hypothesized that developmental neurotoxicity (DNT) results from disturbance of some biological processes, such as differentiation, proliferation, apoptosis, and neurite growth (Bal-Price et al., 2012; Kadereit et al., 2012).

Trichloroethylene (TCE) is a common volatile organic solvent that has been widely used in industrial applications and consumer or commercial products such as a cleaning and degreasing agent in ink and varnishes. It is also a common environmental pollutant usually found in air, soil, and water. Many studies have shown that TCE is associated with serious health hazards as well as neurodevelopmental abnormalities due to gestational exposure to this chemical (Environmental Protection Agency [EPA], 2014).

Accordingly, TCE is highly suggested to be classified as a developmental neurotoxicant. However, this claim needs to be validated by more detailed DNT testing such as the three-dimensional (3D) neurosphere system. This model represents an *in vivo*-like microenvironment which could reflect the basic developmental processes of the growing brain and improved the ability to verify the neurotoxic effects of chemicals during early life exposure (Choi et al., 2013; Dingle et al., 2015).

The mechanism by which developmental exposure to TCE induces neurotoxicity is still unclear (Blossom et al., 2017). In a previous work, our group tested the neurotoxicity of a higher dose of TCE (1 μM) which is claimed to be safe in human (Abdraboh et al., 2017). In the present work, we investigate the neurotoxic effects of a much lower dose of TCE (0.25 μM) on the same system of 3D neurospheres.

MATERIALS AND METHODS

The present work was approved by the Institutional Review Board, Faculty of Medicine, Mansoura University (code MS/896). Nine newborn rats (1 day after birth), Sprague Dawley strain, regardless of sex, were obtained from the animal house of Medical Experimental Research Center (MERC). All chemicals and reagents were purchased from Sigma-Aldrich Company, St. Louis, MO, United States unless declared otherwise.

Cell culture media included the following: (a) Growth medium: Dulbecco's modified Eagle medium and Ham's F-12 (1:1 MIX) (Lonza, cat. no. BE12-719F, Basel, Switzerland) supplemented with 10% Fetal bovine serum "FBS" (Hyclone, San Angelo, TX, United States), 1% L-glutamine (Gibco, Carlsbad, CA, United States) and 1% Penicillin–streptomycin–Amphotericin B Mixture (Lonza, cat. no. 17-745E, Basel, Switzerland). (b) Differentiation medium: [Dulbecco's modified Eagle medium and Ham's F12 (3:1) enriched with neural growth factors 1% B27, 1% N2 supplement, 20 ng/mL Recombinant human Fibroblast growth factor basic (Invitrogen/Gibco, Carlsbad, CA, United States), 2–5% FBS and 1% Penicillin–streptomycin–Amphotericin B Mixture].

Preparation of Neurospheres

Neurospheres were obtained as described previously (Abdraboh et al., 2017) by aseptically dissecting out the cortices from the brains of nine newborn rats and isolating rat neural progenitor cells. In brief, the cortices were chopped into very tiny pieces on separate sterilized Petri dishes. Then, 10 ml trypsin EDTA solution were added to each tissue and incubated for 45 min at 37°C with constant shaking. To inactivate trypsin, 10 ml of growth media were added to each sample, and pipetted up and down 10 more times. Then, the brain tissues were filtered using sterilized mesh filter. Cell suspensions (1×10^6 cells) were transferred to disposable conical tubes and centrifuged at 2000 RPM for 10 min to precipitate the pellets. Each pellet was resuspended in 15 ml growth medium and transferred into a tissue culture flask in humidified 5% CO_2 incubator at 37°C for 24–48 h (Iwamaru et al., 2013; Louis et al., 2013).

Trichloroethylene Exposure

Trichloroethylene (cat. no. 79-01-6): (density: 1.463 g/mL, purity: ≥99%, Technical grade) was obtained. Thereafter, the nine tissue culture flasks containing the neurospheres were randomly divided into two groups; Group 1: neurospheres served as a control group and received no treatment. Group 2: neurospheres were treated with 0.25 μM trichloroethylene (Environmental Protection Agency [EPA], 2014).

Assessment of the Effects of TCE on Neurospheres

It was performed by 40× objective through evaluation of three neurospheres per field in eight randomly distributed visual fields per culture well in at least three biological replicates per concentration in a blind manner.

Cell Proliferation

The neurospheres were photographed at different durations (0, 3, 7, and 14 days). Then, each sphere size was determined by software analyses (Cell Profiler, version 2.1; Broad Institute, freely downloaded from http://www.cellprofiler.org). The diameter of each neurosphere was measured in μm and exported to excel file further to statistical analysis.

Cell Differentiation

Images of the plated neurospheres were evaluated regarding distinct neuronal morphology with fasciculation of neurites that radiate from the central aggregation of neuronal perikarya. In addition, the cell capacity to differentiate into dopaminergic neurons was challenged. It was then assessed through immunostaining against anti-tyrosine hydroxylase (TH) antibody (Novus Biologicals, United States, 1:200 dilution: Cat.No. #NB300–109).

Detection of Cell Viability, Necrosis, and Apoptosis by Flow Cytometry (Wlodkowic et al., 2010)

- Initially, cell viability was estimated using Trypan Blue Exclusion test. Thereafter, to evaluate the aforementioned parameters more precisely, Annexin V kit, Propidium Iodide and 1X Binding Buffer (cat. No. 556547 BD Pharmingen FITC "fluorescein isothiocyanate" apoptosis Kit, Princeton, NJ, United States) were used.
- The cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer. Five μl Annexin V (FITC label) and 5 μl Propidium Iodide (PI) were added. Gently, the cells were mixed using vortex and incubated for 15 min at room temperature (25°C) in the dark. 200 μl of 1X Binding Buffer was added. The cells were evaluated for the cell cycle by Flow Cytometer (BD Accuri^{TM} C6,

Piscataway, NJ, United States) within 1 h. The intact membrane of living cells excludes cationic dyes, such as PI which can stain the nucleus in case of lost membrane integrity while Annexin V (apoptotic marker) can bind to the phosphatidylserine present on the surface of apoptotic cells. In early apoptosis, phosphatidyl serine is exposed on the cell surface which is detected by Annexin V. Due to their extensive membrane damage; necrotic cells are quickly and brightly stained with PI and will appear as a peak at very high fluorescence values. Apoptotic cells will be dimly stained and show a much lower uptake of PI than that seen with necrotic cells.

- Cell death was evaluated as follows: dot plots were generated and divided into four quadrants (UR, upper right; UL, upper left; LR, lower right; LL, lower left). The LL quadrant represented the living non-apoptotic cells (negative for both annexin V and PI). The living early apoptotic cells were shown in the LR quadrant (annexin V positive cells but negative for PI). The UL quadrant demonstrated the necrotic cells (negative to annexin V and positive for PI). Whereas, the late apoptotic cells were shown in the UR quadrant (both annexin V and PI positive cells).

Statistical Analysis

Data were assessed for normality using Shapiro–Wilk test, then statistical analyses were performed. Differences between mean values were assessed for statistical significance using a two-tailed Student's *t*-test (GraphPad Prism 5.0 software, La Jolla, CA, United States). For all tests, *P*-value 0.05 was deemed significant.

RESULTS AND DISCUSSION

In the present work, we assessed how the rat neural progenitor cells were affected by TCE in a minimal dose (0.25 μM) at different timelines. The morphological changes such as proliferation and differentiation of neurospheres were evaluated to validate the neurotoxic effects of TCE. In addition, to clarify the mechanism of involved cytotoxicity, the cell viability, apoptosis, and necrosis were analyzed using flow cytometry.

Noteworthy, we utilized a higher concentration of TCE (1 μM) in a previous report (Abdraboh et al., 2017). This dose was proved to cause a significant time-dependent reduction in the proliferative capacity of neurospheres, failure of the cells to differentiate into astrocytes as well as a significant decrease of cell viability at 1 and 2 weeks duration. These findings could be extrapolated to the human population as the minimal occupational exposure among degreasing workers was found to be about (131 mg/m^3) which is equivalent to 1 μM TCE (Environmental Protection Agency [EPA], 2014). Also, this concentration is the representative dose of the environmental concentration of TCE on surface water (1 μg/L) according to United States Environmental Protection Agency report (Agency for Toxic Substances and Disease Registry [ATSDR], 2016).

In the present study, we tried to assess the neurotoxic effect of a much lower dose of TCE than the previously investigated (Abdraboh et al., 2017). Based on a preliminary pilot study, we chose a TCE concentration of 0.25 μM that is equivalent to the average daily life human exposure occurring through inhalation and ingestion which is suggested to be 33 μg per day (Environmental Protection Agency [EPA], 2014).

Neurospheres were obtained by the same protocol described in our previously published work (Abdraboh et al., 2017). **Figure 1** shows the growing 3D neurospheres after 3 days. Then, the effects of TCE on the proliferation of neurospheres were evaluated through measurement of the cell diameter and assessment of the sequence of its increase as well as their size variation with the progress of time in the culture medium as illustrated in **Table 1**. The present findings reveal a normal pattern of proliferation and progressive increase in the diameter of the cells in the control group throughout the study period (from day 0 till day 14). Whereas, TCE-exposed neurospheres show reduced proliferation as evidenced by the decreased cell diameter.

More or less similar, those results reported by Wang et al. (2001) who tested the vaporous toxicity of TCE (20–80 μl) on Chinese hamster ovarian (CHO-K1) cells (Wang et al., 2001). They found that there was a dose-dependent decrease in cellular proliferation. They suggested that proliferation arrest was dependent on GSH metabolism.

Interestingly, neurite outgrowth is relevant to study the DNT of chemicals as it is a critical process occurring during the development of the nervous system, which when disrupted, this may lead to serious adverse neurodevelopmental disorders

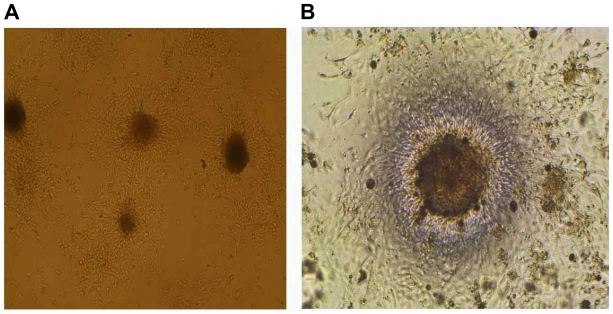

FIGURE 1 | Image illustrating the growing neurospheres after 3 days of the study using low **(A)** and high **(B)** magnification.

TABLE 1 | Neurospheres diameter (proliferation) in the studied groups.

Groups/ Day	Group 1 (control)	Group 2 (TCE-treated group) (TCE: 0.25 μM)
0	161 ± 22.3	165 ± 30.1
3	299 ± 26.5	281 ± 39.7
7	642 ± 18.9	418 ± 27.7*
14	812 ± 27.8	402 ± 23.5*

*N.B. TCE, trichloroethylene. All values were expressed as Mean ± SD. *Statistically significant at P < 0.05 (between control and test groups).*

(Harrill et al., 2013; Krug et al., 2013). Hence, neurite outgrowth could serve as a preliminary assessment tool for neuronal differentiation (Ryan et al., 2016). In addition, for more distinctive assessment of neurosphere differentiation, the capacity of the cells to differentiate into dopaminergic neurons was investigated.

In this context, the present work revealed that neurospheres in the control group have distinct neuronal morphology with fasciculation of neurites that radiate from the central aggregation of neuronal perikarya. Paralleled to those morphological clues of differentiation, control cells demonstrate their ability to differentiate into dopaminergic neurons (15% of cells) as seen in **Figure 2**. On the other hand, TCE treated cells failed to show this differentiation capacity.

The increased size of the neurospheres in the control group is likely due to the differentiation of the cortical neural progenitor cells into mature nerve cells, which then extend axons and dendrites leading to the increased diameter of the neurospheres with the progress of time (Choi et al., 2013). This was again confirmed by differentiation of 15% of cells into dopaminergic neurons compared to the TCE treated cells which fail to differentiate.

Several mechanisms of TCE-induced neurotoxicity are suggested. For instance, Grandjean and Landrigan (2014) explained the arrest of proliferation and failure of neurogenic differentiation by decreased neurotrophic factors (Grandjean and Landrigan, 2014) which are important mediators for these processes and disturbance of these factors is claimed to be involved in neurodevelopmental disorders of the CNS (Sajdel-Sulkowska et al., 2011).

In addition, Blossom et al. (2013) observed that the abnormal behavior and neurotoxic effects in mice exposed to TCE at doses of 0.01 and 0.1 mg/ml in water could be due to oxidative stress with global DNA hypomethylation (Blossom et al., 2013).

Our second objective in the present work is to assess cytotoxicity in the neuroprogenitor cell culture exposed to TCE. It is documented that flow cytometry is the technique of choice to assess viability, apoptosis, and/or necrosis on a single cell basis (Galluzzi et al., 2015). Generally, the scatter analysis of the cell population allows a sufficient distinction between viable and non-viable cells. Additionally, staining by Annexin V and propidium iodide (PI) which is a DNA-binding dye that does not penetrate the intact cell membrane could be beneficially combined with the scatter method to recognize various types of cells. Annexin-V-positive and PI-negative cells are considered as early apoptotic while the double positive cells are classified as late apoptotic while the necrotic cells are Annexin negative and PI positive (Wlodkowic et al., 2010; Stepanek et al., 2011).

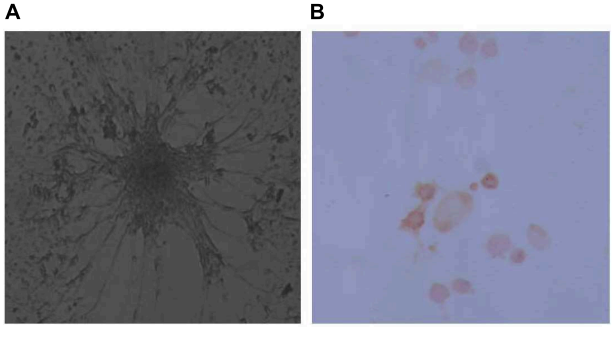

FIGURE 2 | Image showing differentiation of neurospheres as evidenced morphologically **(A)** and through staining by Tyrosine Hydroxylase **(B)** to show the ability to derive dopaminergic neurons from the rat progenitor cells.

Figure 3 and **Table 2** demonstrate the results of flow cytometric analysis of the cultured neurospheres. It is observed that cell viability (both Annexin V and PI negative) is significantly lower in the neurospheres exposed to TCE (0.25 μM) than that of the control cells. This finding is supported by the report of Zhu et al. (2005) who found that the normal human epidermal keratinocytes (NHEK) treated with various concentrations (0.01–31.6 mM) of TCE revealed a dose-dependent decrease in cell viability.

One of the important parameters studied in the present research is apoptosis (programmed cell death) which is a crucial process for neurodevelopment. It prevents redundant and unused neurons from disarrangement and cluttering of the developing brain (Creeley and Basavarajappa, 2016). On the other hand, necrosis had been claimed to be an unregulated mode of cell death but recently the term "necroptosis" has been used to describe a programmed, caspase-independent cell death with necrotic morphology. Its mechanism is suggested to be due to activation of the same receptors involved in apoptosis (Karch and Molkentin, 2015).

In this regard, our results reveal increased early apoptosis although it was statistically insignificant whereas late apoptosis and necrosis are significantly increased in the TCE-treated neurospheres (0.25 μM) when compared to the control cells. Accordingly, these findings observed during cell cycle analyses indicate an evident toxic effect of the examined low dose of TCE (0.25 μM) on neurospheres through induction of different types of cell death (**Figure 3** and **Table 2**).

In harmony with the present findings, an occupational study was done in lock industry workers who were exposed to TCE and its metabolites. The authors reported a significant increase of apoptosis in the collected blood samples in association with a significant up-regulation of pro-apoptotic P53 and Bax (Varshney et al., 2015). In addition, TCE exposure remarkably interferes with mitochondrial signaling through activation of caspase-dependent apoptotic cell death (McDermott and Heffron, 2013) which supports the current findings.

Furthermore, our results are in accordance with those reported by Ali et al. (2016) who studied the cytotoxic effect of low doses of TCE (0.5–32 μM) in human epidermal keratinocytes. The authors observed a significant increase in the number of cells undergoing apoptosis in the TCE-exposed groups. The findings supported the results of the present work concerning apoptosis; however, the probability of necrosis is excluded.

It is worth mentioning that apoptosis and necrosis represent two pivotal types of cell death. Apoptosis is an active process

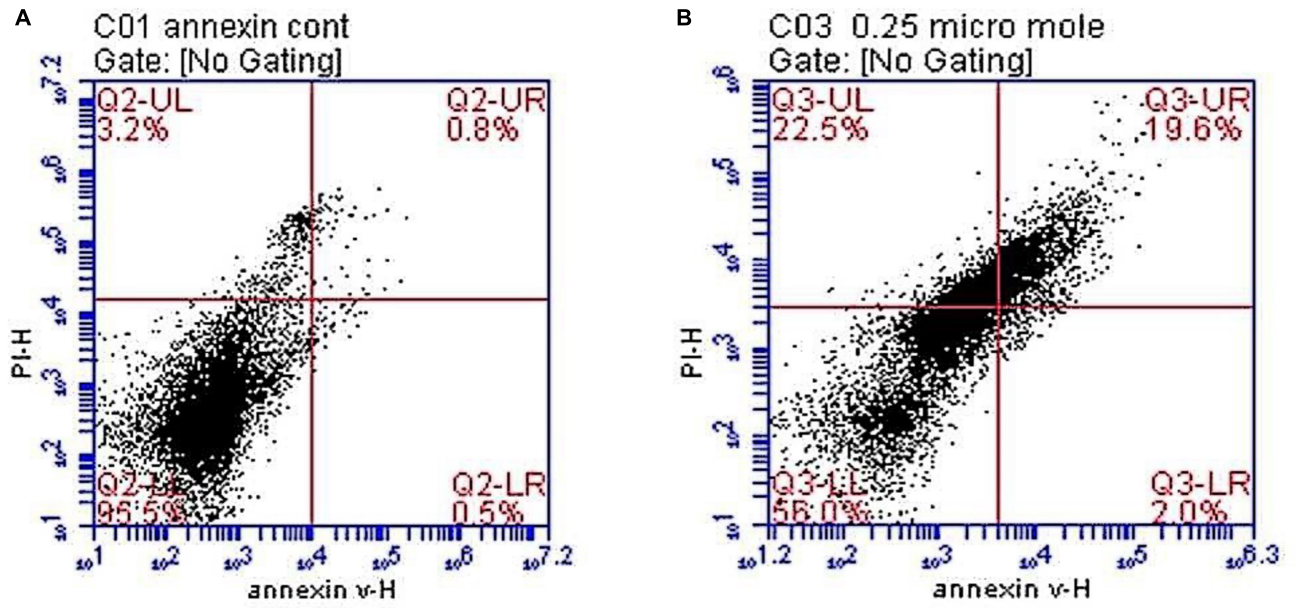

FIGURE 3 | Histograms showing flowcytometric analysis of the neuronal cell cultures in control and test groups. **(A)** A histogram showing that most of the neurospheres are viable in the control group. **(B)** A histogram showing decreased cellular viability, increased necrosis, early and late apoptosis in neurospheres exposed to trichloroethylene (TCE) 0.25 μM [viable cells in the lower left quadrant (LL), early apoptotic cells in the lower right (LR) quadrant, late apoptosis is seen in the upper right (UR) while necrosis is shown in the upper left (UL) quadrants].

consisting of highly organized molecular events whereas necrosis is a passive uncontrolled cell rupture mediated by extremely exogenous damage. Early apoptotic cells preserve the integrity of plasma membrane to prevent the release of the potentially harmful cellular contents outside. Late apoptosis (or secondary necrosis) occurs if the early apoptotic cells are not taken up by phagocytes, which does not happen *in vitro*. Loss of the membrane integrity is a gradual process. First, the membrane of a late apoptotic cell becomes permeable for small molecules (e.g., PI) and subsequently opens also for macromolecules (Patel et al., 2006; Stepanek et al., 2011; Galluzzi et al., 2015). Accordingly, early apoptosis is insignificant at the start of the experiment but with cumulative exposure to TCE, the apoptotic process is gradually increased and the cells suffer from late apoptosis and/or necrosis due to ultimate damage of the cell membrane.

TABLE 2 | Comparison between the studied groups regarding viability, necrosis, and apoptosis in neurospheres by flowcytometry.

Group/ Parameter	Group 1 (control)	Group 2 (TCE -treated group) (TCE: 0.25 μM)	*P*-value
Viable cells	95.47 ± 0.45	57.17 ± 1.26	0.0005**
Early apoptosis	0.36 ± 0.23	1.60 ± 1	0.2
Late apoptosis	1.5 ± 1.57	19.06 ± 1.29	0.001**
Necrosis	2.67 ± 1.29	22.17 ± 1.04	0.00001**

N.B. TCE, trichloroethylene. All values were expressed as Mean ± SD.
Double negative cells for Annexin V and Propidium iodide (PI) = viable cells, no plasma membrane rupture, Annexin V + , but negative for PI = early apoptotic cells, Double positive cells for both Annexin V and PI = late apoptosis, Positive for PI, but negative for annexin V = necrotic cells (plasma membrane rupture).
*P-value: **highly significant < 0.001.*

Moreover, the occurrence of necrosis in the neurospheres exposed to TCE in our work could be explained by the fact that apoptotic cells ultimately shut down metabolism after a long period of *in vitro* culture (Riss and Moravec, 2004) and exhibit some morphological forms associated with necrosis. These cells became secondary necrotic in the absence of phagocytosis and could develop features of primary necrosis (VandenBerghe et al., 2013). Additionally, numerous cell culture models and diverse study designs could also contribute to the controversial findings in various studies.

Interestingly, extrapolation of the current observations to humans exposed to TCE could be problematic. It is worth to mention that previous studies estimating serum TCE levels in human beings were highly variable due to different populations, occupational versus non-occupational exposure and various measurement methodologies. For example, in an occupational study in the United States, TCE has been estimated in the blood of 157 metal workers who had an average concentration of 2.5 μg/L (range: 0–22 μg/L) (Pfaffenberger et al., 1984). On the other hand, analysis of TCE levels in samples taken from 290 subjects revealed that the mean concentration was 0.013 μg/L besides that 88% of samples were found to be below the limit of detection (Jia et al., 2012).

As previously mentioned, pharmacokinetic modeling of TCE exhibits complicated conversion of *in vivo* to *in vitro*

concentrations (U.S. Environmental Protection Agency [U.S. EPA], 2011). We used a much lower TCE concentration in the present study compared to our previously published work (Abdraboh et al., 2017), however, the used dose (0.25 μM which is equivalent to 32.85 μg/L) is still higher than the reported human serum concentrations (Pfaffenberger et al., 1984; Jia et al., 2012).

CONCLUSION

The present work confirmed the potential neurotoxic effects of a very low dose of TCE (0.25 μM) in 3D neurosphere model through the affection of neural proliferation, neurite outgrowth, and differentiation in addition to disturbance of cell viability and induction of apoptosis and necrosis. The neurotoxicity of this dose of TCE which is relevant to the daily life exposure of humans to this ubiquitous pollutant is alarming and necessitates more detailed research on much lower concentrations than that investigated in the current study.

AUTHOR CONTRIBUTIONS

MS: concept and study design. MS and DE-N: practical work and data acquization. MS, RA-R, and SE: data analysis. MS, DE-N, RA-R, and SE: writing manuscript.

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frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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Opposite Roles of Wnt7a and Sfrp1 in Modulating Proper Development of Neural Progenitors in the Mouse Cerebral Cortex

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The Wingless (Wnt)-mediated signals are involved in many important aspects of development of the mammalian cerebral cortex. How Wnts interact with their modulators in cortical development is still unclear. Here, we show that Wnt7a and secreted frizzled-related protein 1 (Sfrp1), a soluble modulator of Wnts, are co-expressed in mouse embryonic cortical neural progenitors (NPs). Knockout of Wnt7a in mice causes microcephaly due to reduced NP population and neurogenesis, and Sfrp1 has an opposing effect compared to Wnt7a. Similar to Dkk1, Sfrp1 decreases the Wnt1 and Wnt7a activity *in vitro*. Our results suggest that Wnt7a and Sfrp1 play opposite roles to ensure proper NP progeny in the developing cortex.

Keywords: Wnt7a, Sfrp1, cerebral cortex, neural progenitors, antagonist

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INTRODUCTION

During development of the mammalian CNS, billions of neurons are produced from proliferating NPs (Rakic, 2009). In the cerebral cortex, NPs are expanded through symmetric and asymmetric division at the VZ and SVZ (Haubensak et al., 2004; Gotz and Huttner, 2005; Homem et al., 2015). The proper control of proliferation, survival and differentiation of NPs is the key step for normal cortical formation (Rakic, 2007, 2009; Zhao et al., 2008; Sun and Hevner, 2014).

A number of signaling pathways that regulate the switch and balance between proliferation and differentiation of NPs have been defined, including the Notch, Sonic hedgehog, fibroblast growth factor, TGF-β/Smads, and Wnt pathways (Chenn and Walsh, 1999; Rowitch et al., 1999; Hirabayashi et al., 2004; Joksimovic et al., 2009; Aguirre et al., 2010; Menendez et al., 2011; Rash et al., 2011). Wnt signaling pathways play crucial roles in neurogenesis (Kuwabara et al., 2009; Durak et al., 2016). For example, the canonical Wnt/β-catenin pathway is required for NP self-renewal and differentiation (Chenn and Walsh, 2003; Kalani et al., 2008;

Abbreviations: CNS, central nervous system; CP, cortical plate; CRD, cysteine-rich domain; E0.5, embryonic day 0.5; Fz, frizzled; IP, intermediate progenitor; ISH, *in situ* hybridization; IUE, *in utero* electroporation; NP, neural progenitor; P0, postnatal day 0; PFA, paraformaldehyde; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; Sfrp1, secreted frizzled-related protein 1; shRNA, short hairpin RNA; SVZ, subventricular zone; TGF-β, transforming growth factor-β; VZ, ventricular zone.

Bengoa-Vergniory et al., 2014; Delaunay et al., 2014; Bengoa-Vergniory and Kypta, 2015; Garriock et al., 2015). Among the Wnt signaling molecules, *Wnt7a* has been shown to be critical in axonal remodeling, guidance, synaptogenesis and neurotransmitter release in the hippocampus (Hall et al., 2000; Cerpa et al., 2008; Ciani et al., 2011, 2015). *Wnt7a* controls neurogenesis through regulating genes involved in both cell cycle control and neuronal differentiation (Qu et al., 2013; Long et al., 2016).

Furthermore, three distinct receptor families have been reported to mediate the Wnt signaling: Fz, RoR, and Ryk (van Amerongen et al., 2008; Angers and Moon, 2009). In the nervous system, Fzs regulate a range of functions from neuronal differentiation to cell polarity, axon guidance, and cell survival (Van Raay et al., 2005; Prasad and Clark, 2006; Liu et al., 2008; Kilander et al., 2014; Zhou and Nathans, 2014; Morello et al., 2015; Chailangkarn et al., 2016). Moreover, Sfrps are a family of secreted factors that modulate Wnt-induced β-catenin pathway through selectively sequestering specific Wnts in different neurons by possessing the Wnt-binding frizzled CRD (Dann et al., 2001; Bovolenta et al., 2008; Nathan and Tzahor, 2009; Lavergne et al., 2011). For example, both *Sfrp1* and *Sfrp2* can be the dominant negative inhibitors of *Wnt3a* to inhibit proliferation in the developing chick neural tube (Galli et al., 2006), and *Sfrp2* can negatively regulate the Wnt signaling in the CNS of *Pax6* mutant mice via inhibiting *Wnt7b* (Kim et al., 2001a). *Sfrp1* knockout mice display abnormal cortical morphogenesis (Esteve et al., 2018). However, the precise regulation of Wnts and their antagonist Sfrps in mammalian cortical neurogenesis is still unclear.

In this study, we show that *Wnt7a* and *Sfrp1* are co-expressed in the VZ of mouse embryonic cerebral cortices. Knockout of *Wnt7a* causes microcephaly due to reduced numbers of NPs and decreased neurogenesis. *Sfrp1* showed overexpression leads to a decrease in the NP population. Similar to the known Wnt antagonist *Dkk1*, *Sfrp1* directly blocks the *Wnt1* and *Wnt7a* activity *in vitro*. Our results indicate that opposite effects of Wnt7a and Sfrp1 play an important role in expansion of cortical NPs.

MATERIALS AND METHODS

Animals and Genotyping

The *Wnt7a* knockout mice (*Wnt7a* KO, $Wnt7a^{-/-}$) were generated by mating female *Wnt7a* heterozygous mice ($Wnt7a^{+/-}$) with male *Wnt7a* heterozygous mice ($Wnt7a^{+/-}$). Mice that only have the mutant allele ($Wnt7a^{-/-}$) were *Wnt7a* KO mice, wild-type (WT) mice were used as controls. To achieve knockout of *Wnt7a*, a double-selection gene-replacement construct was designed to insert a neo gene into a Nael site in the second exon of the *Wnt7a* gene (Parr and McMahon, 1995; Ashrafi et al., 2012).

For staging of embryos, midday of the day of vaginal-plug formation was considered as E0.5; the first 24 h after birth were defined as P0. Animal use was overseen by the Animal Facility at Weill Cornell Medical College (Protocol number #2011-0062), and was performed according to the institutional ethical guidelines for animal experiments.

Mouse tail-tip biopsies were used for genotyping by PCR reactions using the following primers: for *Wnt7a* KO, forward: 5-CTCTTCGGTGGTAGCTCTGG-3 and reverse-1: 5-TCACGTCCTGCACGACGCGAGCTG-3 (product size: 350 bp); for WT, reverse-2: 5-TCCTTCCCGAAGACAGTACG-3 (product sizes: 560 bp).

RNA Sequencing (RNA-Seq)

Total RNAs for RNA-seq were extracted from three individual E12.5 mouse cerebral cortices using TRIzol (Invitrogen, United States) according to manufacturer's instructions. The ribosome RNA (rRNA) removal, generation of cDNA library and high-throughput sequencing were performed on the Ion proton platform (Life Technologies, United States) from the NovelBio Bio-Pharm Technology Company (Shanghai, China). Three sets of raw reads were obtained, and data were deposited in Gene Expression Omnibus (GEO[1]) under the series number GSE116056. After removing contaminated and low-quality sequences, all reads were mapped onto the Ensembl mouse reference genome. Gene expression level were calculated by RPKM (reads per kilo-bases per million mapped reads).

In Situ Hybridization

In situ hybridization was performed as described: following fixation with 4% PFA, acetylation with acetylation buffer (1.3% triethanolamine, 0.25% acetic anhydride, 20 mM HCl), treatment with proteinase K (5 μg/ml, IBI Scientific) and pre-hybridization (1 × SSC, 50% formamide, 0.1 mg/ml Salmon Sperm DNA Solution, 1 × Denhart, 5 mM EDTA, pH 7.5), brain sections were hybridized with DIG-labeled LNA probes at Tm −22°C overnight. After washing with pre-cooled wash buffer (1 × SSC, 50% formamide, 0.1% Tween-20) and 1 × MABT, sections were blocked with blocking buffer (1 × MABT, 2% blocking solution, 20% heat-inactived sheep serum) and incubated with anti-DIG antibody (1:1, 500, Roche) at 4°C overnight. Brain sections were washed with 1 × MABT and Staining buffer (0.1 M NaCl, 50 mM $MgCl_2$, 0.1 M Tris-HCl, pH 9.5), stained with BM purple (Roche) at room temperature until ideal intensity was reached. The antisense RNA probe (*Sfrp1*, *Wnt7a*, *Wnt7b*, *Pax6, Ngn2,* and *Hes5*) was labeled using the DIG RNA labeling Kit (Roche, Switzerland) following the manufacturer's instructions.

Nissl Staining and Measuring Brain Size

Brain sections (14 μm) were processed through incubation in the subsequent solutions in the following order: ethanol/chloroform (1:1, overnight), 100% ethanol (30 s), 95% ethanol (30 s), distilled water (30 s, twice), cresyl violet (3–5 min), distilled water (2 min, three times), 50% ethanol (2 min), 95% ethanol (5–30 min), 100% ethanol (5 min, twice), xylene (3 min, twice). Thereafter, the sections were mounted with a coverslip.

[1]http://www.ncbi.nlm.nih.gov/geo/

The *Wnt7a* KO and WT brain images were captured in one picture, and the thickness of the cortex and CP was measured separately. The relative thickness of the cortex and CP in the KO was normalized from dividing the mean length of KO by that of the WT groups. At least three brains, and two chosen areas in each brain section were measured and averaged in each group. All data are presented as mean ± SEM. *P*-values were calculated using unpaired Student's *t*-test.

RNA and qRT-PCR

The RNAs for RT-PCR from five stages of samples (E12.5, E13.5, E14.5, E15.5, and E17.5), were extracted by TRIzol (Invitrogen, United States), with three mouse cerebral cortices from each age group. Experimental protocols of embryo treatment used here were approved by Weill Cornell Medical College's animal care and use committee. The procedures were carried out in accordance with the approved guidelines. After RNA extraction, the cDNA for RT-PCR was synthesized using high-capacity cDNA Reverse Transcription kit (Applied Biosystems). The qRT-PCR reactions were carried out in the Bio-Rad CFX-384 system, using the reaction mixture SYBR Green Mix (Bio-Rad, United States) with the aforementioned cDNA samples.

β-Actin was used as an internal control, and was used to normalize the relative mRNA expression level. Each group had three biological repetitions, and all experiments were performed in triplicate, and each experiment was repeated at least twice. The qRT-PCR primers are: *Wnt7a*, forward: 5′-CCGAAATGG CCGTTGG-3′ and reverse: 5′-CGATGCCGTAGCGGATGT-3′ (PCR product: 251 bp); *Sfrp1*, forward: 5′-CAACGTGGGCT ACAAGAAGAT-3′ and reverse: 5′-GGCCAGTAGAAGCCGA AG AAC-3′ (product size: 249 bp); β-actin, forward: 5′-GGCT GTATTCCCCTCCATCG-3′ and reverse: 5′-CCAGTTGGTAA CAATGCCATGT-3′ (product size: 245 bp). All data are presented as mean ± SEM. *P*-values were calculated using unpaired Student's *t*-test.

Tissue Preparation, Immunohistochemistry, and Analysis

Immunohistochemistry was performed as described: mouse brains were fixed in 4% PFA in phosphate-buffered saline (PBS) over night, incubated in 25–30% sucrose in PBS, embedded in OCT and stored at −80°C until use. Brains were sectioned (14–16 μm) using a cryostat. For antigen recovery, sections were incubated in heated (95–100°C) antigen recovery solution (1 mM EDTA, 5 mM Tris, pH 8.0) for 15–20 min, and cooled down for 20–30 min. Before applying antibodies, sections were blocked in 10% normal goat serum (NGS) in PBS with 0.1% Tween-20 (PBT) for 1 h. Sections were incubated with primary antibodies at 4°C overnight and visualized using goat anti-rabbit IgG–Alexa-Fluor-488 and/or goat anti-mouse IgG–Alexa-Fluor-546 (1:300, Molecular Probes) for 1.5 h at room temperature. Images were captured using a Leica digital camera under a fluorescent microscope (Leica DMI6000B) or a Zeiss confocal microscope.

The following antibodies were used: bromodeoxyuridine (BrdU) (1:50, DSHB), Ki67 (1:500, Abcam), Pax6 (1:30, DSHB), Tbr1 (1:2500, Abcam), Tbr2 (1:2000, kindly provided by Robert Hevner, University of Washington, Seattle, WA, United States), Ctip2 (1:1000, Abcam), Satb2 (1:1000, Abcam), GFP (1:600, DAKO), Neun (1:300, Chemicon), Wnt7a (1:1000, Abcam) and Sfrp1(1:1000, Abcam).

Cell counting in the mouse brain sections was performed on a fixed width (200 μm bin) of a representative column in the cortical wall. All sections analyzed were selected from a similar medial point on the anterior-posterior axis. Cell counting was performed in minimal three chosen areas in each brain, and at least three brains were analyzed in each group. Cell counting in each chosen area was repeated at least three times and a mean was obtained. All data are presented as mean ± SEM. *P*-values were calculated using unpaired Student's *t*-test.

Plasmid DNA Constructs

To clone *Sfrp1*, *Dkk1* and *Wnt7a* coding sequences into *pCAGIG* for IUE, *Sfrp1*, *Dkk1* and *Wnt7a* coding sequences from *pGEM-T* was attached to d2EGFP, a destabilized variant of the wild-type GFP, and then subcloned *d2EGFP-Sfrp1*, *-Dkk1* and *-Wnt7a* coding sequence fragments into *pCAGIG*.

Full length coding sequences (CDSs) for *Sfrp1*, *Dkk1* and *Wnt7a* were cloned using the following primers: *Sfrp1*, forward: 5′-ATTCCGCTCGAGCGGGTCGCCGAGCAACATG GGCGTC-3′ and reverse: 5′-ATTCCTTAAGGCCTTCCCCAG TCCGCCCCAG-3′ (PCR product: 954 bp); *Wnt7a*, forward: 5′-GCACTCGAGCAGCGGGGACTATGACCCGGAAAGCGC-3′ and reverse: 5′-CATTCACTTGCACGTATACATCTCCG TG-3′ (PCR product: 1,053 bp); *DKK1*, forward: 5′-CGGAATTC GGAGATGATGGTTGTGTGTGC-3′ and reverse: 5′-GGTTT AGTGTCTCTG GCAGGTGTG-3′ (PCR product: 826 bp).

The *Sfrp1*, *Dkk1* coding sequences were subcloned into the *pcDNA3.1* vector for the *TOPflash* and *FOPflash luciferase* reporter (Promega, United States) assay.

RNA Interference Design and Efficiency Analysis

To knockdown *Sfrp1*, 4 different *Sfrp1* specific *short hairpin RNA* (*Sfrp1-shRNA*) were designed and cloned into the *pSilencer* vector, separately. To analyze interference efficiency, Neuro2A cells were plated into 6-well plates in triplicate, and were transfected with four *Sfrp1-shRNA* using Lipofectamine 3000 (Invitrogen, United States). Cells were cultured for 2 days and the endogenous *Sfrp1* knockdown efficiency was verified by qRT-PCR. The *shRNA* with the highest knockdown efficiency was selected to perform further IUE in cerebral cortices.

The following oligos were used to clone *Sfrp1-shRNA*: *Sfrp1-shRNA1*, 5′-CACCGCTACAAGAAGATGGTGCTGC TTCAAGAGAGCAGCACCATCTTCTGGTAGCTTTTTTG-3′ (Target site: GCTACAAGAAGATGGTGCTGC, 498–519); *Sfrp1-shRNA2*, 5′-CACCGCCACAACTTTCTCATCATGGTTCAAG AGACCATGATGAGAAAGTTGTGGCTTTTTTG-3′ (Target site: GCCACAACTTTCTCATCATGG, 1,077–1,098); *Sfrp1-shRNA3*, 5′-CACCGCCATTCACAAGTGGGACAAGTTCAAG AGACTTGTCCCACTTGTCCCACTTGTGAATGGCTTTTTT

G-3′ (Target site: GCCACAACTTTCTCATCATGG, 1,130–1,151); *Sfrp1-shRNA4*, 5′-CACCGCAGTTCTTCGGCTTCTACTGTTCAAGAGACAGTAGAAGCCGAAGAACTGCTTTTTTG-3′ (Target site: GCAGTTCTTCGGCTTCTACTG, 715–736); for negative control, 5′-CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTG-3′.

In Utero Electroporation

In utero electroporation was performed in E12.5 embryos according to the published protocol (Saito and Nakatsuji, 2001; Saito, 2006; Ito et al., 2014). Briefly, plasmid DNA was prepared using the EndoFree Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions, and diluted to 2 μg/μl. DNA solution was injected into the lateral ventricle of the cerebral cortex, and electroporated with five 50-ms pulses at 35V using an ECM830 electro square porator (BTX). Embryos were allowed to develop to E13.5. Animals with their brains electroporated, as detected by the GFP fluorescence under a fluorescent dissection scope (Leica, MZ16F), were selected for further analyses. Cell counting was performed in minimal three chosen areas in each brain, and at least three electroporated brains for each construct were analyzed. Cell counting in each chosen area was repeated at least three times and a mean was obtained.

TOPflash and *FOPflash Luciferase* Reporter Assay

The coding sequences of the *Wnt7a* and *Wnt1* were amplified by PCR from mouse cDNA. Reporter genes were cloned into *TOPflash* and *FOPflash* vector (Promega, United States). For transfections, mouse Neuro2A cells were suspended in DMEM and plated into 24-well plates in triplicate at 1.5×10^4cells/100 mL. Adherent cells were co-transfected with 100 ng/mL *luciferase* reporter containing the reporter gene and 60 ng/mL vector (pcDNA3.1 blank vector, pcDNA3.1-*Dkk1* and pcDNA3.1-*Sfrp1*) using Lipofectamine 3000 (Invitrogen, United States). After 48 h, cells were harvested and *luciferase* activity was measured using the *luciferase* reporter assay system (Cat. #E1910, Promega, United States) according to the manufacturer's protocol.

The relative *luciferase* activity was normalized from the mean of pcDNA3.1 blank vector, separately. Each group had three biological repetitions, and experiments were performed in triplicate and each sample was repeated at least three times. All results are presented as mean ± SEM. *P*-values were calculated using unpaired Student's *t*-test.

Statistical Analysis

All experiments using cultured cells and mouse embryos were repeated at least with three biological replicates. All results are presented as mean ± standard error of the mean (SEM). *P*-values were determined by unpaired Student's *t*-test for assessing the significance of differences between two treatments (See each figure for details). *P*-values <0.05 were considered significant. Significant differences were denoted as $^{*}P$-values < 0.05, $^{**}P$-values < 0.01, $^{***}P$-values < 0.001.

RESULTS

Wnt7a and *Sfrp1* Are Co-expressed in NPs in the VZ

To screen genes that are highly expressed in the mouse E12.5 cerebral cortices, RNA sequencing (RNA-seq) was performed. 30,827,078 and 29,345,746 and 32,038,052 raw sequencing reads, and 28,547,544 and 27,289,172 and 29,753,653 clean reads, respectively, were obtained from three individual E12.5 cortices (Supplementary Table S1). The mapping rates of clean reads are 92.2%, 93.4%, and 92.6% (Supplementary Table S2). Among these genes, *Wnt7a*, *Wnt7b,* and *Sfrp1* showed high expression (RPKM >500) (Supplementary Figure S1A and Supplementary Table S4). Moreover, *Wnt7b*, *Wnt7a,* and *Wnt5a* displayed higher abundant expression levels than other Wnt genes (Supplementary Tables S3, S4).

To verify the RNA-seq data, we examined expression patterns of *Wnt7a*, *Wnt7b,* and *Sfrp1*, and compared them with those of NP markers such as *Pax6*, *Ngn2,* and *Hes5*, and other *Sfrp*s such as *Sfrp2, Sfrp4,* and *Sfrp5* in the mouse cortex at E12.5 using ISH (**Figure 1A** and Supplementary Figure S1B). We found that both *Wnt7a* and *Sfrp1* are expressed in the VZ of the E12.5 cortex (**Figure 1A**). Moreover, expression of *Wnt7a* and *Sfrp1* was co-localized with that of *Pax6*, *Ngn2* and *Hes5*, suggesting that *Wnt7a* and *Sfrp1* are largely expressed in NPs (**Figure 1A**). Conversely, *Wnt7b* was highly expressed in newborn neurons, and other *Sfrp*s such as *Sfrp2* displayed low expression in the cortex (**Figure 1A** and Supplementary Figure S1B).

Next, we investigated whether expression levels of *Wnt7a* and *Sfrp1* progressively change over embryonic stages at E12.5, E13.5, E14.5, E15.5, and E17.5 using qRT-PCR. *Wnt7a* displayed ascending expression from E12.5 to E15.5 (**Figure 1B**). *Sfrp1* expression showed a gradual decline from E12.5 to E17.5 (**Figure 1C**). Compared to *Wnt7a*, *Sfrp1* displays overlapping expression with *Wnt7a* in the VZ and opposite expression levels, implying distinct roles of Wnt7a and Sfrp1 in cortical development.

Wnt7a Positively Regulates Proliferation of NPs and Promotes Neurogenesis

Because of *Wnt7a* expression in the cortical VZ, we investigated whether *Wnt7a* regulates NP proliferation by analyzing cortical development in *Wnt7a* knockout mice (*Wnt7a* KO). The body size of *Wnt7a* KO was indistinguishable from that of WT mice. The cortical size and brain size were measured at P0, P5, and P20 (**Figures 2A–C** and Supplementary Figure S2). Compared to WT, the cortical size and brain size of *Wnt7a* KO mice were greatly reduced from P0 to P20, suggesting a progressive brain deterioration (**Figures 2A–C** and Supplementary Figure S2). Moreover, the thickness of the cortical wall was significantly reduced in the brain sections with Nissl staining in *Wnt7a* KO mice (**Figures 2B,C**). Interestingly, the ratios of cortical size versus brain size were similar between WT and KO, suggesting that the overall brain size is reduced in *Wnt7a* KO mice (**Figure 2C** and Supplementary Figure S2).

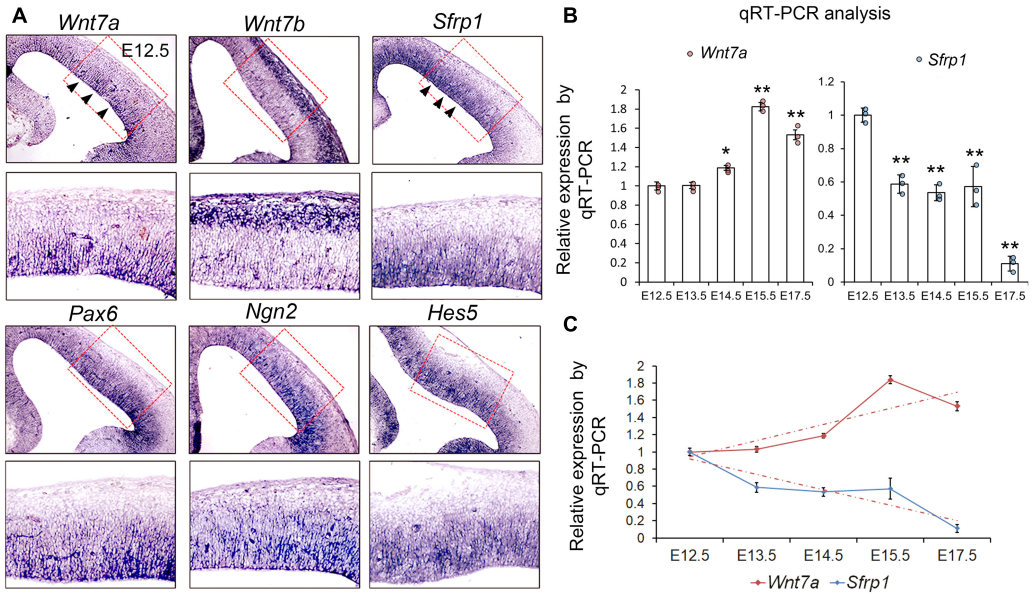

FIGURE 1 | *Wnt7a* and *Sfrp1* are co-expressed in neural progenitors and show opposite expression trends. **(A)** In coronal sections of mouse E12.5 cerebral cortices, *Wnt7a*, *Sfrp1*, *Pax6*, *Ngn2,* and *Hes5* were expressed in the ventricular zone (arrowheads). Conversely, *Wnt7b* was expressed in newborn neurons. Red boxes show high power views. **(B)** qRT-PCR analysis of *Wnt7a* and *Sfrp1* expression levels at different embryonic stages (E12.5, E13.5, E14.5, E15.5, and E17.5). All comparisons were made with that of values at E12.5. Values of histogram represent mean ± SEM, and each dot represents a data point in each biology repeat ($n = 3$, $^*P < 0.05$; $^{**}P < 0.01$; unpaired Student's *t*-test). **(C)** Opposite expression trends between *Wnt7a* and *Sfrp1* at different embryonic stages (from E12.5 to E17.5) measured by qRT-PCR.

We then examined whether the NP population was changed in E13.5 *Wnt7a* KO mice using immunohistochemistry. NPs can be detected by labeling cells in the G1, S, G2, and M phases using the anti-Ki67 antibody. The number of Ki67$^+$ cells was significantly decreased in the E13.5 *Wnt7a* KO cortex, compared to the control (**Figures 2D,E**). The numbers of Sox2$^+$ and Pax6$^+$ radial glial cells (RGCs), and Tbr2$^+$ IPs were also reduced, suggesting an early reduction of NPs (**Figures 2F–K**). Moreover, because Pax6$^+$/Tbr2$^+$ cells are under transition from RGCs to IPs, we quantified the number of Pax6$^+$/Tbr2$^+$ cells. While a significant decrease in the number of Pax6$^+$/Tbr2$^+$ cells was detected in E13.5 *Wnt7a* KO cortex, the percentages of Pax6$^+$/Tbr2$^+$ cells versus total Pax6$^+$ cells and Pax6$^+$/Tbr2$^+$ cells versus total Tbr2$^+$ cells were unchanged, indicating that *Wnt7a* deletion doesn't affect transition of RGCs to IPs (Supplementary Figures S3A–D). In addition, even though the total number of Tbr2$^+$ cells was reduced, the percentage of Tbr2$^+$ cells versus total DAPI$^+$ cells remained the same in WT and *Wnt7a* KO cortices, suggesting that reduction in IPs is in proportion with that of total cells (Supplementary Figures S3E,F).

Next, we examined whether the early loss of NP population is maintained at E15.5. Compared to the controls, the numbers of BrdU$^+$, Ki67$^+$, Sox2$^+$, Tbr1$^+$, Pax6$^+$, and Tbr2$^+$ cells were greatly reduced in E15.5 *Wnt7a* KO cortices, suggesting that the deletion of *Wnt7a* causes a progressive loss of NPs (**Figures 3A–F** and Supplementary Figures S4A,B).

Because the overall organization of cortical layers is becoming clear, and neuronal production is evident at P0, P0 pups were collected to analyze brain phenotypes without sacrifice of the mother. We examined the expression of Tbr1 (layer VI), Ctip2 (layer V) and Satb2 (layer II, III, and IV) in P0 *Wnt7a* KO and control cortices (Molyneaux et al., 2007). The relative positioning of layer markers in the CP was similar to that of the WT, suggesting that overall cortical layer organization is not greatly affected by *Wnt7a* deletion (**Figures 3G,I**). Despite concordance of the position of layer markers, each layer examined was thinner in the *Wnt7a* KO cortex than that in the control, with significantly fewer mature NeuN$^+$ neurons found, and great reductions in the number of Tbr1$^+$ and Satb2$^+$ neurons (**Figures 3G–J**). The Citp2$^+$ neurons showed no appreciable decrease in *Wnt7a* KO mice (**Figures 3I,J**). Moreover, the percentages of Tbr1$^+$ and Satb2$^+$ cells versus DAPI$^+$ cells were unchanged in WT and KO cortices, indicating that the reduction in newborn neurons is in proportion with that of total cells (Supplementary Figure S4C).

Taken together, our results indicate that knockout of *Wnt7a* causes reduced NPs and production of newborn neurons.

Sfrp1 Negatively Regulates Proliferation of NPs

We next examined whether altering *Sfrp1* expression in the cortex has a similar or an opposite effect on NPs as deleting *Wnt7a* expression. The full length cDNA for *Sfrp1* was cloned (*pCAGIG-Sfrp1*) and was ectopically expressed in E12.5 cortices by using IUE, and embryos were analyzed after 24 h. Overexpression of *Sfrp1* resulted in a decreased number of GFP$^+$ NPs that are

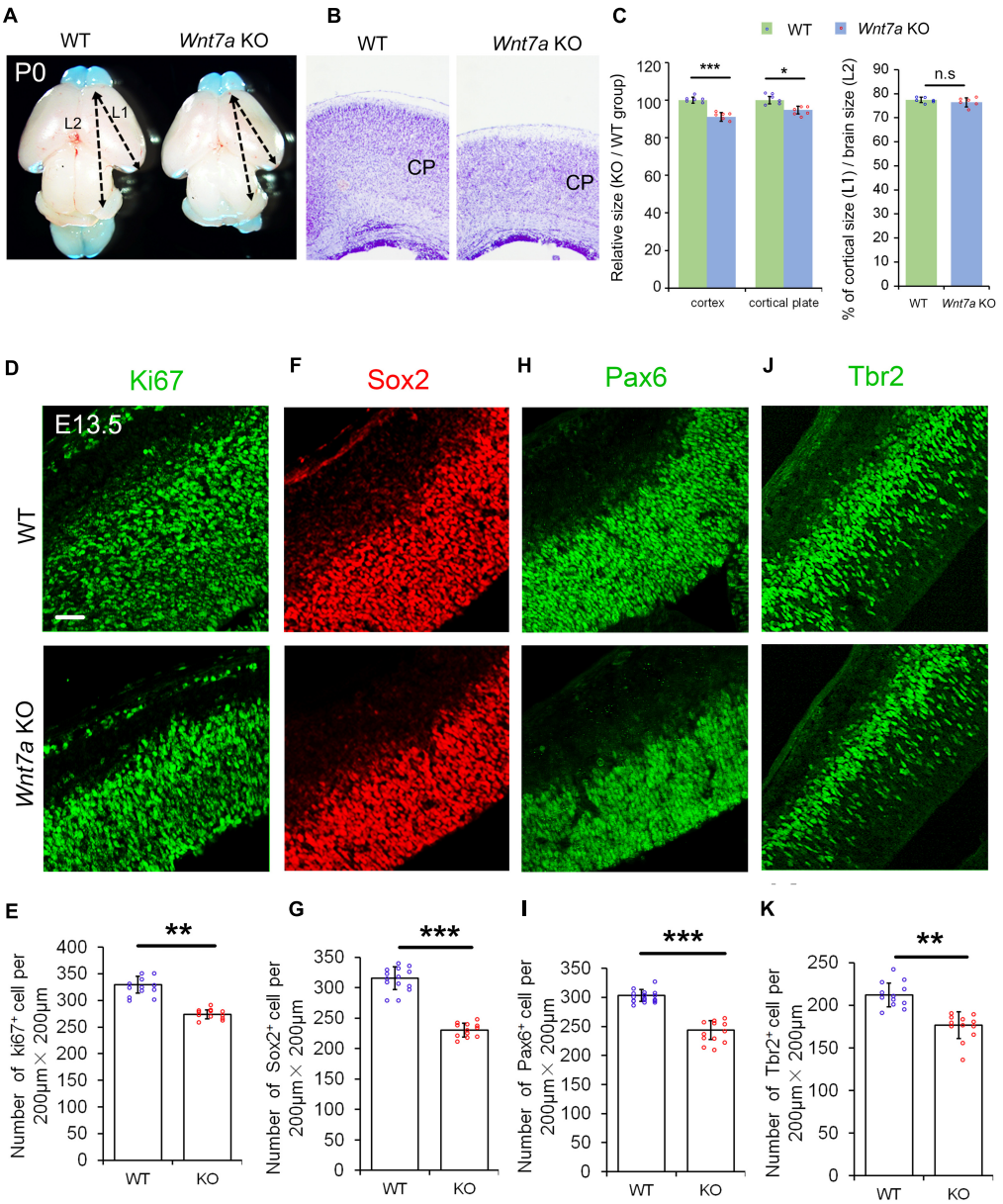

FIGURE 2 | *Wnt7a* positively regulates brain size and proliferation of NPs. **(A)** The cortex of P0 *Wnt7a* knockout (KO) mice was greatly reduced compared to wild type (WT) controls. The arrowheads show the most rostral and caudal regions in the cortex. "L1" represent the cortical length, and "L2" represent the brain length. **(B)** The cortical wall in P0 *Wnt7a* KO mice were thinner than that in WT mice, detected by Nissl staining. CP: cortical plate. **(C)** The relative thickness of the cortex and cortical plate in the KO was normalized from dividing the mean length of *Wnt7a* KO by that of the WT groups. Values of histogram represent mean ± SEM, and each dot represents a data point of the relative thickness in each section or length in the brain images. $n = 3$ brains, at least two sections from each brain. $^*P < 0.05$;$^{***}P < 0.001$; ns, non-significant; unpaired Student's *t*-test. **(D–K)** The numbers of $Pax6^+$ and $Tbr2^+$ neural progenitors were greatly reduced in the E13.5 *Wnt7a* KO cortex. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 μm bin). $n = 3$ brains, at least four sections from each brain. $^{**}P < 0.01$; $^{***}P < 0.001$; unpaired Student's *t*-test). Scale bar: 50 μm.

double-positive for $BrdU^+$, $Pax6^+$, $Sox2^+$ and $Tbr2^+$, compared to those of electroporation of the control (*pCAGIG*) in E13.5 cortices, suggesting a decrease of NPs after Sfrp1 overexpression (**Figure 4**).

To test whether the endogenous *Sfrp1* limits the NP numbers *in vivo*, we used *shRNA* designed to outcompete endogenous *Sfrp1* transcripts. The *Sfrp1* knockdown efficiency were verified in mouse Neuro2A cell by qRT-PCR (Supplementary Figure S5). The construct of *shRNA* (*Sfrp1-sh4*) that shows the highest knockdown efficiency among four tested shRNAs was used to perform IUE. Greater proportions of GFP^+ NPs expressed BrdU, Pax6 and Sox2 were found in the VZ/SVZ following electroporation of the *Sfrp1-sh4* (Supplementary Figures S6A–F). $Tbr2^+$ NPs displayed no appreciable increase (Supplementary

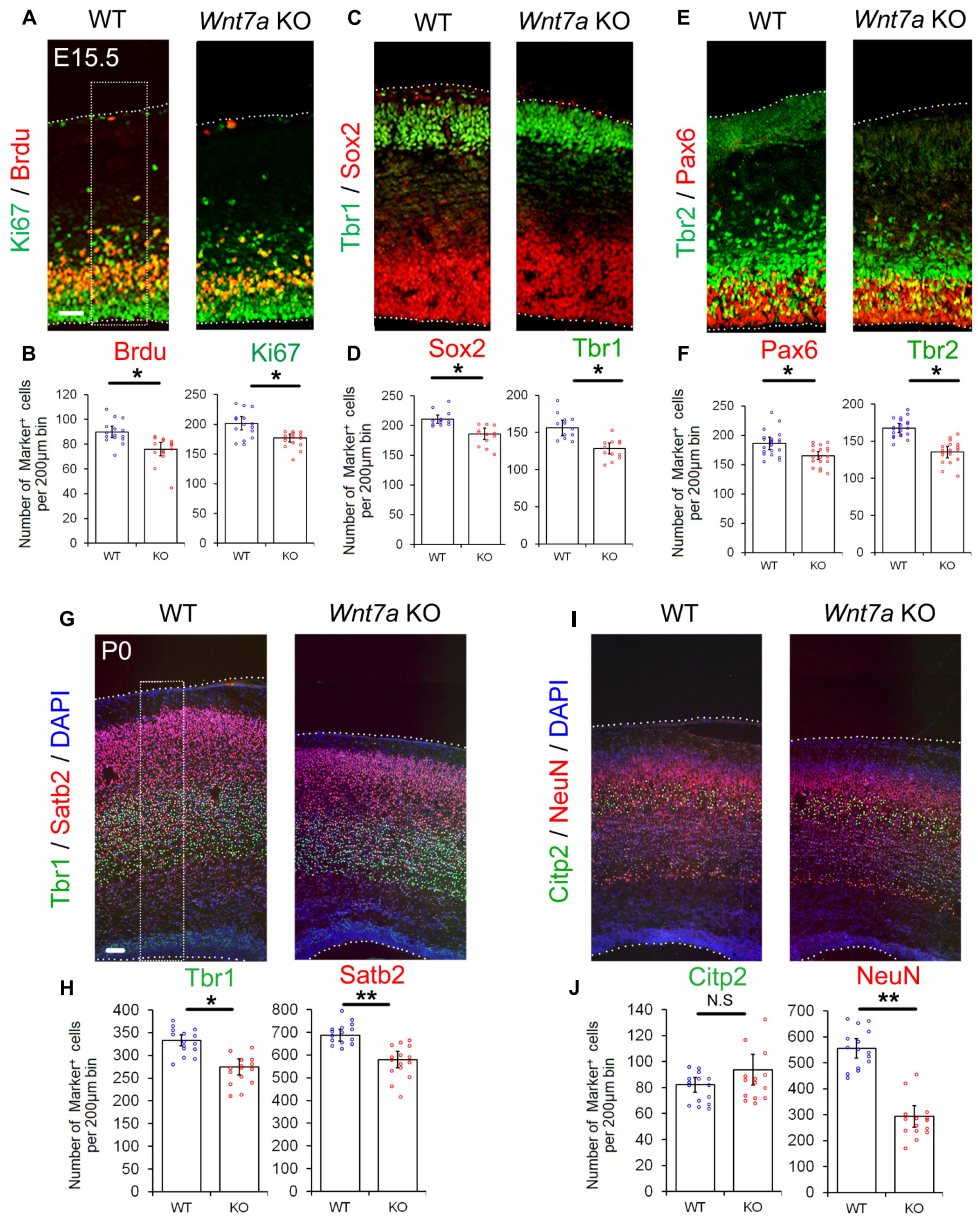

FIGURE 3 | *Wnt7a* promotes neurogenesis at E15.5 and P0. **(A–F)** Compared to controls (WT), *Wnt7a* knockout (KO) cortices at E15.5 displayed a reduction in the numbers of BrdU$^+$, Ki67$^+$, Sox2$^+$, Tbr1$^+$, Pax6$^+$, and Tbr2$^+$ cells. The dashed box represents the cell counting area. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 μm bin). $n = 3$, at least four sections from each brain. *$P < 0.05$; unpaired Student's *t*-test. **(G–J)** In P0 *Wnt7a* KO cortices, the numbers of Tbr1$^+$ and Stab2$^+$ neurons were greatly reduced. NeuN$^+$ neurons but not Citp2$^+$ neurons were also reduced. The dashed box represents the cell counting area. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 μm bin). $n = 3$, at least five sections from each brain. *$P < 0.05$; **$P < 0.01$; ns, non-significant; unpaired Student's *t*-test. Scale bar: 100 μm.

Figures S6G,H). These results indicate that *Sfrp1* negatively modulates NP proliferation.

Sfrp1 Has an Opposite Role of *Wnt7a* in Regulating NP Proliferation

Based on opposite effect of *Wnt7a* and *Sfrp1* on NP development, we suspected that Wnt7a might be regulated by its antagonists during cortical development. Previous studies have shown that *Dkk1* is an antagonist of *Wnt7a* (Fortress et al., 2013). To examine how the *Wnt7a* antagonist may regulate NP development in the cortex, we over-expressed both *Wnt7a* and *Dkk1* in the VZ of cortex using IUE. While *Wnt7a* promoted expansion of NPs, as shown by an increased number of BrdU$^+$ and Pax6$^+$ cells, over-expression of *Dkk1* and *Wnt7a* in the VZ dampened *Wnt7a* effects, suggesting an antagonistic regulation of Dkk1

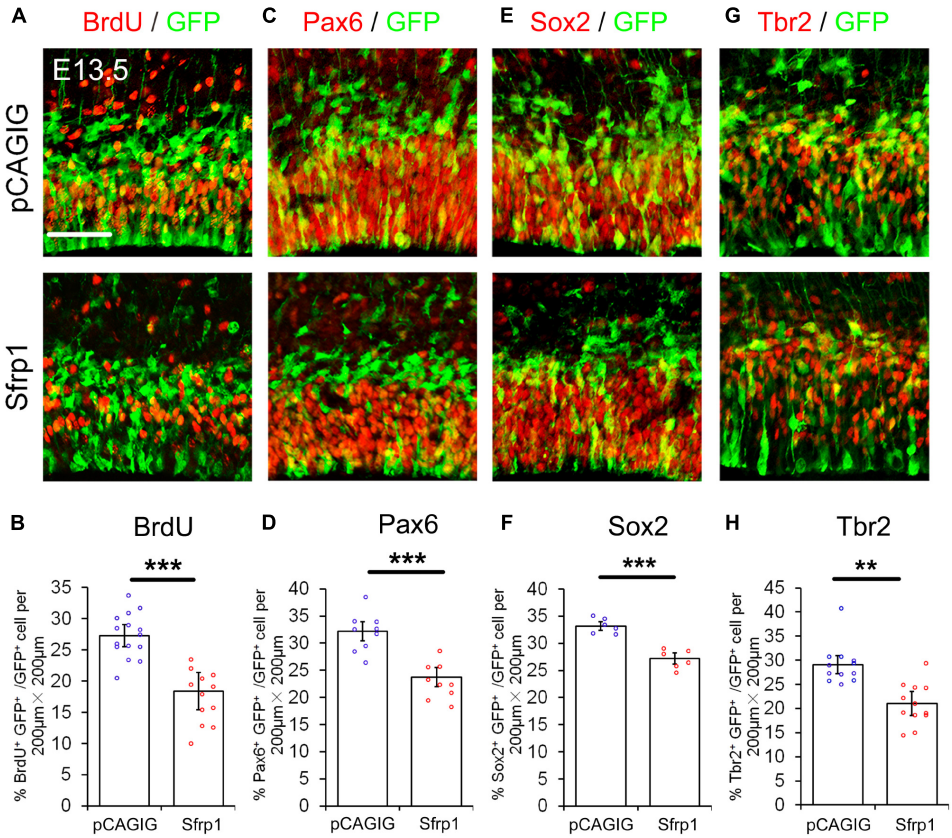

FIGURE 4 | Sfrp1 negatively regulates proliferation of NPs at E13.5. **(A,C,E,G)** Overexpression of *Sfrp1* in E12.5 cortices using *in utero* electroporation, analyzed at E13.5, caused the reduction of BrdU$^+$/GFP$^+$, Pax6$^+$/GFP$^+$, Sox2$^+$/GFP$^+$, and Tbr2$^+$/GFP$^+$ neural progenitors. **(B,D,F,H)** The proportion of cells labeled with individual progenitor markers and GFP versus cells labeled with GFP was quantified. Values represent mean ± SEM, and each dot represents a data point of the marker$^+$ GFP$^+$/GFP$^+$ % in each section (200 μm × 200 μm). $n = 3$, at least two sections from each brain. $^{**}P < 0.01$; $^{***}P < 0.001$; unpaired Student's *t*-test. Scale bar: 50 μm.

(Supplementary Figure S7). Moreover, increasing *Dkk1* dosage caused a greater decrease in the number of BrdU$^+$ and Pax6$^+$ cells, suggesting a dosage-dependent antagonistic regulation of *Dkk1* on *Wnt7a* (Supplementary Figure S7).

If *Sfrp1* also has the functions as a *Wnt7a* antagonist, it should have a similar effect to *Dkk1* in NP development. With this in mind, *Wnt7a* and *Sfrp1* were both overexpressed in the cortex using IUE. Similar to *Dkk1*, *Wnt7a-Sfrp1* overexpressed in the VZ caused a reduction of BrdU$^+$ and Pax6$^+$ cells (**Figure 5**). Moreover, increasing the dosage of *Sfrp1* had a more profound activity in suppressing *Wnt7a* effect on NP expansion (**Figure 5**).

Our results suggest that similar to *Dkk1*, *Sfrp1* acts as an antagonist of *Wnt7a* and negatively regulates expansion of NPs.

Sfrp1 Inhibits *Wnt7a* Activity in *TOPflash Luciferase* Reporter Assay

Based on the dosage-dependent regulation of *Sfrp1* on *Wnt7a*, we tested whether *Sfrp1* could down-regulate the *Wnt7a* activity. To validate *Sfrp1-Wnt7a* interaction, we used the *TOPflash luciferase* reporter assay containing the active TCF/LEF binding sites, which is the classical method to identify canonical Wnt/β-catenin activity (**Figure 6A**) (Veeman et al., 2003). If the canonical Wnt signaling is activated, the β-catenin will be associated with the TCF/LEF transcription factors to promote the *Firefly luciferase* activity. The mutant TCF/LEF binding site of *FOPflash* was used as the control (**Figure 6A**).

Wnt1 is a known molecule of the Wnt signaling and is crucial for early development of the CNS (Leal et al., 2011; Cai et al., 2013). As the positive control, we first tested whether *Dkk1* and *Sfrp1* can block *Wnt1* in Neuro2A cells. Compared to the *FOPflash* group, the *luciferase* activity of *Wnt1* in *Dkk1* overexpression treatment was significantly decreased in the *TOPflash* group (**Figure 6B**). Agreed with *Dkk1*, the *luciferase* activity of *Sfrp1* overexpression showed a similar decrease (**Figure 6B**).

Next, we tested whether *Sfrp1* can inhibit *Wnt7a* in a similar fashion to how Wnt1 is negatively regulated in the aforementioned experiment. We found that the *luciferase* activity of *Wn7a* was decreased appreciably in both *Sfrp1* and *Dkk1* overexpression treatment, suggesting that *Sfrp1* acts like the known antagonist *Dkk1*, and blocks the *Wnt7a* signal (**Figure 6C**).

In summary, Sfrp1 has an attenuating role in Wnt signaling by blocking *Wnt1* and Wnt7a *in vitro*.

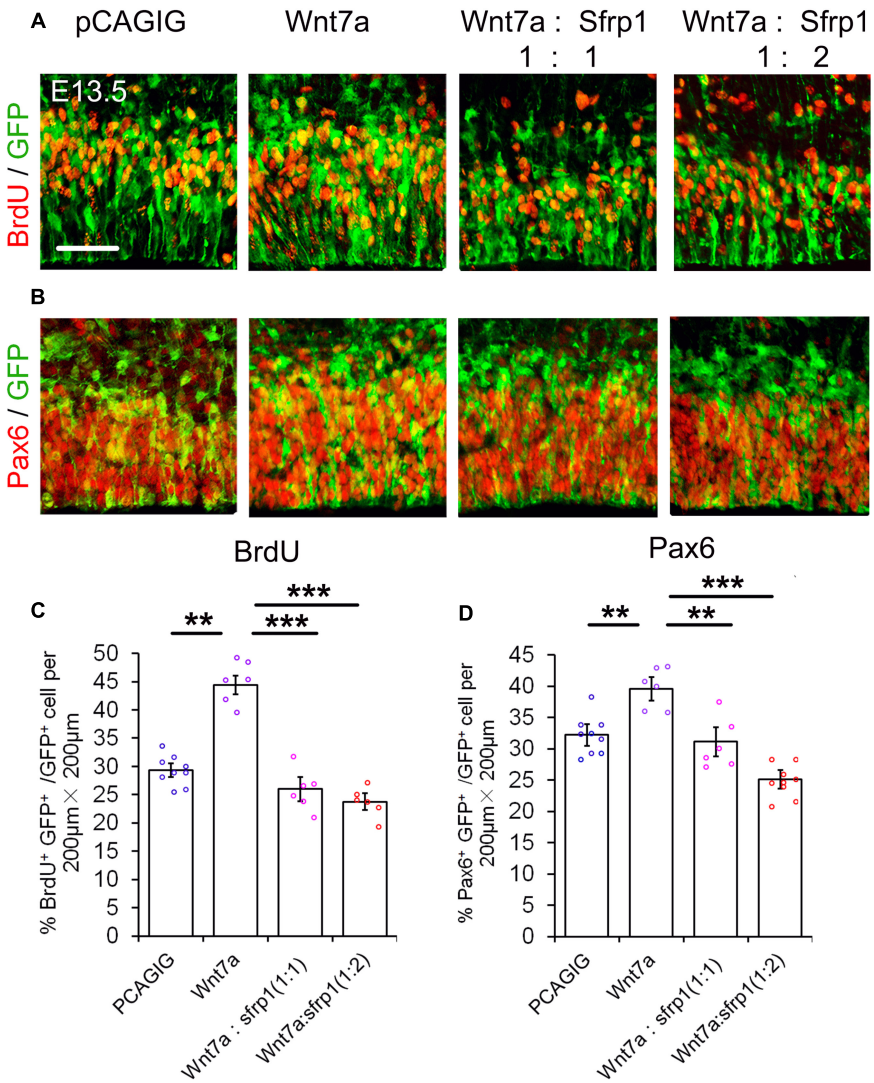

FIGURE 5 | Sfrp1 suppresses *Wnt7a* activity in neural progenitor proliferation dosage-dependent manner. **(A,B)** Co-expression of *Sfrp1* and *Wnt7a* dampened the effect of *Wnt7a* in expanding neural progenitors at E13.5. **(C,D)** The numbers in BrdU$^+$/GFP$^+$ and Pax6$^+$/GFP$^+$ neural progenitors showed a decreasing trend with a proportional increase of *Sfrp1* (*Wnt7a*:*Sfrp1* = 1:1 vs. *Wnt7a*:*Sfrp1* = 1:2). Values represent mean ± SEM, and each dot represents a data point of the marker$^+$ GFP$^+$/GFP$^+$ % in each section (200 μm × 200 μm). $n = 3$, at least two sections from each brain. $^{**}P < 0.01$; $^{***}P < 0.001$; unpaired Student's *t*-test. Scale bar = 50 μm.

DISCUSSION

The maintenance of normal cortical formation and size is essential for brain function. The Wnt signaling plays critical roles to regulate cell cycle control, neuronal differentiation and tissue repair (Chenn and Walsh, 2003; Kalani et al., 2008; Piccin and Morshead, 2011; Delaunay et al., 2014). The precise antagonistic regulation of Wnt members by Wnt modulators also controls cortical neurogenesis. Our study shows that *Wnt7a* and *Sfrp1* are co-expressed in cortical NPs and their opposite role is essential for controlling NP expansion and neuronal production.

Among the many signals known to influence the CNS development, the Wnt signal has attracted great attention. Wnt/β-catenin signaling acts upstream of a complex and dynamic temporal network to control progenitor fate (Draganova et al., 2015): long-term overexpression of *Wnt3a* leads to cortical dysplasia by inducing early differentiation of IPs into neurons and the heterotopias of these newborn neurons (Munji et al., 2011). Studies have shown the role of *Wnt7a* in axon development and guidance, as well as synapse formation and maintenance (Hall et al., 2000; Cerpa et al., 2008; Ciani et al., 2011, 2015). Investigations of *Wnt7* in the early step of neurogenesis in the cerebral cortex have just begun (Qu et al., 2013; Long et al., 2016). Transcriptome sequencing data from us and others have shown that *Wnt7b, Wnt7a,* and *Wnt5a* are the most abundant Wnt factors in the E12.5, E16.5, and E17.5 cortices (Wang et al., 2016; Nguyen et al., 2018). Moreover, we have found that *Wnt7a* is highly expressed in the VZ and *Wnt7b* in the

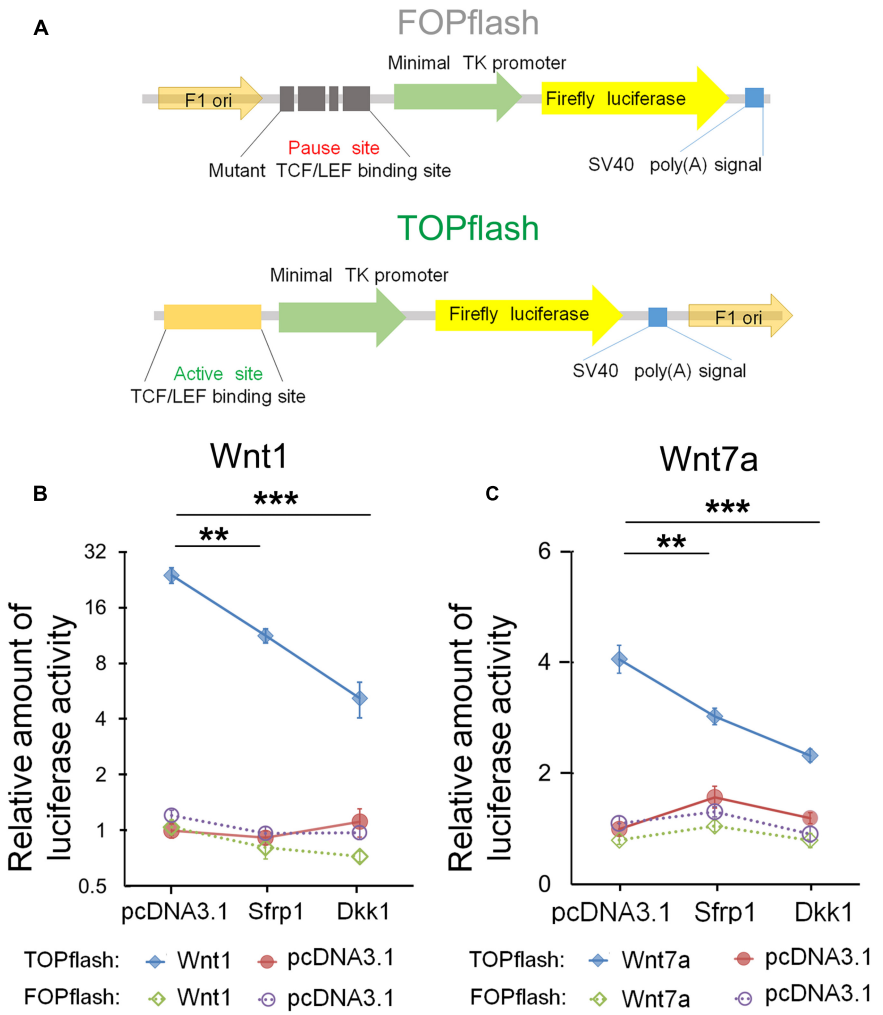

FIGURE 6 | Sfrp1 inhibits *Wnt7a* activity in the *TOPflash luciferase* reporter assay. **(A)** *TOPflash* is a *luciferase* reporter of β-catenin-mediated transcriptional activation with active TCF/LEF binding sites, which affect the *firefly luciferase* expression. The control plasmid is *FOPflash*, which contains mutant TCF/LEF binding sites. **(B,C)** After transfection of the *pcDNA3.1-Sfrp1* and *pcDNA3.1-Dkk1*, a statistically significant decrease in *luciferase* activity of *Wnt1* and *Wnt7a* was observed in comparison with controls. Values represent mean ± SEM. $n = 3$, $^{**}P < 0.01$; $^{***}P < 0.001$; unpaired Student's *t*-test.

intermediate zone and CP, which is consistent with the RNA-seq results from isolating specific cellular zones and layers in E14.5 and E15.5 cortices (Ayoub et al., 2011; Belgard et al., 2011; Aprea et al., 2013; Liu et al., 2016). How distinct expression patterns of different Wnts are established in developing cortices remains unclear. Differential expression of Wnt7a and Wnt7b in the cortical layers may determine their different roles in cortical neurogenesis (Stenman et al., 2008; Durak et al., 2016): *Wnt7a* promotes neurogenesis by regulating genes involved in cell cycle control and neuronal differentiation (Qu et al., 2013); the increased *Wnt7b* modulates neuronal differentiation by regulating T-domain transcription factors Tbr1 and Tbr2 (Papachristou et al., 2014).

Moreover, we have shown that the deletion of *Wnt7a* expression causes microcephaly by reducing the population of NPs and newborn neurons. These data are consistent with previous reports demonstrating that *Wnt7a* positively regulates NPs and neurogenesis (Qu et al., 2013; Long et al., 2016; Wang et al., 2016). Recent research has shown that *Wnt7a* regulates the asymmetry of spindles in neuroepithelial cells in the VZ, which is linked to asymmetric cell division (Delaunay et al., 2014). The embryonic ventral midbrain of *Wnt7a* KO mice displays reduced $Sox2^+$ progenitors (Fernando et al., 2014). We have also found that $Sox2^+$ progenitors are decreased in the cerebral cortex at E13.5. Decreased expansion of cortical NPs is likely a major cause of microcephaly in *Wnt7a KO* mice. Among Wnt molecules, Wnt7a is a known regulator in the beta-catenin signal pathway (mmu04310) functioning in different biological processes (Daneman et al., 2009; Ciani et al., 2011; Qu et al., 2013; King et al., 2015). Wnt molecules are associated with Hippo signaling pathway, Integrin signaling and Notch signaling (Qu et al., 2013; Ciani et al., 2015; Wang et al., 2016). These pathways likely cooperate to regulate cortical development.

Sfrps are a family of receptors known to possess a Wnt-binding frizzled CRD, and abnormal expression of *Sfrp1* leads to CNS functional disorders (Esteve et al., 2011, 2018). *Sfrp1* is a key member of the Sfrp family that can bind directly to Wnts via their regions of homology to Fz. In the CNS, *Sfrp1* can block dopamine neuron development, dendritic development and hippocampus formation (Rosso et al., 2005; Miquelajauregui et al., 2007; Kele et al., 2012). In this study, we have found that *Sfrp1* is expressed in the VZ of the mouse embryonic cerebral cortex, which is consistent with the observation of its expression restricted to the proliferative zone in the CNS (Augustine et al., 2001). Similar to the known antagonist Dkk1, we have found that overexpression of *Sfrp1* reduces the NP population, and *Sfrp1* significantly decreases the number of NPs in a dosage-dependent manner, suggesting an opposite role of Sfrp1 in cortical development compare to Wnt7a (Adamska et al., 2004; Kim et al., 2008; Osada et al., 2010). In the recent study of *Sfrp1* knockout mice, the authors observed an increase in the number of $BrdU^+/Tbr2^+$ cells in E12.5 $Sfrp1^{-/-}$ cortex (Esteve et al., 2018). We think that the reason we did not detect an increase of $Tbr2^+$ cells when Sfrp1 is knocked down, it is likely due to the efficiency of shRNA of Sfrp1, compared to the gene knockout. Moreover, recent studies have shown that Sfrps interact with the Wnt signaling, Hedgehog signaling, BMP and Notch signaling (Katoh and Katoh, 2006; Mii and Taira, 2009; Misra and Matise, 2010; Esteve et al., 2011, 2018). It is likely a combined effort of Sfrp1 with other signals contributes to cortical development.

Sfrps is a physiological Wnt-signaling scavenger that binds directly to Wnts due to their similarity to the receptor Frizzled, thus, it is capable of regulating the availability of Wnt proteins (Finch et al., 1997; Rattner et al., 1997; Baarsma et al., 2013; Cruciat and Niehrs, 2013). The exclusive repression of the Wnt pathway is possible by selective Sfrps in cortical development (Mikels and Nusse, 2006; Lacour et al., 2017). *Sfrp1* and *Sfrp3* are expressed in opposing anterolateral to caudomedial gradients, and regulate normal temporal advancement of neuronal birth and maturation in anterior and lateral cortical regions by selectively modulating Wnts (Kim et al., 2001b). Previous studies have shown that Dkks inhibit the canonical Wnt pathway by internalizing LRP5/6, whereas Sfrps inhibit both the canonical and non-canonical pathways by binding Wnt ligands or Frizzled (Dees et al., 2014; Majchrzak-Celinska et al., 2016). The future study will be to investigate whether Sfrp1 directly binds to Wnt7a or through other mechanisms in the cortex.

The reciprocal control of *Wnt7a* and *Sfrp1* may be a dosage-dependent compensatory mechanism to maintain normal cortical formation during early development. Our study reveals that an optimal expression level of *Wnt7a* and *Sfrp1* is critical for proper establishment of the NP population. Further work will be dedicated to explore the precise regulation of how different Sfrps mediate canonical Wnt signaling pathway in NP proliferation and differentiation during embryonic cortical development. Our findings suggest that dysregulation of the Wnt signaling can lead to developmental defects similar to human cortical malformation disorders such as microcephaly.

AUTHOR CONTRIBUTIONS

TS: conceived and designed the experiments. NM, SB, TL, and TM: experiment. NM, SB, SH, ZW, GH, and TS: result analysis. NM and TS: wrote the paper. NM and TS: edited paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00247/full#supplementary-material

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REVIEW
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Zebrafish Models of Neurodevelopmental Disorders: Past, Present, and Future

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Zebrafish are increasingly being utilized as a model system to investigate the function of the growing list of risk genes associated with neurodevelopmental disorders. This is due in large part to the unique features of zebrafish that make them an optimal system for this purpose, including rapid, external development of transparent embryos, which enable the direct visualization of the developing nervous system during early stages, large progenies, which provide considerable tractability for performing high-throughput pharmacological screens to identify small molecule suppressors of simple behavioral phenotypes, and ease of genetic manipulation, which has been greatly facilitated by the advent of CRISPR/Cas9 gene editing technologies. This review article focuses on studies that have harnessed these advantages of the zebrafish system for the functional analysis of genes that are strongly associated with the following neurodevelopmental disorders: autism spectrum disorders (ASD), epilepsy, intellectual disability (ID) and schizophrenia. We focus primarily on studies describing early morphological and behavioral phenotypes during embryonic and larval stages resulting from loss of risk gene function. We highlight insights into basic mechanisms of risk gene function gained from these studies as well as limitations of studies to date. Finally, we discuss advances in *in vivo* neural circuit imaging in zebrafish, which promise to transform research using the zebrafish model by illuminating novel circuit-level mechanisms with relevance to neurodevelopmental disorders.

Keywords: zebrafish, neurodevelopmental disorders, autism spectrum disorders, epilepsy, schizophrenia, model system, genetics, neural circuits

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INTRODUCTION

In recent years, there has been growing interest in the use of zebrafish as a model system for the functional analysis of genes in neurodevelopmental disorders, which are a group of disorders characterized by alterations in behavior, cognition, communication, and/or motor function during development (American Psychiatric Association, 2013). This is due in large part to the unique features of this system, which offer distinct advantages over more traditional model systems (McCammon and Sive, 2015; Ijaz and Hoffman, 2016; Kozol et al., 2016). For example, zebrafish have transparent embryos that develop externally and rapidly, allowing for the direct visualization of neurodevelopmental processes and neural activity in an intact, functioning nervous system. In addition, zebrafish are highly tractable and produce large progenies, which facilitate the conduct of high-throughput pharmacological screens at a scale that would not be feasible in rodent models.

Further, with advances in CRISPR/Cas9 gene-editing techniques in zebrafish (Hwang et al., 2013; Moreno-Mateos et al., 2015), it is now possible to generate zebrafish mutants carrying loss-of-function mutations in a gene of interest relatively rapidly and at a low cost. Given this ease of genetic manipulation, zebrafish are emerging as an optimal system for modeling the growing list of risk genes in neurodevelopmental disorders, keeping pace with the rapid rate of gene discovery in these disorders (Allen et al., 2013; Purcell et al., 2014; Sanders et al., 2015). Therefore, zebrafish have considerable potential for advancing our understanding of the roles of risk genes in the developing brain and elucidating basic biological mechanisms underlying neurodevelopmental disorders.

Despite the limitations of modeling human disorders in zebrafish, given their evolutionary divergence, several lines of evidence point to a remarkable degree of conservation, suggesting that studies in zebrafish are likely to have translational relevance to humans. First, at a structural level, zebrafish have the same major subdivisions of a vertebrate brain as mammals—forebrain, midbrain, hindbrain and spinal cord (Guo, 2009) While there are notable structural differences, such as the development of the telencephalon, which forms by a different process in zebrafish (eversion) than mammals (invagination), many brain regions in zebrafish and mammals, including the thalamus, optic tectum and cerebellum, display structural homology and are reviewed in detail in Kozol et al. (2016). In addition, early developmental genes share similar expression patterns in the brains of zebrafish and mammals, and the major neurotransmitter systems in the mammalian brain, including GABA, glutamate, dopamine, norepinephrine, serotonin, histamine and acetylcholine, are present in zebrafish (Guo, 2009). Second, there is evidence for conservation of pharmacological pathways (Burgess and Granato, 2007b; Renier et al., 2007; Rihel et al., 2010). For example, a large-scale screen of psychoactive compounds found that drugs targeting conserved neurotransmitter systems elicit similar effects on sleep in zebrafish and mammals (Rihel et al., 2010). Third, approximately 80% of risk genes associated with human disorders have an orthologous version in zebrafish, revealing considerable genetic conservation (Howe et al., 2013). Fourth, there is evidence that the neural circuits underlying basic behaviors, such as acoustic startle, prepulse inhibition, sleep and arousal, are conserved, suggesting that findings in zebrafish are likely to be relevant to our understanding of related circuits in mammals (Prober et al., 2006; Burgess and Granato, 2007a,b; Schoonheim et al., 2010; Lovett-Barron et al., 2017). These studies highlight the potential of zebrafish, with their optical transparency and amenability to whole-brain, *in vivo* imaging, for elucidating the roles of risk genes in neurodevelopmental processes and neural circuit function.

In this review article, we will focus on genetic models of the following neurodevelopmental disorders: autism spectrum disorders (ASD), intellectual disability (ID), epilepsy and schizophrenia, in zebrafish. Specifically, we will focus on early phenotypes (morphological and behavioral), rather than adult behaviors, which have been addressed in detail in other reviews (Meshalkina et al., 2018; Shams et al., 2018). We will highlight insights into basic mechanisms of risk gene function and potential drug candidates identified in zebrafish models, as well as the limitations of studies to date. Finally, we will discuss advances in functional imaging of zebrafish brain activity, which has the potential to illuminate new roles for risk genes in conserved neural circuits.

ADVANCES IN GENE TARGETING METHODS IN ZEBRAFISH

Zebrafish first emerged as an optimal model system for studying vertebrate development through their use in large-scale forward genetics screens, leading to the discovery of hundreds of genes involved in early developmental processes (Granato and Nusslein-Volhard, 1996). Until recently, one of the challenges of using zebrafish as a model for reverse genetics was the limited availability of methods for generating mutants in a gene of interest. While mouse "knockouts" are generated by isolating embryonic stem cells, related methods are more challenging in zebrafish. For this reason, generating zebrafish mutants relied for a long time on Targeted Induced Local Lesions in Genomes (TILLING), which involves screening thousands of zebrafish carrying random mutations induced by the chemical N-ethyl N-nitrosourea (ENU) to identify a damaging mutation in a target gene (Moens et al., 2008). Limitations of TILLING include the time-consuming process of screening zebrafish libraries and the relatively low likelihood of identifying the desired mutation, though more recent large-scale ENU and retroviral mutagenesis projects using next-generation sequencing have improved the efficiency of this approach (Kettleborough et al., 2013; Varshney et al., 2013; Pan et al., 2015). Because zebrafish have a duplicated genome with at least two orthologs of many human risk genes that display sub-functionalization (Kozol et al., 2016), the lack of methods for rapidly generating targeted mutations greatly restricted the use of zebrafish as a genetic model.

However, the introduction of targeted nuclease technologies, including zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), transformed the field, enabling the rapid induction of damaging, heritable mutations in a gene of interest in zebrafish (Doyon et al., 2008; Meng et al., 2008; Sander et al., 2011; Dahlem et al., 2012). ZFNs and TALENs are chimeric fusion proteins designed to bind to a target site within a gene and produce double-stranded breaks that are repaired inefficiently by non-homologous end joining, resulting in insertion-deletion mutations. Despite their advantages over TILLING, a number of limitations prevented their widespread use, including challenges in predicting the efficiency of gene disruption and identifying a target site within an early exon of a gene of interest, along with the high cost of commercially available ZFNs. While TALENs improved upon many of these features, offering increased flexibility and lower cost, both methods were soon supplanted by CRISPRs.

Clustered regularly interspaced short palindromic repeats (CRISPRs) hijack an adaptive immune mechanism used by bacteria for protection against viruses (Jinek et al., 2012). To generate zebrafish mutants, a single guide RNA (sgRNA)

recognizing a target genomic sequence is introduced into zebrafish embryos along with mRNA encoding the enzyme, Cas9 (Hwang et al., 2013). Following sgRNA-directed cleavage of DNA by Cas9, inefficient non-homologous end joining leads to insertion-deletion mutations, as with ZFNs and TALENs, though CRISPRs offer superior flexibility and efficiency over these earlier gene-editing methods (Hwang et al., 2013). Further, their low cost and ease of use have made this technology accessible to most laboratories, facilitating the rapid generation of zebrafish mutants and leading to a paradigm-shift in the use of zebrafish as a reverse genetics tool. While a limited number of studies to date have used ZFNs or TALENs to generate zebrafish mutants of genes associated with neurodevelopmental disorders, it is likely that a growing number of studies will harness CRISPRs for this purpose in the near future.

In contrast, the majority of zebrafish studies of neurodevelopmental disorder-associated genes to date have used morpholinos, which are modified antisense oligonucleotides that cause a transient "knockdown" of target gene expression by blocking mRNA splicing or translation (Nasevicius and Ekker, 2000; Draper et al., 2001). Given their low cost, ease of use, and until recently, the limited availability of methods for rapidly generating genetic mutants, morpholinos have been a commonly used method for analyzing gene function in early development in zebrafish. However, morpholinos have several notable drawbacks, including their transient effects, which limit the ability to investigate gene function beyond early stages, as well as their tendency to induce off-target effects (Eisen and Smith, 2008). For example, some morpholinos activate p53 via an unknown mechanism, resulting in widespread apoptosis, which may lead to nonspecific phenotypes, such as changes in head size or brain structure (Robu et al., 2007; Eisen and Smith, 2008). In addition, a growing number of studies are finding a lack of concordance between the phenotypes of genetic mutants and morpholino-induced knockdowns of the same gene, which in most cases are due to the off-target effects of morpholinos (Kok et al., 2015; Lawson, 2016). Therefore, morpholino-induced phenotypes must be interpreted with caution.

Given these limitations, early guidelines were established for confirming the specificity of morpholino-induced phenotypes, including: (i) using two morpholinos targeting distinct sites; and (ii) demonstrating rescue of the phenotype by introducing mRNA lacking the morpholino target site (Eisen and Smith, 2008). With the careful use of these controls, morpholinos have been used successfully to investigate the early developmental roles of genes in zebrafish (Eisen and Smith, 2008). However, many experiments fail to follow these guidelines (Lawson, 2016). Further, studies have found discrepancies between morphant and mutant phenotypes even when these guidelines have been followed (Stainier et al., 2017).

To address these issues, a group of leaders in the zebrafish scientific community recently established new guidelines for the use of morpholinos, requiring that all morpholino-induced phenotypes be confirmed in genetic mutants where possible, which is now feasible due to CRISPRs (Stainier et al., 2017). These guidelines further state that morpholinos may continue to be used for rapid gene "knockdown" only in cases where the phenotype under investigation has been recapitulated in a mutant (Stainier et al., 2017). While there are cases where genetic compensation has been shown to occur in mutants (Rossi et al., 2015; El-Brolosy and Stainier, 2017), the current guidelines require demonstrating the absence of the morpholino-induced phenotype in the mutant background as evidence for compensation (Stainier et al., 2017). Moving forward, following these guidelines will be critical, particularly for the interpretation of phenotypes in zebrafish models of neurodevelopmental disorders, given that morpholinos alone can induce nonspecific neural effects (Shams et al., 2018). At the same time, as mutants become the "gold standard" for reverse genetics studies in zebrafish, it will be equally important to confirm that germline mutations result in loss-of-function (Shams et al., 2018).

ZEBRAFISH MODELS OF NEURODEVELOPMENTAL DISORDERS

Here, we discuss findings from studies that used zebrafish to investigate the function of genes that are strongly associated with ASD, epilepsy, ID and schizophrenia, or to screen for the functionality of newly identified risk genes or variants (**Tables 1–4**). Importantly, the genetics of these disorders is complex, likely involving hundreds of risk genes, and is characterized by considerable pleiotropy, with the same genes or genomic regions conferring risk to a range of disorders (State and Šestan, 2012). For clarity, we have categorized genes in **Tables 1–4** by the disorder to which they are most closely associated in the literature, noting overlapping associations where applicable, though studies of the biological functions of these genes are likely to be relevant across diagnostic boundaries. While zebrafish have also been used to study other neurodevelopmental disorders, including attention-deficit/hyperactivity disorder (ADHD; Lange et al., 2012a,b), Bardet-Biedl syndrome (BBS; Zaghloul et al., 2010; Heon et al., 2016; Lindstrand et al., 2016), and maple syrup urine disease (MSUD; Friedrich et al., 2012), we focus on ASD, epilepsy, ID and schizophrenia, which have been the subject of most zebrafish studies to date and highlight the advantages of this system for the functional analysis of risk genes. Moreover, while an increasing number of studies are investigating complex behaviors in adult zebrafish, such as social behaviors, it is important to observe that there are limitations of face validity, such that it is not possible to recapitulate fully the symptoms of neurodevelopmental disorders in zebrafish (or any animal model), and this is not a prerequisite for demonstrating the relevance of the model. Therefore, in this review article, we focus our discussion on embryonic and larval phenotypes, which highlight the unique strengths of the zebrafish system for illuminating conserved roles of risk genes in basic biological pathways and brain circuits underlying simple behaviors, which are likely to have translational relevance.

Autism Spectrum Disorder

ASDs are a devastating group of neurodevelopmental disorders characterized by marked impairments in social behavior and communication, and by the presence of restricted, repetitive

TABLE 1 | Zebrafish models of autism spectrum disorders (ASD)-associated genes and genetic syndromes.

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
CHD7	***chd7***	CHARGE	*MO*	• Abnormal somite segmentation (13 somite) • Small eyes, pericardial edema, abnormal otoliths (48 hpf) • Abnormal vascular patterning (48 hpf), cranial neural crest (34–36 hpf), cranial neuron (48 hpf), and retinal (72 hpf) development; decreased bone mineralization (8 dpf, 14 dpf)	Zebrafish mRNA rescues eye, heart, and otolith abnormalities	Patten et al. (2012)
			MO	• Developmental defects as in Patten et al. (2012) • Decreased cell proliferation (25 hpf) • Abnormal craniofacial cartilage (4 dpf)	*fbxl10* MO reduced developmental defects, cell proliferation deficit	Balow et al. (2013)
			MO **CRISPR** **Mutant**	• ***chd7−/− mutants and chd7 MO:*** - Pericardial edema, cardiomegaly (3 dpf); abnormal pigmentation (4 dpf) - Reduction of vagal innervation of GI tract in foregut more than hindgut (5 dpf) - Decreased GI emptying (5 dpf)	Prokinetic drugs do not rescue GI emptying	Cloney et al. (2018)
CHD8	***chd8***	ASD	*MO;* *F0* *CRISPR*	• Macrocephaly (increased interorbital/intertectal distance (4.3 dpf) in MO; increased interorbital distance in F0 CRISPR) • Developmental marker expression changes: expansion of *chordin* (shield stage); increased mid/forebrain neuronal progenitor marker *otx2* (tail bud) and *dlx2* in prethalamus (24 hpf; MO) • Decreased GI motility (MO) and # of enteric neurons (6 dpf; MO and F0 CRISPR)	–	Bernier et al. (2014)
			MO	• Ectopic HuC/D expression in anterior forebrain (2 dpf) • Increased cell proliferation in brain (2 dpf)	–	Sugathan et al. (2014)
CNTNAP2	***cntnap2a/b***	ASD EP CDFE	**ZFN** **Mutant**	• Decreased forebrain GABAergic neurons (4 dpf) • Increased sensitivity to drug-induced seizures; nighttime hyperactivity (4–6 dpf) • GABA-modifying drugs induce differential behavioral responses • Increased sensitivity to behavioral activation by NMDA antagonists	Estrogenic compounds identified in drug screen rescue nighttime hyperactivity	Hoffman et al. (2016)
DYRK1A	***dyrk1aa***	ASD	**TALEN** **Mutant**	• Normal development, morphology (24–48 hpf); normal larval locomotor activity (5–7 dpf) • No difference in brain size (2 weeks old); increased apoptosis in brain (3 weeks old) • *Adult Phenotypes:* Microcephaly; decreased anxiety, social behavior impairments, decreased *c-fos*, *crh* expression in hypothalamic regions after social interaction, social isolation, respectively	–	Kim et al. (2017)
FMR1	***fmr1***	FXS	*MO* *OE*	• ***fmr1 MO:*** - Abnormal mid-hindbrain boundary, altered *dlx2a* expression (24 hpf) - Increased branching of trigeminal and Rohon-Beard neurites (24 hpf) - Axon defasciculation of lateral longitudinal fasciculus (5 dpf) - Fewer trigeminal neurons; craniofacial abnormalities (5 dpf) • ***OE fmr1 mRNA:*** - Decreased branching of trigeminal and Rohon-Beard neurites (24 hpf) - Increased trigeminal neurons	*fmr1* mRNA rescues *dlx2a* expression; MPEP rescues neurite branching, number of trigeminal neurons	Tucker et al. (2006)
			TILLING **Mutant**	• No gross morphological abnormalities; no differences in *dlx2a* expression • No craniofacial abnormalities (5 dpf); no defects in Rohon-Beard neurite branching	–	den Broeder et al. (2009)
MECP2	***mecp2***	RTT	**TILLING** **Mutant**	• Increased contractions during coiling events (25 hpf) • Increased C-bend duration during escape response (51 hpf) • Decreased locomotor activity and thigmotaxis (6 dpf)	–	Pietri et al. (2013)

(Continued)

TABLE 1 | (Continued)

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
MECP2	***mecp2***	RTT	*MO; F0 CRISPR; OE*	• ***mecp2 MO:*** - Increased neural, glial proliferation; decreased differentiation (48 hpf) (MO and F0 CRISPR) - Upregulation of Notch signaling genes; increased brain *her2*, *id1* expression (48 hpf) • ***mecp2 OE:*** - Decreased cell proliferation, increased neuronal differentiation (48 hpf) - Decreased *id1* expression (48 hpf)	*mecp2* mRNA, *her2* MO, *id1* MO rescue proliferation, differentiation phenotypes in *mecp2* MO; *id1* MO rescues *her2* levels in *mecp2* MO	Gao et al. (2015)
			MO; **TILLING Mutant**	• ***mecp2−/− mutant and mecp2 MO:*** - Decreased trigeminal neurite length (24 hpf); less severe in mutant - Downregulation of *sema3f*, *sema5b* and *robo2* (24 hpf); less severe in mutant - MO + mutant does not worsen mutant phenotypes • ***mecp2 MO:*** - Increased apoptosis (16 hpf, 24 hpf); delayed response to tactile stimuli (48 hpf)	Zebrafish *mecp2* mRNA rescues neurite length; *HuC-mecp2*, *sema5b*, *robo2* mRNA rescue neurite length and tactile response phenotypes	Leong et al. (2015)
			MO	• Decreased Rohon-Beard axon length/branching (28 hpf) • Motor neuron axon abnormalities: caudal primary (28 hpf), caudal secondary (72 hpf) • Decreased touch response (28 hpf, 72 hpf) • Increased *bdnf* expression (24 hpf) • Increased presynaptic SV2-stained area, abnormal formation of neuromuscular junction (72 hpf; also in *bdnf OE*); *bdnf* MO decreases presynaptic area in *mecp2* MO	Human *MECP2* mRNA rescues motor axon, touch response phenotypes; *MECP2* lacking MBD domain does not rescue	Nozawa et al. (2017)
			TILLING Mutant	• Body length: decreased at 2 dpf; normal at 7 dpf • GI tract discoloration, abnormal droplets (4 dpf, 7 dpf) • Expression of inflammatory marker *crp:* normal at 3 dpf; increased at 4–5 dpf • Neutrophil number: normal at 3 dpf, increased at 4–5 dpf; increased in GI tract (2–5 dpf) • Dysregulated cytokine expression; decreased *tnfa* expression (6 hpf, 24 hpf–7 dpf) • No *tnfa* activation during acute inflammation (3 dpf)	*mecp2* OE partially rescues *tnfa* expression (24 hpf); *tnfa* OE does not rescue body length, GI-associated neutrophil number (2–3 dpf)	van der Vaart et al. (2017)
SHANK3	***shank3a***	ASD PMS	*MO*	• Abnormal mid-hindbrain boundary, ventricle size (28–30 hpf) • Increased apoptosis in CNS (reversed by p53 MO) (24–28 hpf) • Decreased GABAergic neurons (mid/hindbrain), glutamatergic neurons (hindbrain; 48 hpf) • Abnormal escape responses and seizure-like behaviors (72 hpf)	Human *SHANK3* mRNA partially rescues apoptosis	Kozol et al. (2015)
	shank3b		**CRISPR Mutant**	• Transient developmental delay (24 hpf) • Decreased HuC:RFP levels (24–72 hpf), though difference decreases over time • Decreased locomotor activity (7 dpf) • *Adult Phenotypes:* Increased brain size, decreased locomotor activity/thigmotaxis, social behavior impairments, repetitive behaviors, decreased brain homer1, synaptophysin levels	–	Liu et al. (2018)
SYNGAP1	***syngap1b***	ASD	*MO*	• Abnormal mid-hindbrain boundary, ventricle size (28–30 hpf) • Increased apoptosis in CNS (not reversed by p53 MO) (24–28 hpf) • Microcephaly, developmental delay (48–72 hpf) • Decreased GABAergic neurons in midbrain, glutamatergic neurons in hindbrain (48 hpf) • Abnormal escape responses and seizure-like behaviors (72 hpf)	Human *SYNGAP1* mRNA partially rescues apoptosis	Kozol et al. (2015)

(Continued)

TABLE 1 | (Continued)

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
TSC1	***tsc1a***	TSC	*MO*	• Body curvature, cysts in tubular/glomerular regions of pronephros • Decreased left-right asymmetry; increased TOR activity (24 hpf), ciliary length (20–24 hpf)	–	DiBella et al. (2009)
TSC2	***tsc2***	TSC	**TILLING Mutant;** *OE; Cell transplantation*	• ***tsc2−/−*:** - Deflated swim bladder, enlarged liver, increased TORC1 activity (7 dpf); death by 11 dpf - Increased size of hepatocytes (9 dpf), brain and spinal cord neurons (7.5 dpf) - Forebrain gray and white matter disorganization, ectopic neurons in white matter (7.5 dpf) • ***Mutant tsc2 mRNA OE:*** - Increased TORC1 activity (10–11 hpf); dorsal expansion of hindbrain (27 hpf) • ***Cell transplantation of tsc2−/− cells into wild-type at 4 hpf:*** - Increased TORC1 activity in mutant, not wild-type, cells (7.5 dpf; adult) - Disrupted gray-white matter boundaries (7.5 dpf; adult) - Ectopic wild-type cells in white matter in some chimeric embryos (7.5 dpf) - Mutant cell clusters found in gray-white matter boundary, gray matter (adult)	Zebrafish mRNA rescues elevated TORC1 activity (partial rescue by human mRNA); rapamycin reverses elevated TORC1 activity and enlarged hepatocytes	Kim et al. (2011)

Key: *hpf, hours post fertilization; dpf, days post fertilization;* ****Disorder:*** *ASD, autism spectrum disorder; CDFE, cortical dysplasia focal epilepsy; CHARGE, CHARGE Syndrome; EP, epilepsy; FXS, Fragile X syndrome; PMS, Phelan-McDermid Syndrome; RTT, Rett syndrome; TSC, tuberous sclerosis complex;* *****Methods of Risk Gene Disruption:*** *CRISPR, clustered regularly interspaced short palindromic repeats; CRISPR F0, Mosaic CRISPR-injected embryo; MO, Morpholino; OE, overexpression; TALEN, transcription activator-like effector nuclease; TILLING, targeted induced local lesions in genomes; ZFN, zinc finger nuclease.*

behaviors (American Psychiatric Association, 2013). In recent years, large-scale, whole-exome sequencing studies have led to a rapidly expanding list of reliable, "high confidence" ASD risk genes, which are beginning to reveal common biological mechanisms (Willsey et al., 2013; De Rubeis et al., 2014; Iossifov et al., 2014; Sanders et al., 2015). Despite this progress, how the disruption of these genes leads to the alteration of specific cell types and neural pathways during early stages of brain development remains poorly understood. A growing number of studies have used zebrafish models to investigate the function of "high confidence" ASD risk genes and genes linked to ASD-associated syndromes, such as Fragile X syndrome, tuberous sclerosis complex, Rett syndrome, and CHARGE syndrome (**Table 1**), as well as genes found in the 16p11.2 chromosomal interval (**Table 2**), where copy number variants (CNVs) have been associated with ASD, ID and schizophrenia (Kumar et al., 2008; Marshall et al., 2008; Weiss et al., 2008; McCarthy et al., 2009).

Recent studies of zebrafish models of ASD risk genes are beginning to shed light on relevant neurobiological mechanisms. For example, because excitatory-inhibitory imbalance has been implicated as a potential mechanism underlying ASD (and epilepsy; Rubenstein and Merzenich, 2003), some studies have investigated the extent to which disruption of ASD risk genes alters inhibitory GABAergic and excitatory glutamatergic neurons during early brain development using transgenic lines that label these cell populations. For example, Kozol et al. (2015) found that knockdown of two of the zebrafish orthologs of the ASD-associated genes, *SHANK3* and *SYNGAP1*, led to fewer GABAergic neurons in the midbrain (and hindbrain for *shank3a*) and glutamatergic neurons in the hindbrain. In addition, our group found that zebrafish mutants of both orthologs of the ASD- and epilepsy-linked gene, *CNTNAP2*, display deficits in forebrain GABAergic neurons, but lack regional deficits in glutamatergic neurons (Hoffman et al., 2016). Interestingly, GABAergic deficits were also found in mouse knockouts of *Cntnap2* (Penagarikano et al., 2011), suggesting that this gene affects conserved pathways in fish and mice.

In addition, given that differences in head and brain size, particularly macrocephaly, and changes in neuron number or organization have been described in ASDs (Courchesne et al., 2011; Stoner et al., 2014), a number of morpholino-based studies have examined the effects of decreased ASD risk gene expression on related phenotypes. For example, reduced expression of most zebrafish orthologs of genes in the 16p11.2 chromosomal interval led to structural brain abnormalities at 24 h post fertilization (hpf), including smaller brain ventricles, altered midbrain-hindbrain boundary, or a straight midbrain (Blaker-Lee et al., 2012). In addition, reduced expression of *shank3a* and *syngap1b*, led to alterations in the midbrain-hindbrain boundary, ventricle size, and microcephaly (Kozol et al., 2015). Further, knockdown of the "high confidence" ASD risk gene, *CHD8*, was associated with macrocephaly and increased cell proliferation in the brain (Bernier et al., 2014; Sugathan et al., 2014). However, because morpholinos themselves can cause nonspecific neural

TABLE 2 | Zebrafish models of genes in 16p11.2 interval.

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
21 genes[#]	**22 orthologs**[#]	ASD SCZ ID	*MO*	• Brain or eye abnormalities at 24 hpf (20/22 genes) • Muscle and tail abnormalities at 24 hpf for most genes • Decreased spontaneous movement at 24 hpf (7 genes), touch response at 48 hpf (14 genes) • Abnormal axon tract development at 36 hpf (6 genes); altered pigmentation (8 genes)	Human or zebrafish mRNA rescue phenotypes for all genes (except *mvp*)	Blaker-Lee et al. (2012)
29 genes[##]	***kctd13***	ASD SCZ ID	*OE* (Human mRNA in zebrafish); MO *(kctd13)*	• *KCTD13* is only human mRNA where OE causes head size PT • ***Human KCTD13 mRNA OE in zebrafish***: - Microcephaly; fewer neurons in telencephalon (4.5 dpf) - Increased apoptosis in brain (3 dpf) - Decreased cell proliferation in brain (2 dpf) • ***kctd13 MO:*** - Macrocephaly; increased neurons in telencephalon (4.5 dpf) - Increased cell proliferation in brain (2 dpf) • Mouse *Kctd13* knockdown *in vitro/in vivo*: Increased cell proliferation	Human mRNA + *kcdt13* MO rescue head size phenotypes	Golzio et al. (2012)
KCTD13	***kctd13***	–	**CRISPR Mutant**	• ***Zebrafish and mouse KCTD13 mutants:*** - No changes in head size or cell proliferation (4.5 dpf) - Increased RhoA in brain (adult zebrafish; mice ≥ P18) • Decreased synaptic transmission in mice	RhoA inhibitor rescues synaptic transmission defects in mice	Escamilla et al. (2017)
DOC2A ***FAM57B***	***doc2a*** ***fam57ba***	–	*MO*; **TALEN Mutant**	• Genetic interactions between 16 pairs of genes in 16p11.2 interval, including *fam57ba* and *doc2a*, in MO screen for brain/ventricle morphology phenotypes at 24 hpf • ***doc2a***$^{+/-}$ ***fam57ba***$^{+/-}$**:** - Hyperactivity and seizure sensitivity (7 dpf) - Increased body length and some head size dimensions (12 dpf) • ***fam57ba***$^{-/-}$**:** - Hyperactivity and seizure sensitivity (7 dpf), less than in double heterozygotes - Strong increase in body length and head size (12 dpf) - Increased lipid content	Valproic acid and carbamazepine rescue drug-induced seizures	McCammon et al. (2017)

Key: *hpf, hours post fertilization; dpf, days post fertilization;* ***Disorder:** ASD, autism spectrum disorder; EP, Epilepsy; ID, intellectual disability; SCZ, schizophrenia;* ****Methods of Risk Gene Disruption:** CRISPR, clustered regularly interspaced short palindromic repeats; MO, Morpholino; OE, overexpression; TALEN, transcription activator-like effector nuclease;* [#]***Human Genes in 16p11.2 interval in Blaker-Lee et al. (2012):** ALDOA, ASPHD1, C16orf53, CDIPT, CORO1A, DOC2A, FAM57B, GDPD3, HIRIP3, INO80E, KCTD13, KIF22, MAPK3, MAZ, MVP, PPP4C, PRRT2, SEZ6L2, TAOK2, TBX6, YPEL3;* ***Zebrafish Orthologs:** aldoaa, asphd1, c16orf53, cdipt, coro1a, doc2a, fam57ba, gdpd3, hirip3, ino80e, kctd13, kif22, mapk3, maz, mvp, ppp4ca, prrt2, sez6l2, taok2a, taok2b, tbx24, ypel3;* [##]***Human Genes in 16p11.2 interval in Golzio et al. (2012):** ALDOA, ASPHD1, BOLA2, C16orf53, C16orf54, C16orf92, CDIPT, CORO1A, DOC2A, FAM57B, GDPD3, HIRIP3, INO80E, GIYD2, KCTD13, MAPK3, MAZ, MVP, PPP4C, PRRT2, QPRT, SEZ6L2, SPN, SULT1A3, SULT1A4, TAOK2, TBX6, TMEM219, YPEL3.*

phenotypes, confirmation of these phenotypes in germline mutants is a critical next step.

Several studies have also investigated behavioral phenotypes in zebrafish larvae as a means of elucidating how risk gene disruption leads to alterations in simple behaviors. For example, zebrafish mutants of *MECP2*, the gene responsible for Rett syndrome, display decreased locomotor activity, reduced thigmotaxis (wall preference), and longer touch-evoked escape responses, indicating that loss of *mecp2* affects embryonic and larval behaviors (Pietri et al., 2013). In addition, *shank3a* and *syngap1b* morphant larvae display abnormal escape responses (Kozol et al., 2015), while *shank3b* mutant larvae (lacking the function of the other zebrafish ortholog of *SHANK3*) exhibit reduced locomotor activity (Liu et al., 2018). Mutant larvae of *scn1lab*, an ortholog of the ASD- and epilepsy-associated genes, *SCN1A* and *SCN2A*, exhibit spontaneous seizures (discussed in the "Epilepsy" section), as well as nighttime hyperactivity and increased thigmotaxis (Grone et al., 2017). *cntnap2ab* mutants also display nighttime hyperactivity and increased sensitivity to drug-induced seizures (Hoffman et al., 2016). Further, double heterozygous mutants of two genes in the 16p11.2 interval, *doc2a* and *fam57ba*, show greater hyperactivity and drug-induced seizure sensitivity than single homozygous mutants of each gene, suggesting a genetic interaction (McCammon et al., 2017).

An important advantage of studying larval behavioral phenotypes is their amenability to high-throughput quantitative assays and small molecule screens (Prober et al., 2006; Kokel et al., 2010; Rihel et al., 2010). We capitalized on this approach to investigate rest-wake activity in zebrafish *cntnap2ab* mutants, which display nighttime hyperactivity (Hoffman et al., 2016). By comparing the behavioral "fingerprint" of *cntnap2ab* mutants across a range of rest-wake behavioral parameters with a database of the responses of wild-type fish to over 550 psychoactive compounds (Rihel et al., 2010), we predicted compounds that might rescue the mutant behavioral phenotype and tested a select group of these compounds to identify suppressors (Hoffman et al., 2016). Intriguingly, we found that estrogenic compounds selectively suppress nighttime hyperactivity in *cntnap2ab* mutants, revealing a new neurochemical pathway not previously associated with this gene (Hoffman et al., 2016). In this way, pharmaco-behavioral profiling of zebrafish ASD risk gene mutants represent a promising first-pass screening approach to identify potential pharmacological candidates for further investigation.

Zebrafish mutants also provide an opportunity to investigate risk gene function over the course of development from embryonic stages through adulthood. This is particularly relevant for ASD, where many risk genes are highly expressed in the human brain during embryonic and fetal stages (State and Šestan, 2012). Interestingly, two recent studies found that zebrafish mutants of ASD risk genes display distinct phenotypes at different developmental stages. First, zebrafish mutants of one ortholog of *DYRK1A*, a "high confidence" ASD risk gene located in the Down syndrome critical region, developed normally with no gross morphological or locomotor abnormalities during embryonic and larval stages, yet displayed increased apoptosis in the brain at 3 weeks old, and microcephaly and behavioral abnormalities in adulthood (Kim et al., 2017). Second, mutants of *shank3b*, the second ortholog of *SHANK3*, exhibited transient developmental delay at 24 hpf and fewer CNS neurons at 24–72 hpf, yet this difference diminished over time (Liu et al., 2018). In contrast, adult *shank3b* mutants display increased brain size and behavioral deficits (Liu et al., 2018). These studies highlight the importance of assessing phenotypes along a developmental trajectory.

With regard to ASD-associated syndromes, several studies have investigated signaling pathways in zebrafish models of Rett syndrome. For example, one study found that *mecp2* knockdown led to increased proliferation of neural precursors and decreased neuronal differentiation, which were reversed by simultaneously knocking down *id1* or *her2*, implicating Id1-HER2 signaling as a downstream pathway (Gao et al., 2015). Another study tracked inflammatory phenotypes in *mecp2* mutants over the course of development, finding decreased expression of the proinflammatory cytokine, *tnfa*, as early as 6 hpf, while differences in other cytokines emerge later (van der Vaart et al., 2017). This study further highlights the relevance of assessing phenotypes along a developmental trajectory. Reduction of *mecp2* expression was also associated with abnormalities in both sensory and motor axon outgrowth (Leong et al., 2015; Nozawa et al., 2017). In addition, (Leong et al., 2015) found that the axon guidance molecules, *sema5b* and *robo2*, are downregulated in *mecp2* morphants and mutants, and that co-expression of these genes in morphants rescues decreased trigeminal neurite length and delayed touch responses. Of note, the structural and gene expression phenotypes were more severe in morphants than mutants, but were not worsened by the introduction of the morpholino in mutant embryos, suggesting there may be compensation (Leong et al., 2015). This underscores the importance of directly comparing mutant and morphant phenotypes.

However, zebrafish models of Fragile X syndrome provide a cautionary tale regarding the off-target effects of morpholinos. That is, morpholino-induced knockdown of *fmr1* was associated with multiple neurodevelopmental phenotypes, including alterations in the midbrain-hindbrain boundary, abnormal neurite branching, and craniofacial abnormalities (Tucker et al., 2006). However, none of these phenotypes was replicated in two lines of *fmr1* mutants, which lacked Fmr protein expression by western blot (den Broeder et al., 2009). *KCTD13* offers another example where morphant phenotypes did not replicate in a mutant. *KCTD13* is found in the 16p11.2 chromosomal interval, where deletions are associated with macrocephaly, ASD and ID, and duplications with microcephaly, ASD and schizophrenia. Consistent with this association, Golzio et al. (2012) found that morpholino-induced *kctd13* knockdown and overexpression of human *KCTD13* led to reciprocal phenotypes of macrocephaly and microcephaly, respectively, as well as related changes in cell proliferation in the brains of zebrafish larvae, implicating this gene as a potential driver of head size phenotypes (Golzio et al., 2012). However, a recent study did not identify differences in head size or cell proliferation in zebrafish (or mouse) mutants of *KCTD13* (Escamilla et al., 2017). These studies underscore the importance

TABLE 3 | Zebrafish models of epilepsy and intellectual disability-associated genes.

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
CHD7	***chd7***	DS/ID (* in this study) ASD	*MO*	• Morphological abnormalities: pericardial edema, microcephaly, body curvature, no swim bladder, decreased growth (≥48 hpf) • Abnormal movement (4 dpf) • Epileptiform discharges in tectal field-potential recordings (4 dpf)	–	Suls et al. (2013)
PK1	***pk1a***	EP	*OE*	• Gastrulation defects: Reduced anterior-posterior length (20 hpf); lateral expansion of somites (8–10 somite stage) • OE zebrafish *pk1a* mRNA with human mutation (R104Q) causes less severe defects	–	Bassuk et al. (2008)
			MO	• Increased sensitivity to drug-induced seizures (48 hpf) • Retinal inner plexiform layer defects (76–78 hpf) • Normal visual startle response (5 dpf)	Zebrafish mRNA partially rescues retinal defects; mRNA with human R150H, Y465H mutations do not	Mei et al. (2013)
PSMD12	***psmd12***	ID	*F0 CRISPR*	• Decreased size of optic tecta (3 dpf) • Proximal renal tubule defects, decreased proximal tubule convolution area (4 dpf) • Craniofacial abnormalities (3 dpf)	–	Kury et al. (2017)
RHEB	***rheb***	ID	*F0 CRISPR; OE*	• ***F0 CRISPR:*** - Microcephaly (decreased head area excluding eyes; 5 dpf) • ***OE human RHEB and RHEB carrying human mutations:*** - Macrocephaly (5 dpf)	Rapamycin rescues macrocephaly	Reijnders et al. (2017)
SCN1A	***scn1lab***	DS EP ASD	**ENU Mutant;** *MO*	• Abnormal optokinetic response (OKR; 5 dpf; MO and mutant) • Darkened pigmentation (MO and mutant) • Death by 14 dpf (mutant)	Stimulation of hindbrain saccade generator rescues OKR (morphants)	Schoonheim et al. (2010)
			ENU Mutant	• Spontaneous seizure-like activity • Abnormal forebrain electrographic activity (3–7dpf)	Clemizole identified in drug screen rescues seizure activity	Baraban et al. (2013)
			MO	• Hyperactivity (3–5 dpf); spontaneous electrographic activity (5 dpf) • Increased sensitivity to hyperthermia (5–7 dpf)	Fenfluramine and valproate rescue seizure activity	Zhang et al. (2015)
			ENU Mutant	• Seizure activity	Fenfluramine and dimethadione rescue seizure activity	Dinday and Baraban (2015)
				• Lower levels of serotonin in *scn1lab−/−* head homogenates at 7 dpf • Zebrafish express orthologs of human serotonin (5HT) receptor subtypes at 5 dpf	$5HT_{1D}$, $5HT_{2C}$, $5HT_{2A}$-agonists, fenfluramine rescue seizure activity	Sourbron et al. (2016)
				• Nighttime hyperactivity (5 dpf) • Open field: Increased thigmotaxis, decreased movement (5 dpf) • No differences in GABAergic neurons (5 dpf)	Clemizole, diazepam rescue nighttime hyperactivity, thigmotaxis	Grone et al. (2017)
				• $5\text{-}HT_{2A}$ (*htr2aa/b*) and $5\text{-}HT_{2C}$ (*htr2cl1*) orthologs expressed in larval heads in wild-type and *scn1lab−/−* mutants and in adult wild-type brains • Clemizole binds to serotonin receptors, $5HT_{2A}$ and $5HT_{2B}$, in radioligand binding assay	Trazodone and lorcaserin identified in screen of 5HT compounds rescue seizure activity	Griffin et al. (2017)
STXBP1	***stxbp1a/b***	EP ID	**CRISPR Mutant**	***stxbp1a−/−:*** • Abnormal craniofacial development; failure to hatch; death by 10 dpf • Darkened pigmentation • Immobility; no response to dark flash (5–6 dpf)	–	Grone et al. (2016)

(Continued)

TABLE 3 | (Continued)

Human Gene(s)	Zebrafish Gene(s)	Disorder*	Method**	Phenotype(s)	Rescue	Reference
				• No spontaneous seizure-like behaviors or electrographic seizures • Reduced heart rate and metabolism ***stxbp1b−/−:*** • Darkened pigmentation • No spontaneous seizure-like behaviors • Electrographic seizure events in forebrain field potential recordings • Reduced response to dark flash (5dpf)		
STX1B	***stx1b***	EP	*MO*	• No swim bladder, pericardial edema, tail curvature (5 dpf) • Lack of touch response in ~40% of embryos (4 dpf) • Abnormal behaviors: repetitive fin, increased orofacial, myoclonic-like movement (5 dpf) • Spontaneous epileptiform activity on local field potentials of optic tecta: polyspiking discharges, high frequency oscillations (5 dpf)	CNS-specific expression of human mRNA rescues epileptiform activity; mRNA with a human mutation (V216E) does not rescue	Schubert et al. (2014)
TRAPPC6B	***trappc6b***	EP ASD	*MO*	• Microcephaly (2 dpf, 5 dpf) • Increased apoptosis (24 hpf) • Decreased baseline activity (5 dpf) • Increased sensitivity to drug-induced seizures • Increased neuronal activity at baseline and in response to seizure-inducing drug (5 dpf)	Human mRNA rescues motility deficit	Marin-Valencia et al. (2018)

Key: *hpf, hours post fertilization; dpf, days post fertilization;* ****Disorder:*** *ASD, autism spectrum disorder; DS, Dravet syndrome; EP, Epilepsy; ID, intellectual disability;* *****Methods of Risk Gene Disruption:*** *CRISPR, clustered regularly interspaced short palindromic repeats; CRISPR F0, Mosaic CRISPR-injected embryo; ENU, N-ethyl N-nitrosourea-induced mutant; MO, Morpholino; OE, overexpression.*

of validating morpholino-induced phenotypes in genetic mutants.

Zebrafish models of ASD-associated syndromes also provide evidence for the conservation of molecular pathways. For example, both mutants and morphants of *CHD7*, the gene that is associated with most cases of CHARGE syndrome, display pericardial edema and cardiac abnormalities, consistent with cardiac abnormalities found in affected individuals (Patten et al., 2012; Balow et al., 2013; Cloney et al., 2018). In addition, *chd7* mutants (and morphants) display decreased GI emptying (Cloney et al., 2018), while *chd8* morphants showed reduced GI motility (Bernier et al., 2014), which may be relevant to GI symptoms in individuals carrying mutations in these genes. Further, zebrafish models of tuberous sclerosis complex, including *tsc1a* morphants and *tsc2* mutants, display increased TORC activity (DiBella et al., 2009; Kim et al., 2011). Consistent with findings in mammals, rapamycin reverses elevated TORC1 activity in *tsc2* mutants (Kim et al., 2011). Interestingly, by transplanting cells from *tsc2* to wild-type embryos at the blastula stage, Kim et al. (2011) showed that increased TORC1 activity is cell autonomous, but that mutant cells also induce non-cell autonomous effects, leading to the ectopic localization of wild-type cells in the white matter. Transplanted mutant cells were also found in abnormal clusters at the gray-white matter boundary in adult brains, suggestive of brain hamartomas found in individuals with this disorder (Kim et al., 2011). Together, these studies highlight the potential of zebrafish models of ASD risk genes to reveal conserved pathways with translational relevance to mammals.

Epilepsy

Epilepsy is a common neurological condition characterized by recurrent seizures (Myers and Mefford, 2015). There has been considerable progress in risk gene discovery in epilepsy from studies of Mendelian syndromes in large family pedigrees, as well as through the identification of *de novo* single nucleotide variants and CNVs in affected individuals (Hildebrand et al., 2013). Here, we highlight studies using zebrafish models of genetic epilepsy syndromes (**Table 3**). Zebrafish offer several advantages in this regard. First, zebrafish larvae display robust, seizure-like behaviors, including rapid burst-like and circling movements, following exposure to the GABA-A antagonist, pentylenetetrazol (PTZ; Baraban et al., 2005), providing a quantifiable readout of seizure susceptibility. That is, increased sensitivity to PTZ-induced seizures has been shown in zebrafish models of epilepsy and ASD (Mei et al., 2013; Hoffman et al., 2016; McCammon et al., 2017). Second, zebrafish mutants of epilepsy-associated genes have been shown to exhibit spontaneous seizures as larvae (Baraban et al., 2013; Grone et al., 2016). Third, both drug-induced and spontaneous locomotor seizures are associated with electrographic seizures (Baraban et al., 2005, 2013), and are readily quantifiable in high-throughput assays (Baraban et al., 2013; Hong et al., 2016; Fuller et al., 2018), making zebrafish an optimal model for drug discovery in epilepsy syndromes.

In particular, zebrafish mutants of *scn1lab* have been used as a model of Dravet syndrome, a severe, intractable form of epilepsy,

which in most cases is caused by mutations in *SCN1A* (Baraban et al., 2013). Homozygous *scn1lab* mutants display spontaneous seizures beginning at 4 days post fertilization (dpf), as well as electrographic seizures in forebrain extracellular field recordings, which worsen from 3–7 dpf (Baraban et al., 2013). Interestingly, *scn1lab* mutants were first identified in a forward genetic screen of ENU-mutagenized fish due to their inability to sustain saccadic eye movements during the optokinetic response (OKR; Schoonheim et al., 2010). These mutants have an abnormal pigmentation pattern and die by 14 dpf (Schoonheim et al., 2010). Using these mutants, Baraban et al. (2013) performed a high-throughput screen of 320 compounds, and identified clemizole, a U.S. Food and Drug Administration-approved drug and antihistamine, as a suppressor of both seizure-like behaviors and electrographic seizures. A subsequent study found that clemizole has activity at 5-HT_{2A} and 5-HT_{2B} receptors in a radioligand binding assay, suggesting that a serotonergic mechanism may be responsible for its anti-epileptic activity (Griffin et al., 2017). Interestingly, fenfluramine, an inducer of serotonin (5-hydroxytrypamine, 5-HT) release, which was found to have some efficacy in improving seizures in individuals with Dravet syndrome (Ceulemans et al., 2012), also reduced seizure activity in zebrafish *scn1lab* mutants and morphants, suggesting conservation of pharmacological pathways (Dinday and Baraban, 2015; Zhang et al., 2015; Sourbron et al., 2016).

Based on the serotonergic mechanism of fenfluramine, (Sourbron et al., 2016) tested selective 5-HT receptor agonists in *scn1lab* mutants and found that 5-HT_{1D}, 5-HT_{2C}, and 5-HT_{2A} agonists reverse electrographic seizure activity. Also, Griffin et al. (2017) screened a library of 5-HT-modulating compounds and found that lorcaserin and trazodone rescued seizure activity. Through a compassionate use program, lorcaserin was subsequently prescribed to five patients with Dravet syndrome with intractable seizures. While these patients experienced an initial decrease in seizure frequency, seizures returned to baseline after 3 months in most patients (Griffin et al., 2017). Clearly, larger, double-blind, placebo-controlled trials are needed to fully assess the efficacy of this medication. Another important consideration is how to accurately translate effective dosages between systems. Nonetheless, these studies highlight the strengths of zebrafish as a first-pass screening approach for identifying potential anti-epileptic drug candidates. Indeed, as more epilepsy-associated genes are identified in human studies, it is likely that zebrafish models will continue to be instrumental in this regard. For example, zebrafish mutants of *stxbp1b*, an ortholog of *STXBP1*, which is associated with epileptic encephalopathy syndromes, display electrographic seizures at baseline, suggesting this may be a useful model for these syndromes (Grone et al., 2016).

Screening Risk Genes Associated With Epilepsy and Intellectual Disability

Zebrafish have also been used a genetic tool for rapidly screening the functionality of novel genes and rare variants identified in human genetics studies of epilepsy, ID and other neurodevelopmental disorders (**Table 3,** Bassuk et al., 2008; Gauthier et al., 2010; Suls et al., 2013; Schubert et al., 2014; Kury et al., 2017; Reijnders et al., 2017; Marin-Valencia et al., 2018). These studies assess the extent to which wild-type mRNA or mRNA carrying rare variants identified in affected individuals reverses morpholino-induced or CRISPR F0 phenotypes, providing an *in vivo* readout of the effect of the mutation on gene function. For example, morpholino-induced knockdown of *STX1B*, which was identified by linkage analysis in large pedigrees as carrying damaging mutations in individuals with epilepsy, caused electrographic seizures (Schubert et al., 2014). These seizures were reduced by CNS-specific expression of human *STX1B* mRNA, but not mRNA carrying a patient mutation, demonstrating that this variant represents a loss-of-function. In addition, morpholino-induced knockdown of *TRAPPC6B*, which was identified as a risk gene by linkage analysis and homozygosity mapping in individuals with epilepsy, microcephaly, and ASD from consanguineous families, led to increased baseline neural activity and sensitivity to PTZ-induced seizures in zebrafish larvae (Marin-Valencia et al., 2018).

Another approach to assess the functionality of newly identified human genes or rare variants is overexpression in zebrafish embryos. For example, overexpression of wild-type *pk1a*, the zebrafish ortholog of *PK1A*, caused a more severe phenotype than overexpressing mRNA carrying a mutation identified in individuals with progressive myoclonic epilepsy, suggesting that the mutation alters the *in vivo* function of this gene (Bassuk et al., 2008). In addition, overexpression of mRNA encoding human *RHEB* and versions of the gene containing two missense mutations identified in individuals with ID and macrocephaly, caused macrocephaly in zebrafish larvae, while F0 CRISPR mosaics of this gene displayed microcephaly, suggesting these variants may represent a gain-of-function (Reijnders et al., 2017).

There are several points to consider in the use of zebrafish for screening variants identified in human genetics studies. First, while *in vivo* rescue or overexpression screens may be informative regarding the biological function of an identified variant, the identification of nonspecific neural phenotypes, particularly morpholino-based phenotypes, are not sufficient to establish causation of an identified gene or variant and should not be used a substitute for strong evidence from human genetic studies. Second, while the presence of a phenotype in CRISPR F0 mosaics provides additional support for specificity, it is important to demonstrate in a stable mutant line that the phenotype results from loss of gene function and not nonspecific effects in F0-injected embryos. Third, with advances in CRISPR technology (Auer et al., 2014; Kimura et al., 2014), it will be increasingly feasible to rapidly generate not only loss-of-function mutations in a gene of interest, but "knock-in" models of specific patient mutations, which will be particularly informative given the pleiotropy of genes associated with neurodevelopmental disorders.

Schizophrenia

Schizophrenia is a psychotic disorder characterized by hallucinations, delusions and disorganized thought processes or

behavior, as well as diminished affect, energy and motivation, which severely impacts overall functioning (American Psychiatric Association, 2013). The genetics of schizophrenia are complex, with over 100 common variants identified by genome-wide association studies (GWAS; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), and rare damaging variants and CNVs contributing to risk according to a polygenic model (Walsh et al., 2008; Purcell et al., 2014). This genetic architecture complicates the functional analysis of risk variants in schizophrenia (Fromer et al., 2016). Here, we discuss several studies that used zebrafish to analyze the function of schizophrenia-associated genes (**Table 4**).

While schizophrenia is highly polygenic, *DISC1* is an example of a rare schizophrenia-associated gene, which was discovered in a large Scottish family where a balanced chromosomal translocation segregated with schizophrenia and other psychiatric disorders (schizoaffective disorder, bipolar disorder, major depressive disorder; Millar et al., 2000). Some studies have used zebrafish to investigate *DISC1* function. For example, De Rienzo et al. (2011) found that *disc1* mutants and morphants display abnormal brain morphology at early developmental stages, including small brain ventricles. Full-length human *DISC1* mRNA rescued structural brain phenotypes in morphants, while *DISC1* lacking the GSK3β binding domain did not, suggesting that that loss of Wnt signaling is responsible for these phenotypes. In a subsequent study, Singh et al. (2011) showed that two common variants in *DISC1* identified in individuals with schizophrenia and bipolar disorder that lacked Wnt signaling activity in an *in vitro* assay were unable to rescue structural brain phenotypes in *disc1* morphants, further implicating Wnt signaling as an important pathway downstream of *DISC1*. Another study found evidence for altered hypothalamic development as well as stress responses in zebrafish *disc1* mutants (Eachus et al., 2017). Interestingly, this study found variable phenotypes in two *disc1* mutant lines, such as differences in the time course of expression changes in markers of hypothalamic precursors, even though both lines carry mutations that induce early premature stop codons. This suggests that the specific location of a mutation may alter the expression of a phenotype in mutants. Also, while this study used one of the same mutants as the previous study, no morphological defects were identified in *disc1* homozygous mutants, suggesting that background variation may alter the expression of phenotypes in genetic mutants (Eachus et al., 2017). Together, these studies highlight insights into *DISC1* function gained from zebrafish models.

Additional studies have used zebrafish to rapidly assess the effect of changes in the expression of schizophrenia candidate genes implicated in human genetics studies. By comparing risk variants identified by GWAS with RNA sequencing data from post-mortem brain samples from individuals with schizophrenia, Fromer et al. (2016) identified genomic loci where risk variants might contribute to observed changes in gene expression. By altering the expression of three implicated genes in zebrafish in the same direction as the RNA sequencing result from human brain tissue, this study found that morpholino-induced knockdown of the downregulated gene, *furina*, led to microcephaly and decreased cell proliferation, which was rescued by introducing human *FURIN* mRNA, while overexpression of the upregulated genes, *tsnare* and *cntn4*, led to microcephaly and increased cell proliferation (Fromer et al., 2016). Another study combining GWAS and human gene expression data identified *MAPK3*, which is found in the 16p11.2 interval, as a schizophrenia susceptibility gene. Morpholino-induced knockdown of *mapk3* caused microcephaly, which was reversed by overexpression of human *KCTD13*, another gene in the 16p11.2 interval (Gusev et al., 2018). As discussed earlier, while nonspecific neural phenotypes induced by morpholinos, overexpression, or CRISPR F0 mosaics, may be suggestive of a functional effect, replication of these findings in a stable mutant line is necessary for validation.

FUTURE DIRECTIONS: FUNCTIONAL IMAGING OF NEURAL CIRCUITS

Most studies of zebrafish models of neurodevelopmental disorders to date have focused primarily on early morphological and simple behavioral phenotypes. However, recent advances in functional imaging are likely to transform these studies in the near future, allowing for the assessment of circuit-level phenotypes resulting from risk gene disruption. Progress in brain imaging is due in large part to the development of genetically-encoded calcium indicators (GECIs), such as GCaMP, which provide a rapid readout of activity at the level of a single neuron (Chen et al., 2013). GCaMP can be expressed transgenically in a subset of neurons or throughout the brain of larval zebrafish, which is an ideal system for monitoring neural activity. By harnessing advances in imaging technologies, including two-photon and light-sheet microscopy, a number of studies are beginning to dissect neural circuit mechanisms in the developing zebrafish brain (Ahrens et al., 2013; Portugues et al., 2014; Bianco and Engert, 2015; Dunn et al., 2016; Filosa et al., 2016; Naumann et al., 2016; Thompson et al., 2016). Together with the transparency and relative simplicity of the larval zebrafish brain, these technologies are likely to have considerable translational potential for revealing mechanisms by which the disruption of risk genes leads to alterations in signaling networks in the developing vertebrate brain, resulting in simple behavioral phenotypes.

For example, several studies have used two-photon microscopy to record brain activity in response to visually-evoked stimuli. Two-photon microscopy is a point-scanning method that provides excellent spatial resolution, but is more limited in its imaging speed (Keller and Ahrens, 2015). To image brain activity, zebrafish are immobilized in agarose, while visual stimuli are projected onto a screen to the side or below the fish while brain activity is recorded. Portugues et al. (2014) used this approach to investigate the circuitry underlying the OKR, a reflexive series of eye movements induced by a rotating drum of alternating light and dark stripes. By simultaneously recording brain activity, eye and tail movements during stimulus exposure, this study identified a stereotyped

TABLE 4 | Zebrafish models of schizophrenia-associated genes.

Human Gene(s)	Zebrafish Gene(s)	Disorder	Method**	Phenotype(s)	Rescue	Reference
CNTN4 ***FURIN*** ***TSNARE1***	***cntn4*** ***furina*** ***tsnare1***	SCZ	*MO*; *F0 CRISPR*; *OE*	• ***furina MO:*** - Microcephaly (forebrain-midbrain region; 3 dpf; MO and F0 *CRISPR*) - Decreased cell proliferation - Increased apoptosis • ***tsnare/cntn4 OE:*** - Microcephaly (forebrain-midbrain region; 3 dpf) - Increased cell proliferation - Increased apoptosis	Human *FURIN* mRNA rescues microcephaly and cell proliferation in *furina* morphant	Fromer et al. (2016)
DISC1	***disc1***	SCZ	*MO*; ***TILLING Mutant***	• ***disc1−/− mutant and disc1 MO:*** - Abnormal brain morphology, small brain ventricles, abnormal muscle segments, bent tail (24 hpf); disorganized, decreased/missing axon tracts (36 hpf) - Abnormal pigmentation, bent tail, swim bladder does not inflate (5 dpf, mutant) • Evidence that Wnt signaling pathway underlies phenotypes due to loss of *disc1* in MO: - *DISC1* mRNA lacking GSK3β domain does not rescue brain or muscle phenotypes - β-catenin activation, dominant negative-GSK3β rescue forebrain, axon phenotypes - Activation of noncanonical Wnt pathway rescues muscle/tail phenotypes	Human *DISC1* mRNA rescues abnormal morphology in mutant (24 hpf); CNS-specific zebrafish *disc1* mRNA rescues brain, axon, not tail phenotypes in MO	De Rienzo et al. (2011)
			MO	• Abnormal brain/ventricle morphology, abnormal muscle segments, bent tail, disorganized axon tracts (24 hpf)	Human *DISC1* mRNA rescues; mRNA with human mutations either fully (S704C), partially (L607F), or do not (R264Q) rescue	Singh et al. (2011)
			TILLING Mutant	• Changes in expression of *rx3*, marker of hypothalamic neuron precursors (24 hpf–5dpf) • Increased cell proliferation in hypothalamus at 24 hpf, decreased at 3 dpf • Abnormal numbers of neuroendocrine neurons in hypothalamus (2–3 dpf, 5 dpf) • No change in shoal cohesion after alarm substance or osmotic stress (5 dpf) • *Adult phenotypes:* Increased freezing (open field); no light-dark preference; no increase in bottom dwell time after alarm substance	–	Eachus et al. (2017)
MAPK3	***mapk3***	SCZ	*MO*	• Microcephaly (forebrain-midbrain region; 4 dpf) • Decreased cell proliferation (72 hpf)	Human *KCTD13* OE rescues microcephaly, cell proliferation in *mapk3* MO	Gusev et al. (2018)
SHANK3	***shank3a/b***	SCZ (*in this study) ASD PMS	*MO*	• Microcephaly • Abnormal larval escape responses	Rat mRNA partially rescues escape response; Human *de novo* R1117X mutation does not; R536W partially rescues	Gauthier et al. (2010)

Key: *hpf, hours post fertilization; dpf, days post fertilization;* ***Disorder:** ASD, autism spectrum disorder; SCZ, schizophrenia; PMS, Phelan-McDermid Syndrome;* ****Methods of Risk Gene Disruption:** CRISPR F0, Mosaic CRISPR-injected embryo; MO, Morpholino; OE, overexpression; TILLING, targeted induced local lesions in genomes.*

pattern of brain activity that occurs during the OKR, and found that activity in specific brain regions correlates with sensory or motor signals. In addition, Filosa et al. (2016) used two-photon imaging to interrogate the neural circuitry governing feeding behavior. Interestingly, this study showed that hunger not only makes zebrafish more likely to pursue visual stimuli that resemble their food, but increases the responsiveness of specific cells in the optic tectum to these food-like stimuli, providing a neural correlate for the observed behavior. Other studies have also used two-photon microscopy to examine behavioral circuits, such as those involved in prey capture, predator responses, responses to visual and olfactory stimuli, and the optomotor response, a reflexive behavior that occurs following a perceived change in whole-field motion (Dreosti et al., 2014; Bianco and Engert, 2015; Dunn et al., 2016; Naumann et al., 2016).

In addition, a growing number of studies have used light-sheet fluorescence microscopy for functional imaging of the zebrafish brain. Light-sheet microscopy, which uses a thin "sheet" of light to illuminate samples, offers superior speed over two-photon microscopy (Keller and Ahrens, 2015). For example, this method was used to successfully image over 80% of the neurons in the larval zebrafish brain in approximately 1.3 s (Ahrens et al., 2013). Given its speed, light-sheet imaging was used to perform continuous whole-brain activity recordings at baseline, revealing functional networks of correlated activity in the zebrafish brain (Ahrens et al., 2013). Light-sheet imaging has also been used to study brain activity following exposure to various stimuli. For example, Thompson et al. (2016) found that distinct clusters of neurons in the optic tectum respond to visual, auditory and water flow stimuli, and provide evidence for integration in the processing of these stimuli. One drawback of light-sheet imaging is the potential for retinal activation by the light "sheet" itself (Keller and Ahrens, 2015). Two-photon imaging offers an alternative in this regard, because it provides stimulation outside of the visible range of zebrafish. Another approach is to position multiple light sheets to avoid direct retinal stimulation (Vladimirov et al., 2014). Further, functional imaging in general generates considerably large datasets, which may be difficult to analyze, though computational algorithms have been developed to address this challenge (Keller and Ahrens, 2015).

At the same time, these functional imaging techniques are technically challenging, not high-throughput, and often require immobilizing the fish. To address these limitations, Randlett et al. (2015) developed a technique called mitogen-activated protein kinase (MAP)-mapping, in which fixed brain tissue is stained for phosphorylated extracellular-signaling-regulated kinase (pERK), a marker of active neurons, and then imaged using confocal microscopy. To identify regions of differential activity, images are mapped onto a zebrafish brain atlas (Z-Brain). This approach can be used to obtain a readout of whole-brain activity in freely moving zebrafish either at baseline or in response to a stimulus or drug (Randlett et al., 2015). Another method, developed by Lovett-Barron et al. (2017), called MultiMAP, combines two-photon imaging of zebrafish during exposure to visual stimuli with immunostaining for neuronal cell types. By integrating the functional imaging and immunostained datasets, this method allows for the identification of the specific cell types that were active during a behavioral task. Intriguingly, using this approach, (Lovett-Barron et al., 2017) identified the neuromodulatory cell types controlling alertness in zebrafish, and found that manipulation of related cell types in mice induces similar behavioral effects, providing remarkable evidence for conservation of behavioral circuits in fish and mammals (Lovett-Barron et al., 2017). Therefore, findings in zebrafish models of neurodevelopmental disorders are likely to have translational relevance for understanding related circuits in mammals. Together, these technologies offer considerable promise for illuminating circuit-level mechanisms in zebrafish models of neurodevelopmental disorders.

CONCLUSION

Zebrafish have critical advantages as a model system for investigating the function of genes associated with neurodevelopmental disorders. A growing number of studies are beginning to capitalize on their unique features to illuminate neurobiological and pharmacological pathways underlying ASD, epilepsy, ID and schizophrenia. These studies have utilized the transparency, tractability and throughout of the zebrafish model to identify the effects of loss of risk gene function on the development of specific neuron populations, molecular pathways, and simple behaviors, all of which can be leveraged to screen for novel small molecule suppressors. While many studies to date have used morpholino knockdown technology, which is prone to off-target effects and should not be used as a "standalone tool" (Lawson, 2016), it is important moving forward that the field commit to using genetic mutants to confirm morpolino-induced phenotypes, which is particularly essential for neural phenotypes. While the advent of CRISPR technology has made this goal increasingly feasible, confirming that CRISPR-generated mutants result in loss of function is equally critical. Because splice-site mutations may lead to alternative transcripts that reverse deleterious mutations (Anderson et al., 2017), targeting CRISPRs within a conserved exon, removing most of a target gene using multiple CRISPRs, and demonstrating loss of protein by western blot are recommended steps.

At the same time, one of the challenges in analyzing the function of the growing list of risk genes associated with neurodevelopmental disorders is determining which phenotypes are likely to be relevant to the pathophysiology of these disorders (State and Šestan, 2012). Assessing phenotypes over a developmental time course from embryonic to adult stages will likely provide key insights into when and where risk genes play important roles. In addition, investigating the effect of specific mutations identified in affected individuals by introducing or "knocking in" these mutations using CRISPR will also be instrumental in elucidating how particular variants affect neural development. Moreover, future studies capitalizing on the strengths of zebrafish as a first-pass, high-throughput screening approach have the potential to reveal novel pharmacological candidates for further investigation in these disorders. Given the evidence for conservation of pharmacological and circuit-level

pathways in zebrafish and mammals (Rihel et al., 2010; Lovett-Barron et al., 2017), it is likely that these studies will have translational relevance, though testing compounds identified in zebrafish in rodent models will be an important next step prior to clinical trials in humans. Furthermore, advances in *in vivo* calcium imaging in zebrafish represent an exciting new avenue for investigating the circuit-level roles of risk genes with translational relevance. Taken together, zebrafish represent a promising model system for the discovery of novel biological pathways, pharmacological candidates, and circuit mechanisms with relevance to neurodevelopmental disorders.

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CS, SI and EH contributed to the conceptualization, literature review and writing of this article.

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REVIEW
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De novo Mutations (DNMs) in Autism Spectrum Disorder (ASD): Pathway and Network Analysis

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder (NDD) defined by impairments in social communication and social interactions, accompanied by repetitive behavior and restricted interests. ASD is characterized by its clinical and etiological heterogeneity, which makes it difficult to elucidate the neurobiological mechanisms underlying its pathogenesis. Recently, *de novo* mutations (DNMs) have been recognized as strong source of genetic causality. Here, we review different aspects of the DNMs associated with ASD, including their functional annotation and classification. In addition, we also focus on the most recent advances in this area, such as the detection of PZMs (*post-zygotic mutations*), and we outline the main bioinformatics tools commonly employed to study these. Some of these approaches available allow DNMs to be analyzed in the context of gene networks and pathways, helping to shed light on the biological processes underlying ASD. To end this review, a brief insight into the future perspectives for genetic studies into ASD will be provided.

Keywords: Autism Spectrum Disorder, genetics, post-zygotic mutations, neurodevelopmental disorders, *de novo* mutations, gene networks, pathway analysis, whole exome sequencing

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INTRODUCTION

Autism Spectrum Disorder (ASD) includes a range of NDDs that are characterized by deficits in social communication and interactions, as well as by repetitive behaviors and restrictive interests, with onset in early development (American Psychiatric Association, 2013). The estimated prevalence of ASD in the general population stands at approximately 1%, with males being about three times more likely than females to be affected (Fombonne, 2009; Loomes et al., 2017).

Abbreviations: AAF, alternate allele frequency; ASC, Autism Sequencing Consortium; ASD, Autism Spectrum Disorder; BF, Bayes factor; CADD, combined annotation dependent depletion; CHD, chromodomain helicase DNA-binding family; CNV, copy number variation; DAPPLE, Disease Association Protein–Protein Link Evaluator; DAVID, Database for Annotation, Visualization and Integrated Discovery; DAWN, Detecting Association with Networks; DNM, *de novo* mutation; DZ, dizygotic; FDR, false discovery rate; GCNs, gene co-expression networks; GERP, genomic evolutionary rate profiling; GO, gene ontology; GSEA, gene set enrichment analysis; GWAS, genome-wide association study; ID, intellectual disability; LoF, loss of function; MAF, minor allele frequency; MAGI, merging affected genes into integrated networks; MPC, Missense badness, Polyphen-2 and constraint; MsigDB, molecular signatures database; MZ, monozygotic; NDD, neurodevelopmental disorder; NETBAG, NETwork-based analysis of genomic variation; NGS, next generation sequencing; PCA, principal component analysis; PGC, Psychiatric Genomic Consortium; pLI, prob of being LoF intolerant; PPI, protein–protein interaction; PZM, post-zygotic mutation; RR, relative risk; RVIS, Residual Variation Intolerance Score; SFARI, Simons Foundation Autism Research Initiative; SNP, single nucleotide polymorphism; SNV, single nucleotide variations; SSC, Simons Simplex Collection; SV, structural variant; TADA, transmission and *de novo* association test; WES, whole exome sequencing; WGCNA, weighted correlation network analysis; WGS, whole genome sequencing.

Twin and family studies have demonstrated a genetic contribution to ASD etiology. Indeed, early reports showed a concordance in ASD diagnosis in monozygotic (MZ, 70–90%) and DZ twins (10%), which indicates a heritability of about 90% (Steffeneburg, 1989; Bailey et al., 1995). A recent analysis more precisely estimated heritability to be 83%, which is slightly lower than that reported in the earlier twin studies (Sandin et al., 2017). Moreover, the risk of ASD increases for a child when he has an older affected sibling and as such, the overall risk of recurrence in siblings has been estimated to be around 6.9–18% depending on the study design. This range is also influenced by whether half or full siblings are considered (Ozonoff et al., 2011; Gronborg et al., 2013; Risch et al., 2014).

A substantial fraction of this heritability can be explained by SNPs. The contribution of these common variants to ASD etiology stands at around 50% when it is additively considered (Gaugler et al., 2014). However, early GWAS failed to detect strong signals, in part due to the need for larger samples (Weiss et al., 2009; Anney et al., 2010; Ma et al., 2010). However, subsequent large-scale GWAS identified 12 novel ASD loci, some of them identified as plausible common risk variants in earlier studies (Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017). Moreover, the latest GWAS meta-analysis conducted by the PGC not only represented an incredible effort to increase sample size up to tens of thousands of cases and controls but also, it developed a well-defined quality control and imputation pipeline. For the first time, the results of this ASD GWAS meta-analysis led to the identification of 93 significant genome-wide markers, of which 53 were replicated in independent cohorts (Grove et al., 2017).

Despite the evidence of a significant role for common variants in ASD risk, rare genetic variation ($MAF<1\%$) confers higher individual risk (**Table 1**). Rare variation can be found as small insertions and deletions (indels), CNVs or SNVs. Moreover, these can be inherited from a paternal and/or maternal origin or they may appear *de novo* in the affected subject (De Rubeis et al., 2014). Such DNMs, are mutations identified in the proband that are not found in the genomes of the biological parents. The importance of DNMs in ASD genetics is strongly related to the role of natural selection and allele frequency. Therefore, rare risk alleles tend to be eliminated by purifying selection while common ones show signs of positive selection (Polimanti and Gelernter, 2017). These facts mean that DNMs are most likely to have a strong effect and thus, the discovery of DNMs allows ASD risk genes to be identified. Indeed, exons expressed in the brain that are subject to purifying selection were enriched for DNMs in ASD (Uddin et al., 2014).

The different types of genetic variants, combined with their distinct pattern of inheritance or their *de novo* origin, define the potential genetic risk for ASD. For example, carrying a *de novo* SNV and a specific non-sense mutation in the coding sequence confers around five times more individual risk than carrying a transmitted CNV (Stein et al., 2013). Moreover, children with severe ASD symptoms along with ID are thought to carry more harmful DNMs (Robinson et al., 2014). Hence, there is now considerable interest in identifying novel DNMs associated with ASD.

TABLE 1 | Genetic architecture of ASD.

% Liability due to different classes of mutations		% Of different classes of mutations harbored by ASD probands	
Common variation	49.4%		
De novo variation	3%	*De novo* CNVs	4–7%
		De novo SNVs	7%
Rare inherited variation	3%	Rare variants AR	3%
		X-linked variants	2%
Total	55%	Total	16–19%

The liability in ASD according to the different classes of mutation and the different types of mutations harbored by ASD individuals. Data taken from Gaugler et al. (2014).

DNMs IN ASD GENETICS

Identification of DNMs

Trio genetic association studies (parents and affected proband) have been used since 2007 to study DNMs and to find mutations in the proband that were not present in either parent. By performing such studies on large cohorts of patients and controls, and by analyzing the characteristics of the DNMs identified, it is possible to characterize previously unrecognized ASD genes, the main goal of such studies. In the first studies to detect CNVs using high-resolution microarrays, *de novo* CNVs were more frequent in cases than controls (Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2010; Levy et al., 2011; Sanders et al., 2011) and also more frequent in simplex rather than multiplex families (Marshall et al., 2008; Sebat et al., 2010).

However, the large size of CNVs presents a problem when attempting to detect ASD candidate genes. Indeed, genes disrupted by CNVs may contribute to a moderate risk of ASD, whereas SNVs are more likely to directly indicate genes associated with a high susceptibility for ASD (Sanders et al., 2015). Accordingly, large scale parallel sequencing and specifically, WES has been employed widely to unravel the genetic architecture of ASD (Betancur, 2011; Buxbaum et al., 2013; Sener et al., 2016). Indeed, the vast majority of DNM studies have employed this technology, in conjunction with large sample sizes (thousands of samples) collected from many families (normally trios but also quads) (Neale et al., 2012; De Rubeis et al., 2014; Merico et al., 2017). By comparing DNA sequences obtained from affected children to those from their parents, it is possible to identify DNMs after filtering out sequencing artifacts (Iossifov et al., 2014). This variant calling process requires a detailed bioinformatics pipeline that involves the application of different thresholds to filter for each quality parameter (Patel et al., 2014). This process could be performed following different approaches and accordingly, we can find a more or less restrictive filtering depending on the study. Nevertheless, each single DNM will finally be re-sequenced by other methods, usually Sanger sequencing, to check the accuracy of the findings. We should take into account, that the average rate of DNMs in a set of whole exome data is estimated to be in 1.2×10^{-8} per nucleotide per

generation, and normally ASD studies have observed a similar or slightly higher rate (Conrad et al., 2011).

After this first step, all DNMs located in the coding sequence should be functionally annotated according to the impact that the predicted amino acid substitution has on protein structure and function. Thus, we can find missense DNMs and non-sense DNMs, also referred to as LoF mutations, which can in turn be classified into different subtypes: frameshift, splice site, and stop-gain. It is important to note that although LoF DNMs might be the object of greater attention, the importance of missense DNMs in ASD was recently highlighted. Therefore, such variants may produce a gain of function effect and genes carrying two or more mutations of this type were seen to be more likely to be pathogenic in ASD (Geisheker et al., 2017). Moreover, some studies have reported an overall enrichment of LoF mutations in individuals with ASD compared to their healthy relatives. In particular, heterozygous LoF mutations are present in 20% of probands but in only 10% of unaffected siblings (O'Roak et al., 2011; Neale et al., 2012; Sanders et al., 2012; Ronemus et al., 2014). Missense mutations were also more common in probands than in their siblings when larger cohorts were considered and therefore, it was calculated that missense mutations contribute to at least 10% of ASD diagnosis (Iossifov et al., 2014).

Methods to Assess DNM Pathogenicity

Several tools can be used as functional predictors to assess DNM pathogenicity, such as Polyphen2, SIFT, CADD, and GERP (Cooper et al., 2005; Kumar et al., 2009; Adzhubei et al., 2010; Kircher, 2014). Polyphen2 is without doubt the most widely employed of these, although more recent trends prefer not to focus on just a single method but rather, to consider a combination of several *in silico* scores in order to establish criteria to classify benign and deleterious mutations (Lim et al., 2017). Indeed, an integrative approach was described not long ago that relied on a new functional genome annotation tool called Eigen. This tool provides a meta-score calculated by unifying the information obtained through several annotation methods. Therefore, Eigen provides a better discriminatory ability than other scores like CADD, SIFT, or GERP. As such, Eigen is a powerful and novel annotation tool that was successfully employed on a set of DNMs previously described in ASD and also in other psychiatric disorders like schizophrenia (Ionita-Laza et al., 2016). More recently, other measures of the deleterious nature of mutations have been developed to redefine the impact of DNMs. One of these novel scores is called, MPC (for *Missense badness, Polyphen-2* and *Constraint*), which specifically enables the deleterious effect of missense variants to be predicted. Through the use of MPC, some missense DNMs were shown to have a similar effect as LoF mutations in NDDs, information that will be extremely useful for future ASD sequencing studies (Samocha et al., 2017).

DNMs: Relative Risk, Tolerant, and Intolerant Genes

The contribution of DNMs to the risk of ASD depends on the impact that the amino acid change in the protein coding sequence has on the protein's behavior. Thus, the RR entailed by LoF DNMs will always be larger than that associated with missense DNMs. Moreover, both variants will provide a greater RR when they are considered jointly rather than an inherited LoF mutation alone, for example. This allows a RR to be established for each gene as a function of the class of DNM (De Rubeis et al., 2014). Moreover, some studies also consider the location of the DNM and it was shown that DNMs are more likely to occur in genome locations with a higher rate of mutation that are located close to CNVs (Merico et al., 2017). Another factor that must be taken into account when DNMs are analyzed is that there are genes that are mutation tolerant and intolerant. This means that over the entire human genome some genes are more likely to carry more functional mutations than those expected by chance (tolerant genes), while other (intolerant) genes carry fewer such mutations. Thus, DNMs found in tolerant genes are less likely to influence the development of ASD. A gene-based score RVIS has been developed that allows genes to be ranked depending on their tolerance or intolerance score (Petrovski et al., 2013; Ronemus et al., 2014). Similarly, additional information can be provided by the pLI score (*prob of being LoF intolerant*). Therefore, a gene with pLI $>$ 0.9 is considered to be extremely LoF intolerant, and this is particularly useful when there is more than one LoF mutation in an exome and there is a need to prioritize these causal DNMs (Lek et al., 2016). The interest in this score was successfully confirmed using genetic data from NDDs, including ASD cases (Kosmicki et al., 2017).

As we can see, the discovery, identification and prioritization of DNMs and their respective ASD risk genes, requires a complex workflow. It involves several technical variables that need to be considered in order to identify the DNMs that truly influence ASD risk and to distinguish them from those that are artifacts or that are not pathogenic DNMs.

BIOINFORMATICS APPROACHES EMPLOYED IN THE STUDY OF DNMs

The main aim of the bioinformatics approaches discussed in this section is to start from the genetic information obtained from the genes carrying DNMs, achieving a global vision of the related biological processes that underlie the pathogenesis of ASD (**Table 2**). As detailed below, these tools aim to integrate different sources of genetic and biological information in order to identify the biological processes underlying ASD, as well as new target genes.

Prioritizing Novel ASD Risk Genes Carrying DNMs

The analysis of DNMs has without doubt been a step forward in the discovery of new ASD risk genes. Technically speaking, this type of analysis can only be performed on DNMs. However, it was recently shown that a more robust way to interpret WES data is to analyze DNMs together with inherited variants, given the high heritability of ASD. Therefore, other genetic variants can be added, such as SNPs from case-control studies. This approach came into use when it was seen that the proportion of

TABLE 2 | Bioinformatics approaches that allow WES data (genes carrying DNMs and other genetic information) to be integrated in different pathway and network analyses categorized by the input data necessary, the type of algorithm and the output results.

	Input information	Algorithm	Analysis result	Publications
TADA	DNMs (LoF > missense) + transmitted + case-control variants	Bayesian gene-based likelihood model	Prioritized list of genes depending on the impact of the mutations	De Rubeis et al., 2014 Sanders et al., 2015 Ji et al., 2016
NETBAG	Input data	Likelihood approach including a Bayesian integration of PPIs.	Identifies functional gene networks and phenotype networks	Gilman et al., 2011 Chang et al., 2014
DAWN	List of ASD genes obtained from WES studies scored by TADA	Algorithm based on the "screen and clean" principle (hidden Markov random field + FDR procedure)	Identifies gene networks that are "hot spots" within a co-expression network (RNA-seq data)	Liu et al., 2014
DAPPLE	List of ASD candidate genes	Algorithm based on permutations	Test PPIs across the genes hit by a functional DNM. Allow to redefine a huge list of putative ASD genes in a reduced but most relevant list	Neale et al., 2012 Poultney et al., 2013 Sanders et al., 2015 Parikshak et al., 2013
MAGI	List of ASD genes obtained in WES and case-control studies	Combinatorial optimization algorithm. Maximizes mutations in modules considering gene length and where DNMs are located (LoF and missense)	Creates gene clusters considering the information from PPIs and co-expression networks together	Hormozdiari et al., 2015

Moreover, the most relevant publications employing each of them to study ASD genetics are indicated.

ASD cases that could be explained by considering only DNMs and not other types of genetic variation was really quite small. Moreover, despite analyzing thousands of ASD cases, only tens of LoF DNMs were detected. Therefore, this combined analysis, called TADA, opened the door to expanding the list of ASD candidate genes and it made the analysis of WES data more robust (He et al., 2013; Sanders et al., 2015). This approach has been successfully employed on genetic data from the SSC and the ASC (De Rubeis et al., 2014). TADA uses a Bayesian gene-based likelihood model that weights mutations by type and mode of inheritance in this order: *de novo* LoF > *de novo* Mis3 (missense variants predicted to be damaging by Polyphen) >transmitted LoF. In this way, each DNM is given a predicted impact on the protein function. Moreover, the corresponding gene mutation rate is also considered and these categories can be extended as required for the desired analysis (He et al., 2013). Furthermore, it is possible to obtain expanded or restricted gene lists that consider the load of DNMs by gene and their predicted functional impact. This is possible because TADA generates a gene-level BF that quantifies association and its correspondence to a given FDR or q-value. Thus, TADA allows a prioritized list of genes to be obtained, which is perfect to use as an input for other bioinformatics tools that are optimized to create gene-networks and to unravel new related biological pathways in ASD. Recently, the TADA algorithm was modified (TADAext) allowing data from multiple populations to be employed and related NDDs to be considered together in order to discover common risk genes. As such, TADA helps define and prioritize a list of genes that can be employed as an input for additional analyses, as will be seen below (Nguyen et al., 2017).

Gene-Network and Pathway Analysis Tools

Once gene lists are established and prioritized, several tools can be used to generate gene networks and pathways. NETBAG is one of the latest algorithms that can be successfully employed to create risk gene networks starting from information about DNMs (Gilman et al., 2011). This computational approach was also used in ASD sequencing studies to not only consider data from DNMs (SNVs and CNVs) but also, to combine this with information from other associated genomic regions identified in GWAS studies. As such, NETBAG has been successfully employed with ASD and schizophrenia data (Gilman et al., 2012). Specifically, this tool serves to establish gene clusters that identify distinct biological networks of genes, for example networks that are related to synapse development and/or neuron motility but relying on a previously described phenotype network (Gilman et al., 2011; Pinto et al., 2014). This phenotype network is based on the integration of various protein-function descriptors using Bayesian methods. The network edges will be constructed considering the likelihood that two genes participate in the same genetic phenotype (for example, ASD and/or ID). Among a list of provided genes (from each genetic study), NETBAG will create clusters of strongly connected genes by phenotype depending on the calculated likelihood (Chang et al., 2014). Therefore, the most important characteristic of NETBAG is that the underlying network is created by sets of genes previously associated with ASD and/or ID phenotypes. Once these clusters are formed, specific biological processes related to each one can be added integrating GO, KEGG, and PPI descriptors. Another algorithm that could be very helpful in the search for ASD risk genes and that helps to integrate DNM information, is DAWN. DAWN works in conjunction with a network analysis tool like TADA that sets a score for each gene, and it can identify hotspots (clusters of strong scores) among the complex gene networks that can be established when the whole set of TADA genes is considered. This algorithm works through a hidden Markov random field, a generalization of a hidden Markov model that is widely employed when modeling biological processes. The particular strength of DAWN is that it relies on another type of information to build these new clusters,

integrating transcriptomic data (RNA-seq) analyzed using a WGCNA approach (a method that will be discussed later in more detail). Once the large co-expression network is created, DAWN will help to identify clusters of strongly correlated genes. Therefore, using the TADA scores obtained previously, DAWN will identify ASD risk genes, always performing a multiple testing correction (FDR). DAWN can also incorporate any additional variables as transcription targets if one or more key transcription factor were meaningful to the analysis (Liu et al., 2014, 2015). Therefore, DAWN works in conjunction with TADA but while it is TADA that prioritizes genes carrying DNMs, DAWN moves a step forward by creating gene networks and subnetworks that help to detect novel genes that would not be revealed by using TADA alone. Indeed, DAWN uses TADA scores for different sets of previously published genes. For example, *GRIN2B* is an ASD risk gene reported to be a carrier of multiple LoF mutations (TADA *q*-value 0–0.0025). Consequently, DAWN can establish *ACTN2*, *DLG1*, *CBL*, *AP2A1*, and *DLG4* among others as novel *GRIN2B* connectors, assigning them to a cluster of receptor signaling and protein scaffolding genes (O'Roak et al., 2011; Liu et al., 2014).

Another two complementary strategies that are commonly used in these types of studies are enrichment analysis and PPI networks. GSEA serves to classify genes that are over-represented in a large dataset, identifying those groups significantly enriched or depleted according to another source of external information (e.g., GO terms, KEGG terms, expression data...) and thereby helping to identify a variety of biological signatures among them (Wen et al., 2016). There are several tools and databases that allow GSEA analysis to be run, and one of the most commonly employed is that provided by the Broad Institute website in cooperation with MSigDB. This specific GSEA tool was successfully run in large gene sets like those reported by SFARI, an evolving online database which contains up-to-date information of genes associated to ASD[1]. In addition, hypergeometric distribution can be employed to examine how SFARI genes and other gene sets (GO terms, KEGG) overlap. This tool has led to the characterization of several pathways functionally associated in ASD, such as calcium and MAPK signaling pathways (Wen et al., 2016).

Another GSEA tool is DAVID, an enrichment analysis tool that was employed in ASD genetic studies (Dennis et al., 2003). DAVID is commonly used to consider how informative a gene list obtained from genetic studies is about ASD etiology (Pinto et al., 2014). Thus, DAVID can discover groups of functional-related genes by using different libraries (GO terms for example) to help identify the enrichment of different biological processes from an extended gene list (Huang et al., 2008, 2009; Sanders et al., 2015). Therefore, DAVID and GSEA both allow enriched functionally related gene groups to be discovered and thus, both tools are applied indistinctly for the purpose of ascribing general biological functions to genes. However, DAVID also features some additional options, and it is able to highlight functional protein domains and motifs in those relevant genes.

[1]https://gene.sfari.org/

Another GSEA tool is Enrichr, currently one of the most comprehensive tools that not only includes GO ontologies but also, new gene libraries like target microRNAs, LINCS libraries and even epigenetic data from the RoadMap Epigenomics Project. Moreover, Enrichr also allows the GSEA results to be exported, whether networks, tables or bar graphs, which can be sorted by *p*-values, *q*-values or z-scores for the different terms analyzed (Wen et al., 2016).

The use of PPIs is another strategy that helps to integrate additional information from a different biological hierarchy. PPI data are crucial to define how proteins interact in cellular processes and also, to identify others that could be connected in order to construct an interaction map (McDowall et al., 2009). There are several PPI databases available like BioGRID, STRING, MINT, KEGG, DIP, HPRD, or IntACt (Lehne and Schlitt, 2009). Therefore, ASD genes of interest can be mapped against these PPI networks, identifying connected genes that have not been found previously, or highlighting previously weakly associated ASD genes. Moreover, this approach allows gene sub-networks to be redefined whose involvement in ASD has previously been reported (Corominas et al., 2014). The ultimate aim would be to organize this information to create gene clusters, each of them characterized by cellular processes (Liu et al., 2014). DAPPLE is an algorithm frequently employed in genetic studies of ASD that works using PPI networks. Specifically, DAPPLE searches significant physical interactions between proteins encoded by genes associated with ASD. Moreover, it allows additional genes that have been reported in other independent studies to be introduced in order to expand the interaction network. The perfect strategy is to seed together the interaction network built by DAPPLE with data obtained from several available PPI databases, expanding the known information with new nodes and connectors (Rossin et al., 2011; Neale et al., 2012; Poultney et al., 2013).

Therefore, GSEA allows gene sets to be functionally annotated with their corresponding biological terms and significantly enriched or depleted groups of genes to be identified. However, PPIs represent another source of biological information that can be integrated into bioinformatics tools like DAPPLE, expanding the interaction network to include novel genes.

Characterization of the Biological Processes Underlying ASD Pathogenesis

As explained before, ASD is an extremely heterogeneous disorder, characterized by its genetic variability. It is expected that around 1,000 genes are involved in ASD, meaning that no one gene is likely to explain more than 1% of cases (De Rubeis et al., 2014), which makes functional studies difficult and complicates the identification of high value targets for treatments. One possible solution to help resolve this problem is to look for the common biological mechanisms that could be disrupted in a recurrent manner through the use of integrative systems biology approaches, such as those described in the previous section (Parikshak et al., 2015).

Initial studies focused on testing if the genes disrupted by truncating mutations converge and are related to previously

reported ASD genes. Therefore, it is expected that those genes that interact significantly also share common functions and are probably involved in the same biological pathways (Uetz et al., 2000). A PPI network was constructed based on the data collected by GeneMANIA, considering a list of genes carrying severe mutations (Mostafavi et al., 2008; O'Roak et al., 2012). As such, it was demonstrated that 39% of genes carrying truncating mutations directly interact in this network. This physical interaction between genes is an indicator of their implication in some common biological mechanisms that could underlie ASD pathogenesis. Therefore, those genes carrying truncating mutations are ranked higher. This study is a perfect example of how information about DNMs can be used to identify other potential ASD risk genes using the correct tools and methods, helping to map those interconnected genes in the corresponding biological processes. In this case, the main biological network revealed was a β-catenin/chromatin remodeling protein network (O'Roak et al., 2012).

We performed a similar analysis but choosing only those ASD risk genes carrying DNMs from previous studies and collected in the SFARI database with scores of 1 and 2 (high-confidence and strong candidate genes) (**Supplementary Table 1**). Therefore, 54 genes were used as input in GeneMANIA, revealing 20 related genes and 681 links between them (**Figure 1**). In order to create this network, GeneMANIA employs data from co-expression experiments but also physical interactions, shared protein domains, co-localization and previously reported genetic interactions. Each gene–gene interaction is given a weight and assigned to a corresponding network group (**Supplementary Table 2**). The biological functions of these genes and their corresponding FDRs are also obtained (**Supplementary Table 3**), revealing them to be: neuron cell–cell adhesion, vocalization behavior, glutamate receptor signaling pathway, cognition, and neuron projection.

It should be noted that methodological improvements have allowed genes affected by DNMs and *de novo* CNVs to be included in the same study, leading to the consideration of a higher percentage of ASD heritability. Therefore, these genes cluster together in networks enriched in different biological functions, such as synaptic function, neuronal signaling, channel activity, and chromatin modification (Gilman et al., 2012; Pinto et al., 2014). The same pathways were also identified in subsequent studies, confirming the important role of these processes in ASD neurobiology (De Rubeis et al., 2014; Krishnan et al., 2016).

Accordingly, many of the ASD genes characterized are synaptic genes, including *NLGN3* and *NLGN4X* (Jamain et al., 2003), *SHANK3* (Durand et al., 2006), *NRXN1* (Autism Genome Project Consortium et al., 2007) and *CNTNAP2* (Arking et al., 2008). Therefore, both the development and maintenance of synaptic contacts appear to be a key factor in ASD pathogenesis. Conversely, chromatin regulation also influences neural development and during this process, many events must be precisely orchestrated and mis-regulation can result in cognitive deficits. The modification of chromatin structure controls cell fate and function (van Bokhoven, 2011; Jakovcevski and Akbarian, 2013; Ronan et al., 2013) and dozens of chromatin remodelers have been implicated in ASD and other neurological diseases, including Coffin-Siris syndrome (Tsurusaki et al., 2012), Nicolaides-Baraitser syndrome (Van Houdt et al., 2012), CHARGE syndrome (Vissers et al., 2004), or Rubinstein-Taybi syndrome (Roelfsema et al., 2005). Some of the best studied genes belongs to the CHD. Indeed, functional studies in mice have shown that CHD5 and CHD8 haploinsufficiency causes morphological changes in the brain and behavioral symptoms consistent with ASD (Pisansky et al., 2017; Platt et al., 2017).

A representation of this vast list of ASD genes discovered through the identification of DNMs and those biological processes in which they are involved (see **Supplementary Table 1**) provides a representative gene-list taken from the SFARI database as well as useful additional information.

Another important group of genes overrepresented in ASD networks are FMRP targets, which are defined as gene encoding transcripts that bind to FMRP (Iossifov et al., 2012). This set of genes includes *NLGN1*, *NRNK1*, *SHANK 3*, *PTEN*, *TSC2*, and *NF1*, and it overlaps with the list of candidate ASD genes from the SFARI database (Darnell et al., 2011) that mainly encode synaptic proteins, transcription factors and chromatin modifiers (Korb et al., 2017).

CORRELATION OF DNMs WITH GENE EXPRESSION IN CO-EXPRESSION NETWORKS

Gene co-expression networks (GCNs) represent another tool commonly used in ASD studies. The key point of this approach is to construct gene networks considering not only the genetic data obtained in WES studies but also, to correlate this information with expression data from RNA-seq experiments. Thus, these gene networks allow different temporal-spatial modules to be identified based on expression at different developmental stages and in different brain areas (van Dam et al., 2017). As such, it is possible to achieve the ultimate goal of understanding the genetic causes of ASD and to relate this to gene regulation at different levels. Such information permits the role of DNMs in the pathogenesis of ASD to be better understood, helping to define the molecular pathways and the neural circuits that affect cognition and behavior. Therefore, this complex analytical approach will ultimately construct a spatiotemporal co-expression network of ASD genes.

The generation of co-expression networks involves the application of different statistical approaches, although two main steps are critical and always considered by the corresponding algorithms: calculation of a measure of co-expression (for which different mathematical methods could be used); and the establishment of a significance threshold (Song et al., 2012).

WGCNA constructs networks by using the default Pearson correlation. WGCNA find modules of expression of highly correlated genes and it identifies eigengenes for each module. For this, WGCNA employs a PCA to extract the most representative part of the expression data. Therefore, each module (given by an expression value) corresponds to an eigengene and these

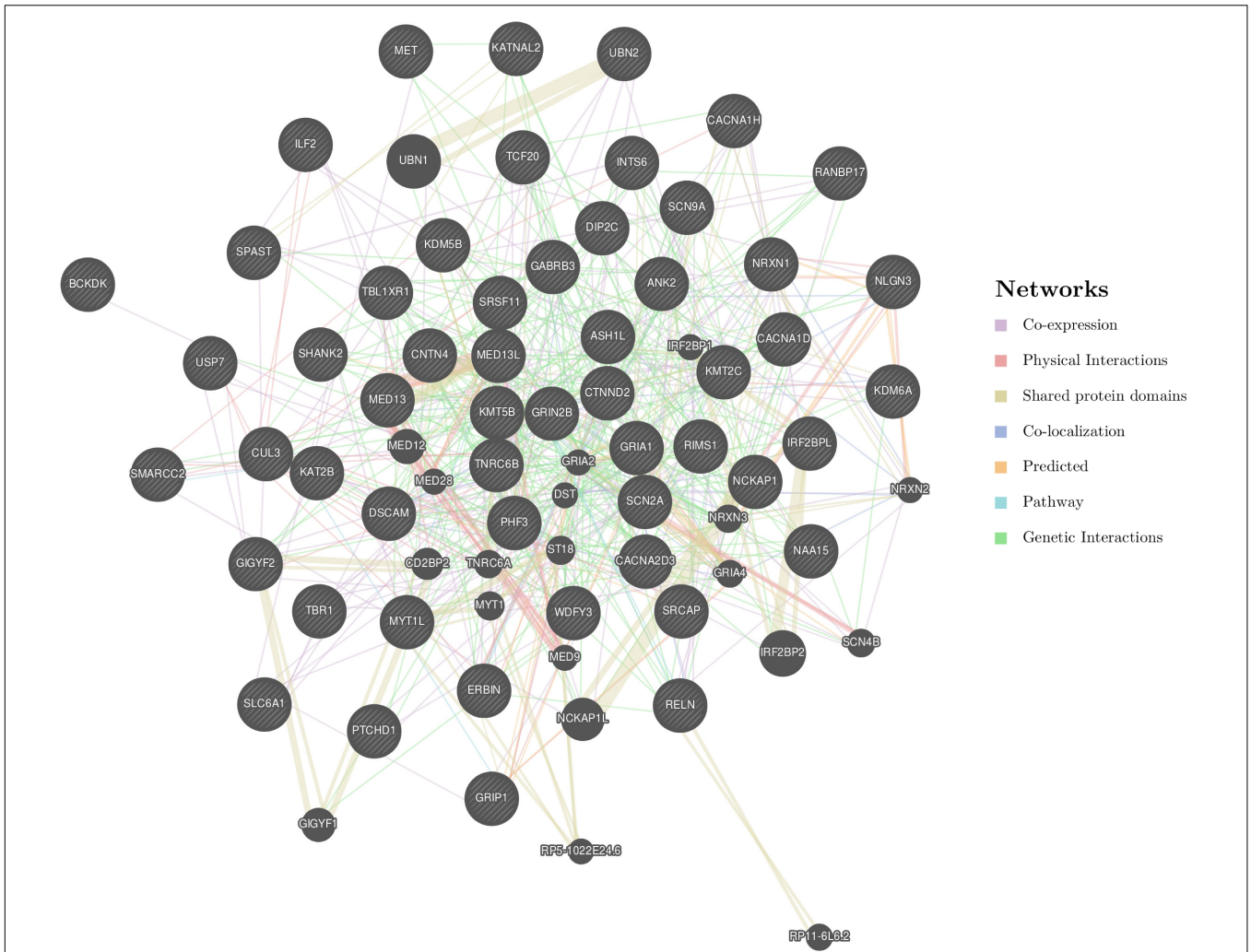


FIGURE 1 | GeneMANIA network created from 54 SFARI genes with scores 1 or 2. The following genes were used as the input: *SPAST*, *CUL3*, *KMT2C*, *NCKAP1*, *RIMS1*, *SRCAP*, *TCF20*, *TNRC6B*, *INTS6*, *BCKDK*, *MET*, *MED13*, *KMT5B*, *ERBIN*, *KAT2B*, *ASH1L*, *SRSF11*, *KDM5B*, *PHF3*, *IRF2BPL*, *MED13L*, *SCN2A*, *TBR1*, *SMARCC2*, *ILF2*, *CNTN4*, *ANK2*, *KDM6A*, *DIP2C*, *GRIA1*, *GRIP1*, *SLC6A1*, *CACNA1D*, *CACNA2D3*, *UBN2*, *SHANK2*, *WDFY3*, *NAA15*, *PTCHD1*, *GABRB3*, *KATNAL2*, *SCN9A*, *CTNND2*, *DSCAM*, *TBL1XR1*, *NRXN1*, *MYT1L*, *USP7*, *RELN*, *NLGN3*, *CACNA1H*, *GIGYF2*, *RANBP17*, and *GRIN2B*. These genes are indicated with stripes. Moreover, another 20 strongly connected genes that were detected by GeneMANIA are also represented.

eigengenes can be employed to construct the related biological networks.

In addition to WGCNA, other methods were recently employed to analyze ASD genomic data, such as MAGI, which represents a further step-forward in the use of this type of tool (**Table 2**). MAGI not only allows expression data (RNA-seq) to be integrated with genetic information (from *missense* or *LoF* mutations to case-control studies) but also, representative biological information from PPIs can also be added (Leiserson et al., 2015). This data integration was successfully employed with WES data from ASD and ID, facilitating the identification of two differentiated modules of genes during brain development, one expressed from 8–14 weeks post-conception, which includes genes related to the Wnt pathway, and another that contains genes related to synaptic function and that is more strongly expressed in postnatal stages (Hormozdiari et al., 2015). The vast majority of ASD co-expression networks have employed the data available at BrainSpan[2], which includes RNA-seq data from sixteen targeted cortical and subcortical structures at different stages of human brain development (prenatal and postnatal development) (Kang et al., 2011).

Expression in brain tissues has been analyzed in different studies, integrating this data with that obtained in genetic studies to identify at which developmental stages and in which brain areas both sources of information overlap. *Post-mortem* brain tissue samples (cases and controls) were analyzed to identify which ASD genes are altered in specific regions. WGCNA was applied to these data to integrate the differences in expression between cases and controls in a systems biology context. Two network modules were enriched in genes highly correlated with

[2]http://www.brainspan.org

ASD: one for genes down-regulated in ASD patients, showing functional enrichment for some GO terms like synaptic function, vesicular transport and neuronal projection; the other containing up-regulated genes with an enrichment of the immune and inflammatory GO categories. The integration of genetics data with co-expression modules has shown that the former may identify potential causes of ASD, while the latter suggests the biological response (Voineagu et al., 2011). Subsequently, a RNA-seq study was performed on a larger ASD cohort, demonstrating similar results. Therefore, altered neural activity and an enhanced microglial response was proposed in ASD brains, highlighting the role of the immune system and synapses in ASD (Gupta et al., 2014). However, the largest cohort of brain samples analyzed to date identified 24 co-expression modules after WGCNA analysis with RNA-seq data. Six modules were associated with ASD, three down-regulated and three up-regulated. Synaptic and neuronal genes were found among the down-regulated modules, while glial function and biological pathways related to inflammatory processes were enriched in the up-regulated modules. Moreover, one of the 24 modules was enriched in DNMs previously associated with ID, while another module was enriched for lncRNAs (Parikshak et al., 2016).

Co-expression networks constructed from publicly available datasets have revealed how ASD genes are differentially expressed during early, mid and late fetal development, indicating that they are directly involved in the development of the prefrontal, temporal, and cerebellar cortex (Willsey et al., 2013; Chang et al., 2014; Krishnan et al., 2016). In particular, strongly associated ASD genes converge in glutamatergic projection neurons located in layers 5 and 6 of human mid-fetal prefrontal and primary motor somatosensory cortex (Willsey et al., 2013). A WGCNA analysis employing an enrichment strategy produced a list of genes from SFARI that mapped into different expression modules (Parikshak et al., 2013). This allowed these genes to be traced to specific neurodevelopmental stages and neuronal cell types. Therefore, the integration of expression data allows ASD risk genes carrying DNMs (and/or other genetic variants) to be correlated with a superior hierarchical level of biological information, expanding our understanding of ASD pathogenesis. Through such studies at the circuit level, ASD genes have been seen to be enriched in glutamatergic neurons in upper cortical layers. It is worth noting that this result is different from the findings obtained in the previous study in which ASD genes converged in layer 5/6 cortical projection neurons. Therefore, these genes converged in modules associated with biological functions like early synaptic development and transcriptional regulation. Interestingly, both modules were enriched in targets of the FMRP gene, indicating that translational regulation could be a link between molecular pathways that are co-expressed during fetal cortical development (Parikshak et al., 2013). Alternatively, a spatial analysis revealed that the activity of ASD genes is widely distributed throughout the brain, which is consistent with the broad spectrum of symptoms associated with ASD. However, some specific areas were apparently more strongly linked to ASD, such as the cerebellum, striatum, amygdala, and thalamus (Chang et al., 2014; Krishnan et al., 2016).

A recent study using co-expression networks and enrichment approaches allowed different types of DNMs to be studied (Shohat et al., 2017). Moreover, different patterns of expression were described in the brain for genes associated with different neuropsychiatric disorders. Enrichment analysis of protein coding genes mapped to those previously described WGCNA modules (Parikshak et al., 2013) in different brain areas and at distinct neurodevelopmental stages. In addition to ASD genes, genes carrying mutations associated with schizophrenia and ID were also tested. Accordingly, genes carrying LoF DNMs in ASD and ID were found to be preferentially expressed in the fetal brain (cortex) and they were related to chromatin organization. However, genes carrying missense DNMs were associated with schizophrenia and they were active in the young adult cortex during adolescence (Parikshak et al., 2013). Therefore, these approaches appear to be able differentiate distinct biological pathways that are associated with ASD, schizophrenia and ID (Shohat et al., 2017).

PATERNAL AGE AND DNMs

A relationship between advanced paternal age and increased ASD risk has been established in different studies (de Kluiver et al., 2016; Janecka et al., 2017). Multiple biological mechanisms can explain this relationship, not only DNMs but also epigenetic changes associated with aging (Atsem et al., 2016). DNMs are typically present in the sperm or egg of one parent and they are then transmitted to the embryo. Thus, these mutations are present in all cells within the offspring. Interestingly, WES data enables the paternal or maternal origin of DNMs to be determined, identifying which parental haplotype carries the same mutation as that found in the proband. Interestingly, it was noted that most of DNMs originate in the father (Iossifov et al., 2012; O'Roak et al., 2012), which may perhaps not be surprising given the ratio in the number of spermatozoa to eggs produced. In addition, the number of DNMs is positively correlated with paternal age and it has been calculated that each additional year of paternal age at the moment of conception results in two extra DNMs in the proband. Conversely, the number of mutations transmitted maternally remains relatively constant over the years (Kong et al., 2012). The number of cell divisions that male germ cells continuously suffer could possibly explain these findings, while female eggs do not actively divide during the female's reproductive years (Crow, 2000). Together, these results are consistent with a hypothesis in which a higher paternal age entails an increased ASD risk in probands due to the higher rate of mutations.

Nevertheless, although the biological hypothesis plausibly explains the relationship between paternal age and ASD risk, it is unlikely to reveal more than a modest genetic risk fraction (10–20%; Gratten et al., 2016). Therefore, there are additional mechanisms to be considered, especially taking into account that offspring of younger parents are also at risk of some mental disorders (McGrath et al., 2014). One alternative hypothesis suggests that delayed fatherhood is correlated with a tendency toward neuropsychiatric illnesses. Therefore, genetic risk factors

for psychiatric disorders that are highly heritable may be shared by older fathers and their offspring (Gratten et al., 2016). Both hypotheses are not mutually exclusive and they reflect how the relationship between risk and paternal age is probably due to a complex interrelated matrix of epidemiological and genetic factors.

POST-ZYGOTIC MUTATIONS (PZMs) AND MOSAICISM IN ASD

PZMs are another type of DNMs that are beginning to generate much interest in ASD genetic studies. PZMs occur during the mitotic cell divisions that generate the embryo after fertilization and as a result, a mosaic individual is created in which a variable number of cells carry the mutation (**Figure 2**; Biesecker and Spinner, 2013). As such, the developmental timing and cell lineages affected will probably determine the severity of the symptoms in these disorders. PZMs are implicated in several brain disorders, including epilepsy, cortical malformations, or RASopathies (Kurek et al., 2012; Lee et al., 2012; Poduri et al., 2013; Jamuar et al., 2014). Indeed, it was shown that some PZMs carried by the X-Linked methyl CpG binding protein 2 (*MECP2*) gene cause Rett's Syndrome. Rett's syndrome is usually lethal in males and dominant in females but in some cases, mosaic mutations have been reported that are compatible with male viability (Pieras et al., 2012).

The detection of PZMs has been a challenge because they are tissue-specific and ASD brain tissue is almost never available. In order to solve this problem, sensitive genotyping techniques are necessary, such as SNP microarrays, NGS and WES studies. The success of these technologies relies on the ability to analyze a large number of cells at once, which helps to increase the probability of detecting mutations in a mosaic state. SNP arrays can detect mosaics when at least 5% of the cells of an individual are carrying the mutation (Conlin et al., 2010), while NGS can also detect mosaic mutations based on the fraction of unusual alleles calculated through the AAF. NGS provides deep sequencing coverage that allows for the observation of a sufficient number of reads with reference and alternate alleles to accurately calculate AAF. In this context, PZMs have been reported when the AAF $\leq$ 40%, shifting from the 50:50 ratio expected for heterozygous germline mutations. Therefore, the deep sequencing coverage of panels of candidate genes allow mutations to be detected that are present in at least 5% of the reads, meaning that 10% of the cells in the individual carry the variant (Jamuar et al., 2014). WES is also sensitive enough to detect PZMs when the AAF is at least 15%, which means that mutations are present in about 25–30% of the cells (Pagnamenta et al., 2012; Genovese et al., 2014).

Despite the potential role of PZMs in the etiology of ASD, the common variant calling pipelines employed in WES lose this valuable source of information due to the application of strict filters to avoid artifacts. Reanalysis of the SSC using novel calling approaches to specifically characterize SNVs that are likely to be PZMs led to a higher proportion of mosaic SNVs (22%) than those reported previously (Krupp et al., 2017). Elsewhere, when WES data was recalled from the same cohort, about 80% of the PZMs detected had not been published before (Lim et al., 2017). Indeed, those variants were validated using three different techniques, proving that PZMs can be better detected by modifying the current pipelines (**Table 3**). In addition, these studies identified PZMs in high-confidence NDD risk genes, such as *SCN2A*, *CTNNB1*, *SYNGAP1*, and *HNRNPU*, evidence that at least a proportion of PZMs predispose to ASD. Moreover, new candidate genes were significantly enriched in PZMs, such as *KLF16* and *MSANTD2* (**Figure 2**).

Detailed analysis of these variants, especially the truncating mutations, revealed novel and uncharacterized pathways and cellular processes that may possibly be involved in ASD pathogenesis (Lim et al., 2017). Surprisingly, an increased burden of synonymous PZMs in probands has been reported, with synonymous mutations enriched in splice sites, indicating that splicing regulation could contribute to ASD pathogenesis. Moreover, around 2.3% of ASD simplex cases harbor a synonymous PZM related to ASD risk. However, missense and LoF PZMs were also associated with ASD, most of them affecting genes expressed in the brain and other high confidence ASD risk genes. Thus, it was estimated that PZMs contribute about 4% to the overall architecture of ASD (Krupp et al., 2017; Lim et al., 2017). The spatiotemporal distribution of these mutations has also been reported, pointing to the amygdala as a brain area of interest that merits further attention in terms of ASD pathogenesis.

In conclusion, preliminary studies have produced strong evidence of the importance of considering PZMs in ASD genetic studies. Therefore, it is necessary to elucidate how PZMs contribute to ASD (and other NDDs), determining the genetic risk that could be explained by them. Thus, different analytical approaches and study designs need to be developed, involving larger cohorts than those analyzed previously and developing improved variant detection pipelines for PZMs.

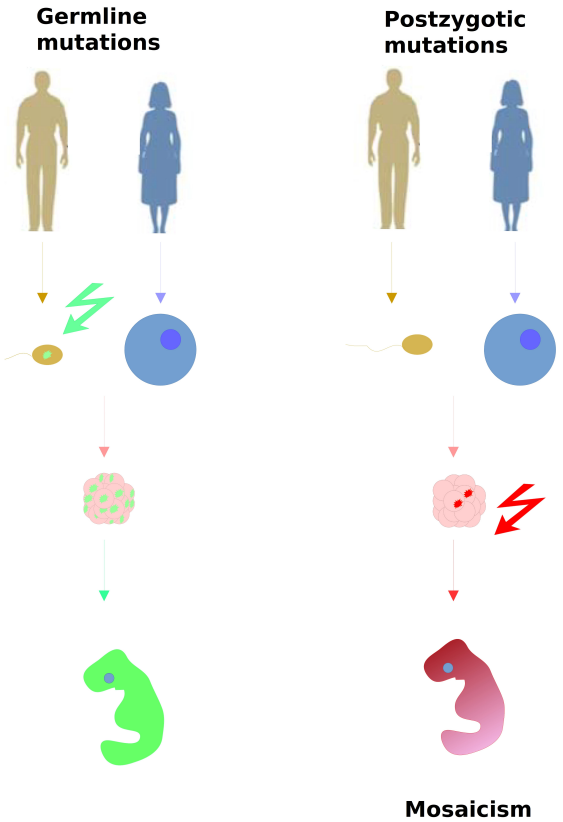
CAVEATS AND FUTURE PERSPECTIVES IN THE STUDY OF DNMs AND ASD GENETICS

Despite the important advances made in the study of ASD genetics over recent years, some caveats still exist regarding the detection of DNMs, which will hopefully be resolved by future studies. The study of PZMs carried out by the ASC

TABLE 3 | Results of the two main studies analyzing PZMs in ASD cohorts.

Study	Krupp et al., 2017	Lim et al., 2017
Number of families analyzed	2264	5947
% Of PZMs detected applying new bioinformatics pipelines	22%	9.7%
% Of mutations not previously published	70.64%	83.3%

Both of them reanalyzed previously published data but applying different bioinformatics pipelines in order to detect PZMs involved in ASD.



Reported genes carrying PZM in ASD

Study	Genes
Lim et al., 2017	*KLF16,MSANTD2,POLA2,SMARCA4, AZGP1,CNGB3,HNRNPU,SCN2A, EPPK1,CARD11*
Krupp et al., 2017	*CHD2,CTNBB1,SCN2A,SYNGAP1, ACTL6B,BAZ2B,COL5A3,SSRP1, UNC79*
Freed and Pevsner, 2016	*KMT2C,NCKAP1,MYH10*

FIGURE 2 | Post-zygotic mutation (PZMs) are acquired after the zygote forms, as opposed to germline mutations that are inherited from the parents. Therefore, PZMs are not present in every cell of the organism, which is therefore a mosaic individual. It was recently demonstrated that PZMs contribute significantly to ASD risk. The most relevant studies focusing on the detection of PZMs are represented along with the genes seen to carry different PZMs.

has helped establish an emergent type of genetic variation that had been dismissed until now (Lim et al., 2017). Subsequently, other studies have focused on this interesting and informative type of DNM (Krupp et al., 2017), although the filtering and variant calling processes used in these studies are quite different, highlighting the need for a single, optimized and unified pipeline. This is without doubt one of the future areas that will benefit from further research. In relation to this, a proportion of *de novo* CNVs are also expected to be postzygotic, yet the repercussion of this type of post-zygotic structural variation in ASD genetic architecture has still to be studied in detail. This will require the implementation of suitable and valid bioinformatics pipelines. Likewise, huge public repositories should be reanalyzed following different pipelines in order to detect PZMs that may have been missed until now, for example the SSC that currently contains 8975 whole genomes. Such efforts will help to highlight new genetic factors involved in ASD pathogenesis.

Another relevant area of study involves the proportion of DNMs in children that are parental mosaic mutations, asymptomatic in the parents yet transmitted to the offspring. The existence of this biological phenomenon was well documented in other genetic diseases and in fact, a genetic test to detect parental mosaicism is included in some routine diagnostic tests (Campbell et al., 2014; Frederiksen et al., 2015). In terms of ASD genetics, the overall incidence of parental somatic mosaicism reported to date is extremely low (6.8% of all DNMs), yet not inexistent (Dou et al., 2017; Krupp et al., 2017). Therefore, future studies on the largest possible number of families, employing different variant detection methods, will be decisive to elucidate the exact role of parental mosaic DNMs in ASD. The identification of genes carrying PZMs and the development of a genetic diagnosis through a simple blood test in parents will also require further research.

There is another type of genetic variation that will require the development of new detection methods for indels (De Rubeis et al., 2014; Brandler et al., 2016). *De novo* indels were previously associated with ASD (*KMT2E* and *RIMS1*) but the systematic analysis of disrupting indels will require the development of robust and more accurate methods (Dong et al., 2014). Therefore, it was demonstrated that the detection of indels could be enhanced by using new algorithms that allow the assembly of DNA sequences to be redefined in order to detect them more accurately. Indeed, through the analysis of samples from the SSC it was demonstrated that disrupting *de novo* indels plays a major role in ASD genetics (Narzisi et al., 2014).

De novo mutations in non-coding regions have become of interest in recent years. Previous WES studies were unable to detect these variants due to the lack of coverage and

sequencing depth across non-coding regions (promoter and regulatory regions). However, there is evidence that ASD genes harbor hotspots of hypermutability in non-coding regions and besides, deleterious mutations across them are subjected to strong negative selection just like the LoF mutations located in the coding region (Michaelson et al., 2012; Warr et al., 2015). Studying non-coding regions demonstrated that promoter regions with *in vivo* enhancer activity in the central nervous system are enriched in DNMs (Turner et al., 2017). The important role of DNMs in NDDs was also demonstrated by targeted sequencing of some selected types of promoter regions, showing that around 1–3% of patients with no genetic diagnosis carry pathogenic DNMs in some of these regions (Short et al., 2018). Another recent study reported rare SVs located in *cis*-regulatory elements of intolerant genes and their inheritance from parents may contribute to ASD in about 0.77% of cases (Brandler et al., 2018). Moreover, when the role of *de novo* SVs (∼5.1%) was assessed, the importance of these variants for future studies was evident. Recently, novel analytic pipelines were developed to integrate DNM information from non-coding and coding regions to characterize the broad spectrum of ASD genetic variability, with non-coding *de novo* indels giving more significant results than those expected by chance (Werling et al., 2018).

These data highlight the current need to perform ASD genetic studies using WGS instead of traditional exome studies. As such, the effort of the SSC in bringing together almost 8975 whole genomes for genetic analysis, including fathers, mothers, affected and unaffected siblings, is noteworthy (Ku et al., 2012; Lelieveld et al., 2015).

Regarding the integration of DNM information into higher biological hierarchies using gene and protein networks, it is also expected that new bioinformatics approaches will shortly allow the implementation of integrative analysis frameworks adapted to ASD biology. These integrative analyses will not only take into account high-throughput data from gene expression and PPI networks but also epigenetic data, information on microRNA regulation, splicing events and even quantitative trait loci when gene information from SNPs is considered together with DNM data. This huge amount of biological information will help define a more detailed and valid map of the neurobiological pathways involved in ASD.

CONCLUSION

Studies into ASD genetics and specifically, DNMs have come a long way in the last few years. However, there are still some gaps to be filled that will require further analysis and the development of novel bioinformatics approaches to tackle them in sufficient detail. The ultimate goal will be to obtain the most complete and detailed biological map of ASD described to date, a map integrating genetic information with other complementary omics data, in order to unravel the complex gene networks and cellular pathways involved in ASD.

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AA-G and CR-F wrote the paper. AC critically revised the work and approved the final content. AA-G, CR-F, and AC participated in the design and coordination of the review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00406/full#supplementary-material

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frontiers in Molecular Neuroscience

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Multiple Critical Periods for Rapamycin Treatment to Correct Structural Defects in *Tsc-1*-Suppressed Brain

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Tuberous sclerosis complex (TSC) is an autosomal dominant neurogenetic disorder affecting the brain and other vital organs. Neurological symptoms include epilepsy, intellectual disability, and autism. TSC is caused by a loss-of-function mutation in the *TSC1* or *TSC2* gene. These gene products form a protein complex and normally suppress mammalian target of rapamycin (mTOR) activity. mTOR inhibitors have been used to treat subependymal glioma (SEGA) that is a brain tumor characteristic of TSC. However, neuropathology of TSC also involves dysregulated cortical circuit formation including neuronal migration, axodendritic differentiation, and synapse formation. It is currently unknown to what extent mTOR signaling inhibitors correct an alteration in neuronal morphology that have already formed prior to the treatment. Here, we address the efficacy of rapamycin treatment on neuronal migration and dendrite formation. Using *in utero* electroporation, we suppressed *Tsc1* expression in a fraction of neuronal progenitor cells during the fetal period. In embryonic brain slices, we found that more *Tsc1*-suppressed cells remained within the periventricular zone, and rapamycin treatment facilitated neuronal migration. Postnatally, *Tsc1*-suppressed pyramidal neurons showed more complex branching of basal dendrites and a higher spine density at postnatal day (P) 28. Aberrant arborization was normalized by rapamycin administration every other day between P1 and P13 but not P15 and P27. In contrast, abnormal spine maturation improved by rapamycin treatment between P15 and P27 but not P1 and P13. Our results indicate that there are multiple critical windows for correcting different aspects of structural abnormalities in TSC, and the responses depend on the stage of neuronal circuit formation. These data warrant a search for an additional therapeutic target to treat neurological symptoms of TSC.

Keywords: tuberous sclerosis complex, neuronal migration, synapse formation, critical period, rapamycin

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder that involves multiple organs including brain, kidney, lung, and heart (Crino et al., 2006). Neurological symptoms of TSC include epilepsy, intellectual disabilities, and autistic behaviors. TSC is caused by loss-of-function mutations in either *TSC1* or *TSC2* (Kandt et al., 1992; European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997). The TSC-1/TSC-2 protein complex (Plank et al., 1998; van Slegtenhorst et al., 1998) negatively regulates the mammalian target of rapamycin (mTOR) pathway, which is triggered by growth factors as well as nutrients and regulates protein synthesis, autophagy, transcription cell growth, cell proliferation, cell motility (Hay and Sonenberg, 2004; Sarbassov et al., 2005).

Neuropathological features of TSC include cortical tubers, subependymal nodules, glioradial fibers, subependymal giant astrocytoma (SEGA). Cortical tubers are hamartomatous tissues and are thought to be a migrational defect of neuronal progenitors (Crino, 2004; Marcotte and Crino, 2006). Neuronal migration has been studied in mouse models of TSC, such as two *Nestin*-promoter driven conditional *Tsc1* knockout mice targeting pyramidal cells, interneurons and glial cells. These models successfully recapitulated pathological features such as subependymal nodule-like lesion (Zhou et al., 2011) or cortical tuber giant cells (Goto et al., 2011).

A recent postmortem study in humans examined non-tuber cortical areas and identified "dyslamination" characterized by an altered radial orientation of pyramidal cells, blurring of laminar boundaries, and disruption of cortical columnar architecture, isolated balloon cells and heterotopic neurons inside subcortical white matter (Marcotte et al., 2012). Indeed, *Emx1-Cre* x $Tsc1^{loxp/loxp}$ mice, which show *Tsc1*-deletion in forebrain pyramidal neurons starting from an early embryonic age, appear to lose cortical lamination without tubers or other obvious focal lesions (Magri et al., 2011; Carson et al., 2012). These findings suggest that TSC brains have diffuse and more subtle abnormalities outside of tubers than previously thought. Furthermore, there are also pathological findings that involve postmitotic neurons or precursor cells at the microscopic level. Specifically, animal models of TSC also showed abnormal axonal growth (Choi et al., 2008; Nie et al., 2010), and dendritic spine pruning (Tang et al., 2014). The mTOR pathway also plays critical roles in synaptic function (Hoeffer and Klann, 2010; Yoshii and Constantine-Paton, 2010). For example, *Tsc2* heterozygous mutant mice have impaired late long-term potentiation (L-LTP) and long-term memory (Ehninger et al., 2008). *Tsc1*-suppressed neurons have impaired α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) currents (Tavazoie et al., 2005) and long-term depression (LTD) (Bateup et al., 2011). These microscopic structural changes and functional alterations underlie neurological disabilities in TSC.

Mutations in *TSC* genes result in overactivation of mTOR. Therefore, mTOR suppression by rapamycin or its derivatives corrects TSC pathophysiology and other mTOR-related disorders (Lipton and Sahin, 2014). For example, everolimus, a rapamycin derivative, reduced the size of SEGA and improved seizure control (Krueger et al., 2010; French et al., 2016). Neuronal circuit formation is a sequence of distinct developmental processes which include neurogenesis axonal growth, dendritogenesis, and synaptogenesis. In the rodent cortex, neurogenesis starts around E11 and ends around E17 (Takahashi et al., 1996; Caviness et al., 2009; Greig et al., 2013), and dendritic arborization occur in the first 2 weeks (Cline, 2001; Wong and Ghosh, 2002). Spine formation and pruning are maximal during the critical period, which starts P16 peaks at P28 and decline from P33 (Hensch, 2005). It is likely that the response to rapamycin is maximal while the abnormal morphology is formed. However, it remains unclear whether each of these cellular processes has a sensitive period to respond to the mTOR inhibitor treatment.

Here, we address the efficacy of rapamycin treatment on neuronal migration, the formation of dendrites and spines. Using *in utero* electroporation, we transferred a DNA construct encoding Cre recombinase tagged with green fluorescent protein (Cre-GFP) into E 15.5 neuronal progenitor cells in a $Tsc1^{fl/fl}$ mouse fetal brain and suppressed the gene expression in a group of cells that are born around the same time. In embryonic brain slices, we found that more *Tsc1*-suppressed cells remained within the periventricular zone and that rapamycin treatment facilitated neuronal migration. Postnatally, the lamination pattern of *Tsc1*-suppressed neurons was widened and scattered more than WT cells. Further, *Tsc1*-suppressed pyramidal neurons showed more complex branching of basal dendrites, which was normalized by rapamycin administrations between postnatal day (P) 1 and P13 but not between P15 and P27. In contrast, abnormal spine maturation improved with rapamycin between P15 and P27 but not between P1 and P13. These results suggest that there is a critical time window during neuronal circuit formation to correct abnormal neuronal morphology in TSC.

MATERIALS AND METHODS

Animal

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of MIT, UIC, and NIH guidelines on the humane care of animals. The protocol was approved by the MIT- and UIC-IACUC. All animal manipulations were approved by the MIT- and UIC-IACUC and were performed in accord with its guidelines. $Tsc1^{loxp/loxp}$ mice (Jackson Laboratory, #005680) were kept under 12 h light/dark cycle. Rapamycin was injected every other day (6 mg/kg/dose) (Meikle et al., 2008).

In utero Electroporation

Timed pregnant mothers were anesthetized with 2–3% isoflurane and oxygen. Following laparotomy, the uterus was externalized and the lateral ventricle of E15.5 embryos was injected with 1–2 μg Cre-GFP alone or in combination with 0.1–0.2 μg DiO-YFP. Using an ECM 830 apparatus (Harvard Apparatus, Holliston MA), brains were electroporated with five 30 V, 50-ms pulses at intervals of 950 ms. After recovery, pregnancies continued, and pups were delivered normally.

Organotypic Slice Cultures

Mouse embryos were electroporated at embryonic day 15 (E15), and acute coronal brain slices (240 μm) were prepared at E17 and E18. Occipital slices were transferred onto slice culture inserts (Millicell) in cell culture dishes (35 × 10 mm; Corning) with Neurobasal medium (Invitrogen) containing the following: B27 (1%), glutamine (1%), penicillin/streptomycin (1%), horse serum (5%), and N2 (1%). Slices were used for imaging (1–2 h after slicing) or for pharmacological treatments (incubated at 37°C in 5% CO_2, for 1 day). A subset of slices was incubated with the medium containing rapamycin (100 μM).

Time-Lapse Imaging

Cre-GFP- and mCherry-positive cells were imaged on an inverted Nikon microscope (TE 2000-S) with a 20× objective lens [numerical aperture (NA) 0.45]. mCherry was added to ensure fluorescent signal detection during serial imaging. During the time-lapse imaging, slices were kept in an acrylic chamber at 37°C in 5% CO_2. We captured time-lapse images with a Cool SNAP EZ camera (Roper Scientific) using NIS-Elements software (Nikon).

ANALYSIS OF NEURONAL MIGRATION VELOCITY

The neuronal migration velocity was measured using a plugin for ImageJ (Mouse Tracker, programmed by P. Malatesta, IST Genova) that allows tracking the cell position over time. Using the coordinates obtained with ImageJ, the velocity was calculated with Excel (Microsoft) (de Anda et al., 2010).

Immunohistochemistry and Confocal Microscopy

Following transcardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (pH7.4), brains are post-fixed, trimmed, embedded in 2% low temperature-melt agarose with PBS and 7% sucrose, and sectioned on a vibratome in the coronal plane at 50 μm for immunohistochemistry and100 μm for Sholl and spine analyses. A cryostat was used for thinner sections of fetal brains. Sections are permeabilized in PBS/4% donkey serum/1% Triton X100 at room temperature 10 min. After rinsing in PBS for 15 min three times, sections are reacted overnight with primary antibodies in PBS/4% donkey serum/0.5%Ttriton X100 at room temp. The following primary antibodies were used; TSC1 (Cell Signaling Technology, #4906); phosphorylated S6 (Cell Signaling Technology, #4858); and Brn2 (Cell Signaling Technology, #12137). After rinsing with PBS (15 min, three times), sections were incubated in an Alexa 568-conjugated secondary antibody overnight at room temp and finally rinsed in PBS (15 min, three times). Images were captured using a 40 x objective lens under identical settings with a Nikon PCM 2000 confocal microscope. The visual cortex was identified on coronal sections using the Paxinos atlas. The *Z*-series of optical sections taken at intervals of 0.5 μm were reconstructed using the same setting. ImageJ was used to measure pixel intensity of the immunolabels in the cytosol. The regions of interest (ROIs) were selected using freehand line tool, then signals in neclei were subtracted. An example of the cytosol is selected with a white box in **Figure 1C** and magnified at the bottom. Averaged pixel intensity was calculated by dividing total pixel intensities with the pixel area of cytosol. Similarly, averaged intensity of Cre-GFP signal was measured. A cell was considered Cre-GFP positive when its averaged Cre-GFP intensity in the nucleus was above 100. Immunolabels of TSC1 and phosphorylated S6 were compared between neurons with and without Cre-GFP.

To measure mTOR activity, immunostaining was performed using an antibody to phosphorylated serine 240/244 of the S6 ribosomal pro- tein (pS6). Serial Z-stacks were acquired under the same settings for the ipsilateral and contralateral hemispheres of coronal sections. Because layer 2/3 cortical neurons project to the contralateral hemisphere, $mRFP^+$ axons were followed to measure pS6 levels in cells of the non-electroporated region. ROIs were generated using an elliptical selection tool, and average intensi- ties for each ROI were determined. The ROI did not include the nucleus that had low pS6 staining. For both cell size and pS6 staining measurement, 3 sections per mouse and 38–111 cells per section were analyzed.

Morphological Analysis

Neurons with a strong signal whose cell body was entirely contained within the slice were selected for morphological analyses in the brains of P28 animals. Z-stacks were stitched together in the XY plane such that the full span of the neurons projections were included in the composite image. Neurolucida 8 software (MBF Bioscience) was used for quantitative analyses of neuronal morphology, including Sholl analysis of apical and basal dendrites, and largest cell body area (Yoshii et al., 2011). Neurons were blindly traced and analyzed based on spherical shells concentric with the somal centroid and spaced at a 5μm interval. The number of intersections and nodes were counted from the soma to the distal end of dendritic branches. For spine density, basal dendritic branches within the interval between 15–40 μm and number of spines were counted to calculate the spine density (number of spines per μm).

Statistics

For immunohistochemical analysis, Wilcoxon Rank sum test was used. For neuronal migration and neuronal morphological analyses, a Student's *t*-test was used for comparison of two groups. One-way ANOVA with *post-hoc* Tukey tests was used for comparisons of more than two groups. $P < 0.05$ were considered significant and indicated as * in graphs. $P < 0.01$ and $p < 0.001$ are indicated as ** and ***. Numeric data are presented as average ± s.d. in the Results section. Error bars in **Figures 1–6** represent s.e.m. In other figures, sidebars represent the range of data set.

RESULTS

To examine the migration of *Tsc1*-suppressed neuronal progenitor cells, we combined a Cre-lox recombination system and *in utero* electroporation. Specifically, we used a $Tsc1^{fl/fl}$ mouse which has exon 17 and 18 flanked with Lox sequences

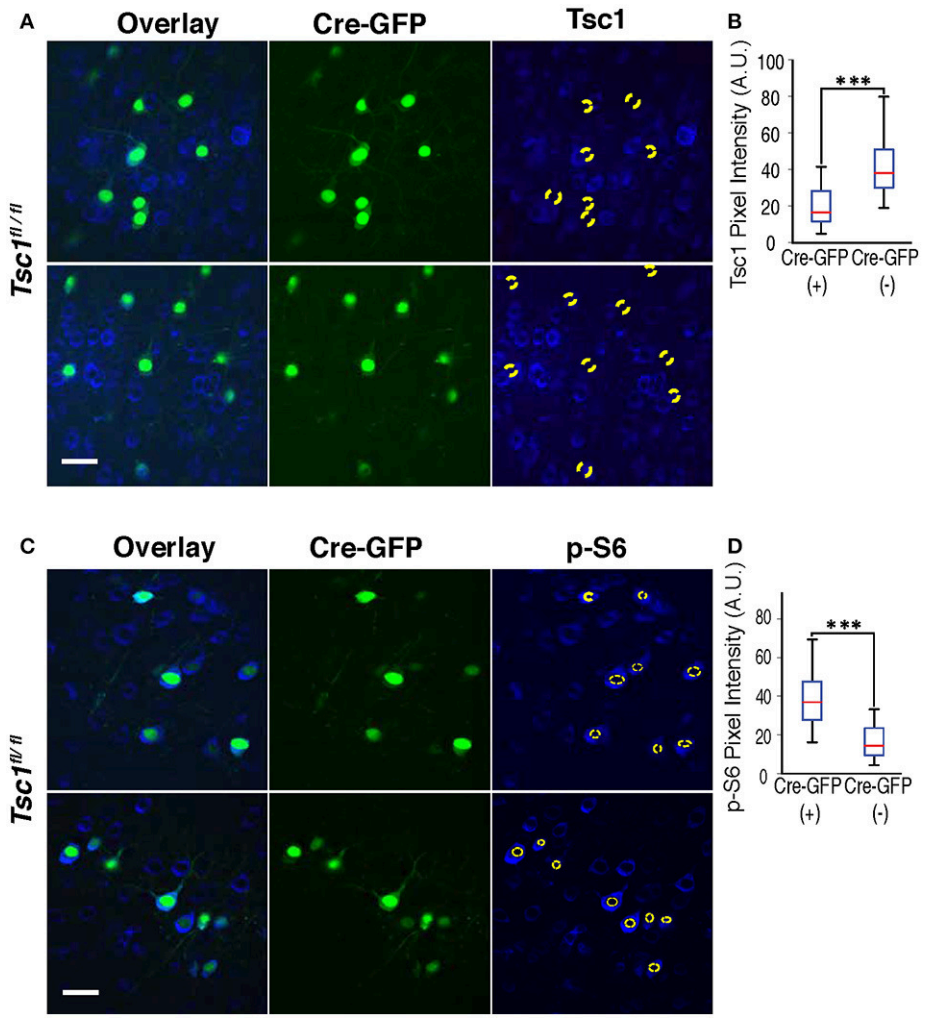

FIGURE 1 | Immunohistochemistry of Tsc1 and p-S6. **(A,B)** In *Tsc1*$^{fl/fl}$ mouse brains, neurons expressing Cre-GFP (indicated with a dotted circle) shows suppression of Tsc1 protein as compared with neurons without Cre-GFP. **(C,D)** Immunohistochemistry of p-S6 protein shows increases in neurons expressing Cre-GFP (labeled with a dotted circle), indicating overactivation of mTOR signaling. A magnification of the neuron encircled by a white box is shown at the bottom and depicts the cytosol (a space between two yellow dashed lines), which is defined as ROI to measure immunolabels. In **(A,C)**, scale bars indicate 50 μm. In **(B,D)**, ***$P < 0.001$, and sidebars show the range of data set.

and electroporated a DNA construct encoding Cre-GFP into wild-type (WT) or mutant fetuses at embryonic day 15.5 (E 15.5).

We performed Immunohistochemistry in both WT and mutant brains at P28 and confirmed that Cre-GFP expressing neurons in the mutant showed suppression of the TSC1 protein (**Figures 1A,B**) and increased phosphorylation of ribosomal protein S6 (p-S6) (**Figures 1C,D**), indicating an enhanced mTOR signaling as a result of suppressed TSC1 function.

Cortical Lamination Is Disorganized in TSC

Next, we examined the distribution of Cre-GFP positive neurons. In WT, Cre-GFP positive neurons were localized in cortical layer 2/3 when the DNA construct was electroporated in E 15.5. Remarkably, mutant brains showed a scattered distribution of Cre-GFP positive neurons (**Figure 2A**) as compared to normal layer 2/3 distribution in WT (**Figure 2B**). A minority of cells remained in deeper layers or the junction of layer 6 and the white matter (see arrowheads in **Figure 2A**) as previously reported (Feliciano et al., 2011). We further analyzed depth ratio by dividing the distance of each cell from the cortical surface with the cortical thickness. *Tsc-1*-suppressed cells showed significantly deeper distribution than WT (**Figure 2C**: $p < 0.001$, $N = 300$ cells each from three WT and five *Tsc1*$^{fl/fl}$ animals). Some *Tsc-1*-suppressed cells were localized in a deeper layer even though they express Brn2, a marker protein for layer 2/3 cortical neurons (**Figure 2A**, see cells indicated by arrows in the *Tsc1*$^{fl/fl}$ panel). This observation is consistent with

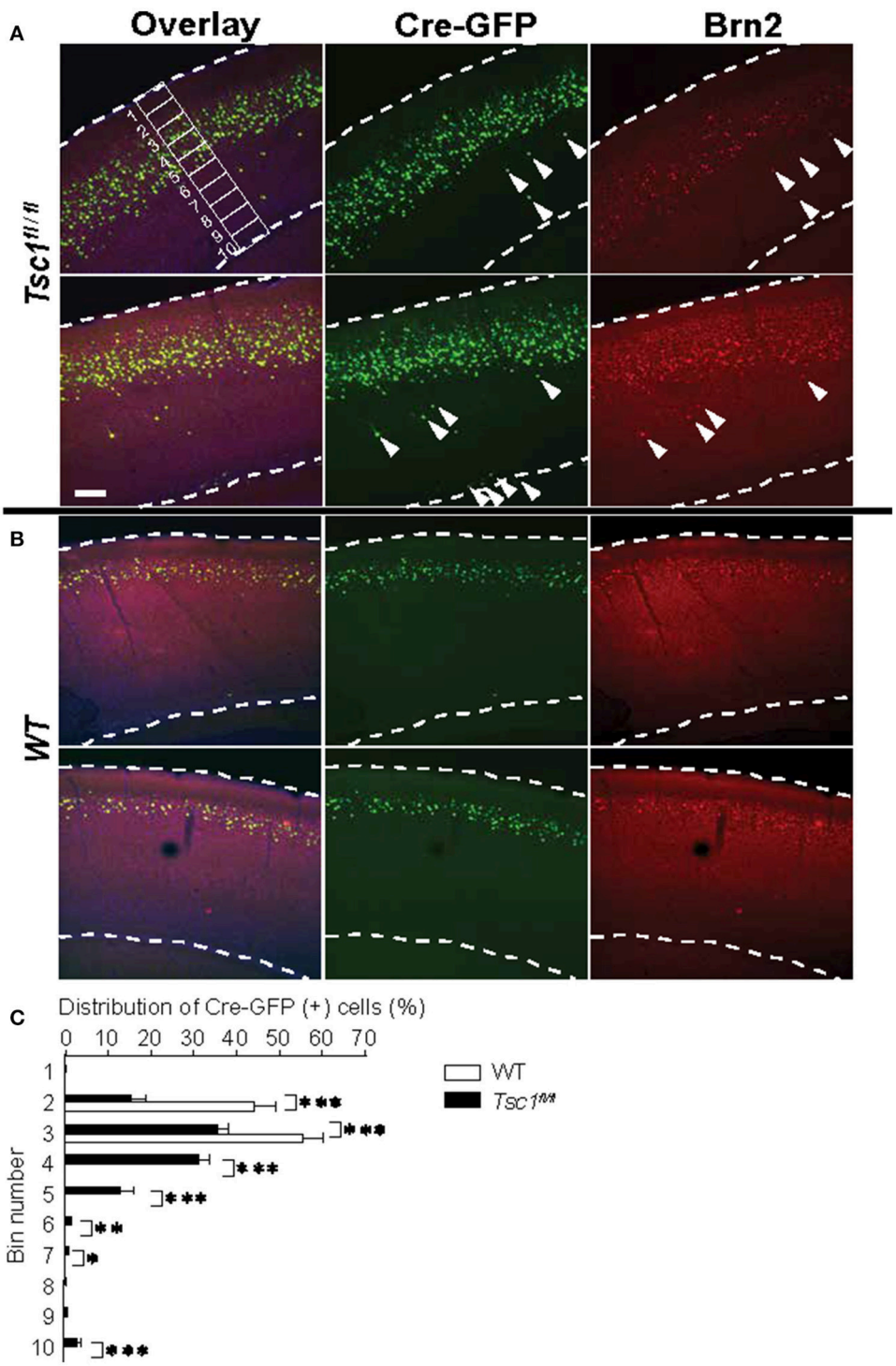

FIGURE 2 | *Tsc1*-suppressed neurons show a scattered distribution and some cells are mislocalized outside of layer 2/3. **(A)** In $Tsc1^{fl/fl}$ mouse brains, the distribution of Cre-GFP (+) neurons is scattered. Arrowheads indicate mislocalized cells and some of them are positive for the layer 2/3 marker Brn2. An example of 10 segments is shown. **(B)** In WT brains, the distribution of neurons that were electroporated with Cre-GFP at E15.5 is consistent with layer 2/3. **(C)** The graph shows averaged percentage of Cre-GFP (+) cell in 10 segments as shown in **(A)**. Cre-GFP (+) neurons in $Tsc1^{fl/fl}$ mouse brains is more widely distributed than those in WT. $^{*}P < 0.05$.$^{**}P < 0.01$.$^{***}P < 0.001$. In **(A,B)**, dashed lines indicate the superficial and ventricular surface of the visual cortex. Scale bar, 50 μm.

disorganized cortical layer formation that is observed in other *Tsc-1* knockout mouse models and postmortem brains of patients with TSC (Magri et al., 2011; Carson et al., 2012).

To further study the neuronal migration defect in the cortex of TSC, we performed live cell imaging of fetal cortical slices. We electroporated a DNA construct encoding Cre-GFP into the $Tsc1^{fl/fl}$ fetal cortices at embryonic day 15.5 (E 15.5). We made cortical slices at E17.5 and incubated them with medium with or without 100 μM rapamycin for 24 h. Then, we fixed the slices and imaged them using a confocal microscope (**Figures 3A,B**). We divided the cortical plate into 10 segments and quantified the distribution of migrating neurons (**Figure 3C**). In WT slices, Cre-GFP positive neurons migrated to the cortical plate at E18.5 *in vitro* (**Figures 3A,C**). Rapamycin treatment did not substantially affect migration of WT neurons. In contrast, ∼70% (see Bin 9 and 10 in **Figure 3C**) of *Tsc1*-suppressed neurons were still localized in the ventricular or intermediate zones at E18.5 (**Figures 3B,C**). Importantly, the mispositioning of neurons at E18.5 can be improved by rapamycin treatment although fewer *Tsc1*-suppressed cells migrated to the superficial region of the cortical plate than WT neurons (see Bin 1 and 3 in **Figure 3C**). We also performed live imaging of slices prepared from E17.5 to measure migration velocities. While the majority of *Tsc1*-suppressed neurons remained in the ventricular or intermediate zones, ∼20–30% of *Tsc1*-suppressed neurons were migrating. We measured velocity of the migrating neurons and found that averaged speed was comparable between WT and *Tsc1*-suppressed cells (**Figure 3D**, **Videos 1**, **2**). These results indicate that *Tsc1*-suppressed post-mitotic neurons remain in the ventricular zone/intermediate zone longer than WT neurons and that rapamycin normalizes the departure timing. However, once neurons leave the ventricular zone/intermediate zone, they migrate properly.

Previous studies show that prenatal rapamycin treatment improves the migrational defect in *Tsc-1* and *-2* conditional knockout mouse models (Anderl et al., 2011; Way et al., 2012). Consequently, we asked whether postnatal administration of rapamycin corrects disrupted mispositioning of layer 2/3 neurons. We injected rapamycin intraperitoneally every other day (6 mg /kg/dose) starting from P1 till P 27 and examined at P28. However, this treatment did not correct mispositioning of *Tsc1*- suppressed neurons, which remained broadly distributed (**Figure 4**). Collectively, these results suggest that the optimal

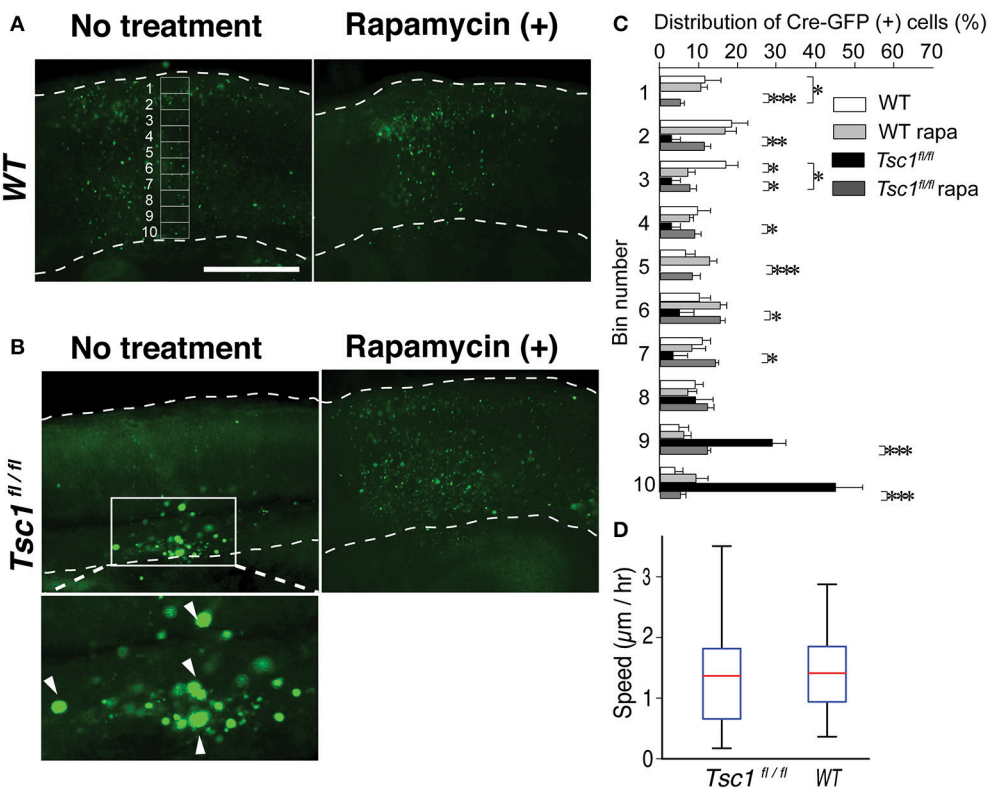

FIGURE 3 | Rapamycin treatment of the fetal brain slice corrected migration of *Tsc1*-suppressed neuronal progenitors. **(A)** Fetal cortical slices that were prepared at E18.5. An example of 10 segments is shown. Scale bar, 50 μm. **(B)** In $Tsc1^{fl/fl}$ slice, Cre-GFP (+) cells without rapamycin treatment appear to be balloon cells (arrowheads in the magnified image) and stay within the ventricular zone. **(C)** In WT slice with or without rapamycin treatment, rapamycin treatment resulted in subtle distribution changes. In $Tsc1^{fl/fl}$ slice treated with rapamycin (100 μM), Cre-GFP (+) cells migrate to the cortical plate and are similar to progenitor cells in WT (except for Bin 1 and 3). Approximately 70% of *Tsc1*-suppressed cells without rapamycin treatment is localized in Bin 9 and 10. **(D)** The velocity of migrating cells is comparable between $Tsc1^{fl/fl}$ and WT brains. In **(C)**, $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

timing to treat the dyslamination defect in the TSC1 cortex is prenatal when the majority of newborn neurons are migrating toward the cortical plate.

Neuronal Morphology of Postnatal Pyramidal Neurons

We examined the neuronal morphology of WT and *Tsc1*-suppressed neurons in the occipital cortex at P28. In agreement with previous studies, *Tsc1*-suppressed neurons showed larger soma size (389.74 ± 70.84 μm^2 in $Tsc1^{f/fl}$ and 194.91 ± 45.6 μm^2 in WT; $p < 0.01$ $n = 9$ from three brains in each genotype). Using Sholl Analysis, we examined both apical and basal dendrite of layer 2/3 pyramidal neurons at P28 (10 neurons from three brains in each condition). In apical dendrites, there were no significant differences in both numbers of intersections and nodes between the two genotypes except for one node at 100 μm, where the average number of intersections measured in the 100 μm shell is significantly different between the two conditions (**Figure 5A**). We were unable to analyze distal segments of apical (tuft) dendrites, which were often cut off in 100 μm sections. In comparison, the Sholl analysis of basal dendritic branches indicates that there were more intersections in the dendritic segments of $Tsc1^{fl/fl}$ neurons than in WT neurons between 15 to 40 μm from the somal centroid (**Figures 5B,C** and **Table 1**).

Next, we asked whether rapamycin corrects aberrant dendritic morphology of layer 2/3 pyramidal neurons. We administered rapamycin intraperitoneally every other day (6 mg /kg/dose) in two different duration: P1 to P13 and P15 to P27. Exuberant branching of proximal basal dendrites was normalized by rapamycin treatment between P1 to P13 but not between P15 and P27 (**Figure 5C**). Finally, we also measured spine density of proximal basal dendrites (10 cells from three brains in each

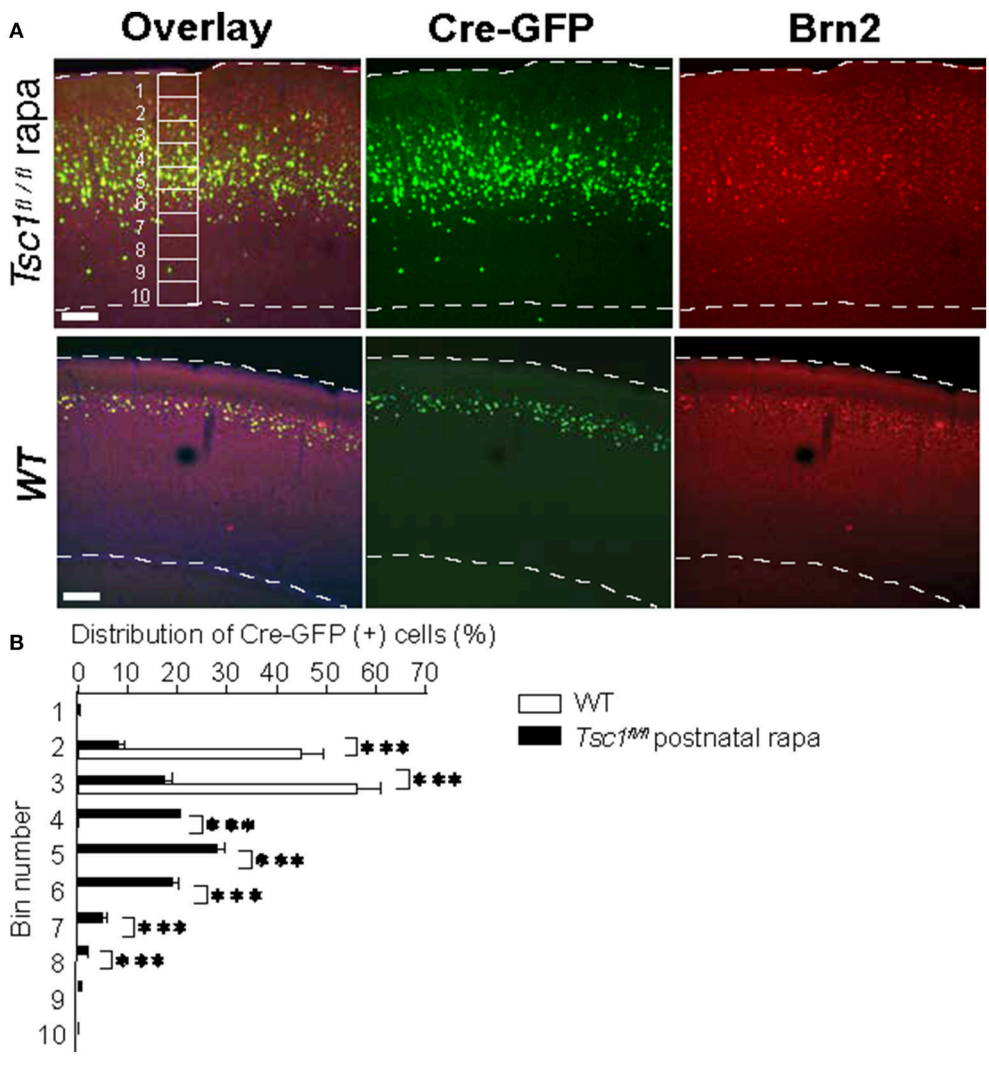

FIGURE 4 | Postnatal administration of rapamycin does not correct the aberrant migration pattern of *Tsc1*-suppressed neurons. **(A)** Representative images of the visual cortices collected from a $Tsc1^{fl/fl}$ mouse with postnatal rapamycin treatment and a WT animal. An example of 10 segments is shown. **(B)** In $Tsc1^{fl/fl}$ animals that treated with rapamycin postnatally, the distribution of neurons electroporated with Cre-GFP at 15.5 is wider than that in WT cortex. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

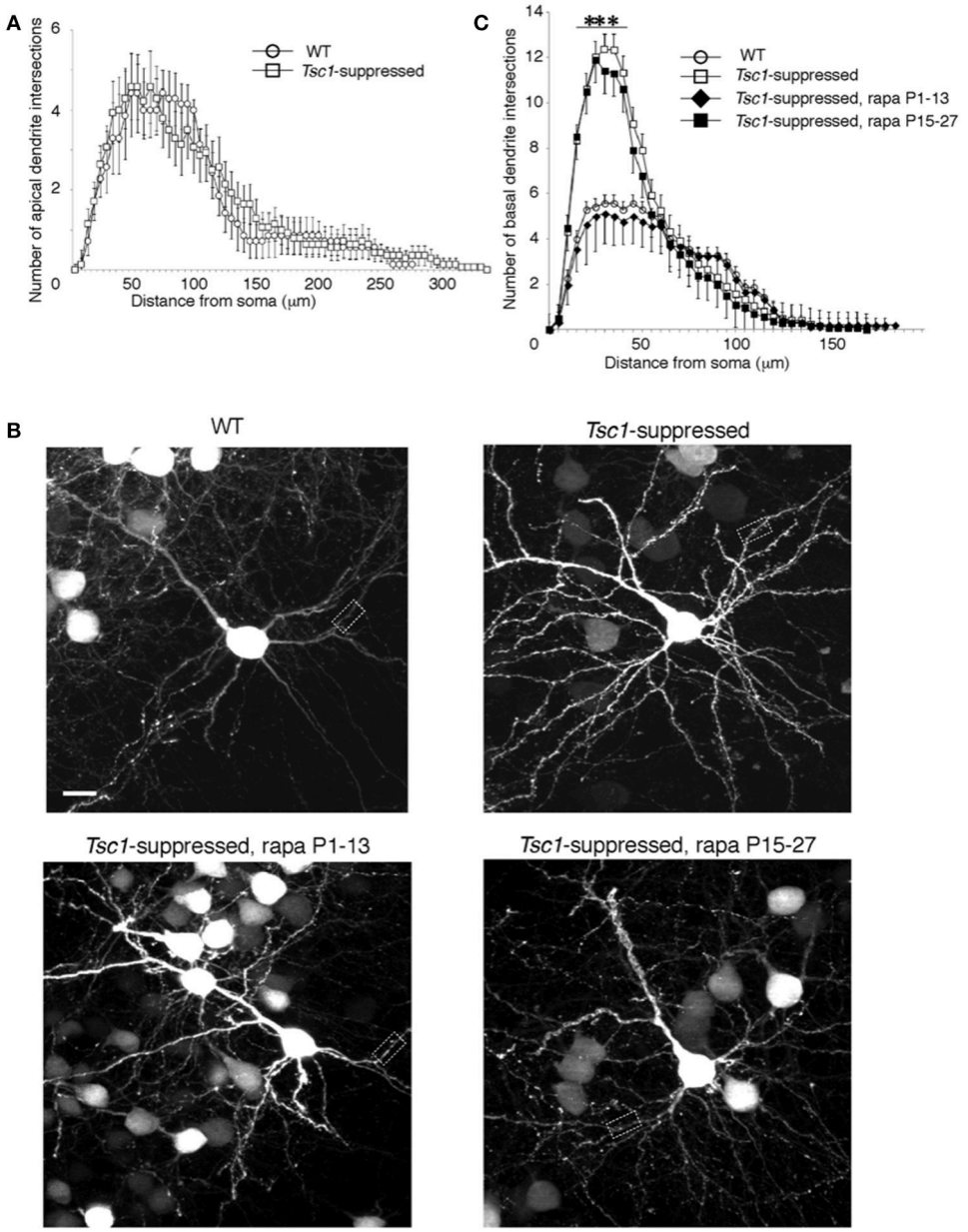

FIGURE 5 | Sholl analysis of electroporated pyramidal neurons. **(A)** There was no significant difference between *Tsc1*-suppressed and WT neurons in numbers of intersections throughout the apical dendrite. **(B)** WT and *Tsc1*-suppressed neurons with and without rapamycin treatment. Dashed boxes are magnified in **Figure 6A**. Scale bar, 10 μm. **(C)** Basal dendrites of *Tsc1*-suppressed neuron had higher intersection numbers than WT cells between 15 and 40 μm from the soma. In *Tsc1*-suppressed neurons, rapamycin treatment between P1 and P13 but not between P 15 and 27 reduced the basal dendrite arborization. ***$P < 0.001$ Error bars represent s.e.m.

group), which is defined as the number of dendritic spines per μm, and found that it was significantly higher in *Tsc1*-suppressed neurons than WT (**Figure 6A** and **Table 1**). The increase in spine density was normalized by rapamycin treatment between P15 to P27 but not between P1 and P13 (**Figures 6A,B**).

These results indicate that the rapamycin effect to normalize abnormal dendritic morphology depends on the timing of developmental maturation. Specifically, exuberant dendritic branching responds to rapamycin treatment within the first two postnatal weeks when dendritic arborization occurs, and aberrant dendritic spine maturation is treatable between P15-27 when spine formation and pruning peaks during the critical period in the visual cortex (Hensch, 2005; Stryker and Stryker, 2012).

DISCUSSION

Neurological symptoms of TSC include epilepsy, developmental delays, and autistic behavior. Classic neuropathological features

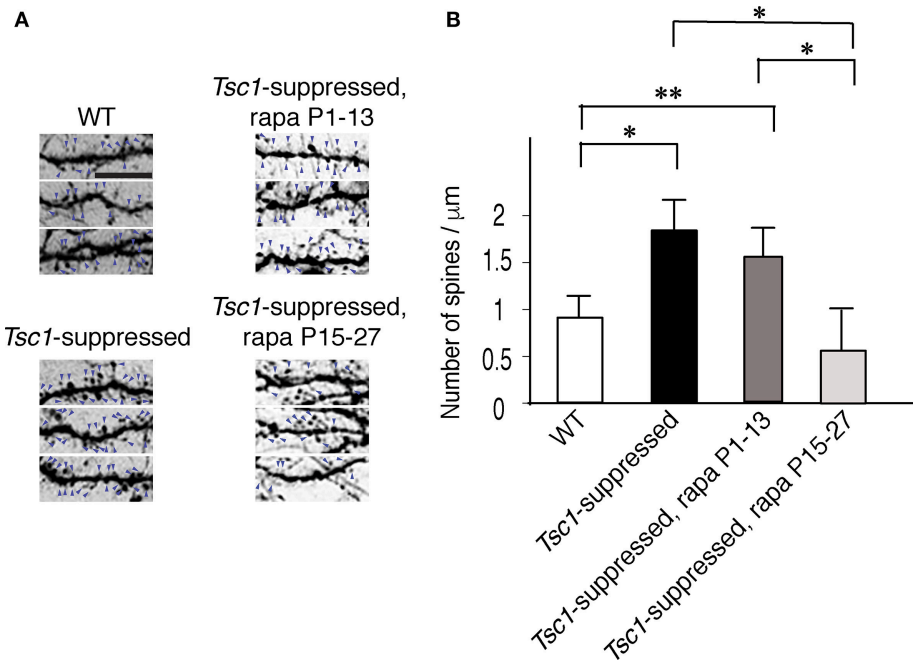

FIGURE 6 | Spine density of basal dendrites in electroporated pyramidal neurons. **(A)** Basal dendritic segments that are encircled with dashed boxes in **Figure 5B**. The signal density of each image is inverted for an improved visualization. Arrows indicate spines. Scale bar, 5 μm. **(B)** *Tsc1*-suppressed neurons have a higher spine density than WT cells at P28. Rapamycin treatment between P15 and P27 reduced spine density in *Tsc1*-suppressed neurons while neurons treated between P1 and P13 did not show a change in spine density. $^{*}P < 0.05$;$^{**}P < 0.01$.

are cortical tubers with giant cells, subependymal nodules, subependymal giant cell astrocytoma, and white matter radial migration lines (Crino et al., 2006). Recent studies also show structural abnormalities and functional deficits at cellular and synaptic levels (Tavazoie et al., 2005; Meikle et al., 2007; Choi et al., 2008; Nie et al., 2010; Goto et al., 2011; Magri et al., 2011; Zhou et al., 2011; Carson et al., 2012; Tang et al., 2014). In the present study, we have systematically analyzed neuronal morphology in TSC and assessed rapamycin effect on neurogenesis, dendritogenesis, and spinogenesis. Using *in utero* electroporation, we suppressed *Tsc-1* expression in a fraction of neuronal progenitor cells. In a mouse brain, neurogenesis starts around E11 and ends around E17 (Takahashi et al., 1996; Caviness et al., 2009; Greig et al., 2013). WT neuroblasts born at E15.5 are properly located in cortical layer 2/3. However, *Tsc-1*-suppressed progenitor cells make a delayed departure from ventricular zone and become scattered postnatally. These *Tsc-1*-suppressed cells also express a marker protein for layer 2/3 despite their malpositioning to a deeper layer. Postnatally, *Tsc1*-suppressed neurons have more complex dendritic arborization and a higher spine density than WT. Importantly, each of these developmental abnormalities that are caused by enhanced mTOR pathway has a specific window of opportunity to respond to rapamycin. Namely, dyslamination must be corrected during neurogenesis, and postnatal rapamycin treatment will not correct the cortical malformation. Similarly, exuberant branching of basal dendrites is rectifiable only during the first 2 weeks postnatally while an increase in spine density responds to rapamycin treatment thereafter. These results suggest that there are multiple critical periods to correct morphological defects in TSC during neuronal circuit formation. Notably, *Tsc2-hGFAP* mouse also exhibits time-sensitive responses to rapamycin: *in utreo* treatment corrects abnormal neuronal migration that results from radial glia dysfunction and postnatal rapamycin administration is necessary to rescue myeliniation defects (Way et al., 2012).

Migration Defect in TSC

Cortical tubers are demarcated hamartomatous tissues that contain dysplastic abnormal and large neurons, including balloon cells. Tubers are thought to be a product of migrational defect. Perhaps the most significant and disabling feature of patients with TSC is chronic and progressive seizures. There is an ongoing controversy concerning how the number or size of cortical tubers (often referred to as "tuber burden") is linked to the severity of neurological disabilities of TSC. There is evidence that a higher cortical tuber count is associated with lower intelligence and increased incidence of infantile spasms (Doherty et al., 2005). Also, EEG discharges highly correlate with tuber locations in magnetic resonance imaging (MRI), and surgical resection of tubers often reduces seizure episodes (Koh et al., 2000). Another study showed an altered expression pattern of glutamate receptors in human cortical tubers (Talos et al., 2008).

While some of the tubers may be epileptogenic, it is not known if this is true of all cortical tubers or dependent on the tuber size, location or disrupted morphology. In fact, other studies

TABLE 1 | Summary of ANOVA analyses.

	Source	Degree of freedom	Sum of square	Mean square	F	*P*-value
Figure 5						
15μm	Between groups	3	218.075	72.692	97.2825	0.0006
	Within groups	36	26.9	0.747		
	Total	39	244.975			
20μm	Between groups	3	380.675	126.892	122.4692	0.0009
	Within groups	36	37.3	1.036		
	Total	39	417.975			
25μm	Between groups	3	453.9	151.3	172.3671	0.0015
	Within groups	36	31.6	0.878		
	Total	39	485.5			
30μm	Between groups	3	486.5	162.167	278	0.0031
	Within groups	36	21	0.583		
	Total	39	507.5			
35μm	Between groups	3	467.275	155.758	247.0176	0.0026
	Within groups	36	22.7	0.631		
	Total	39	489.975			
40μm	Between groups	3	413.3	137.767	124.6131	0.0009
	Within groups	36	39.8	1.106		
	Total	39	453.1			
FIGURE 6						
	Between groups	3	9.139	3.046	21.9933	0.0001
	Within groups	36	4.986	0.139		
	Total	39	14.125			

have observed no solid correlation between tuber burden and the degree or kind of the neurological phenotype: severity of seizures, cognitive disability, or autism (Wong and Khong, 2006). One issue that complicates the clinical view of TSC is that differences may exist depending upon components of the tuber burden (e.g., not only number but also size, location, or morphology of the tubers) (Marcotte et al., 2012) and the severity or type of neurological phenotype. Furthermore, several disabilities may coexist and influence with each other. For example, frequent seizures can exacerbate cognitive and behavioral functions. Consequently, TSC can present with a broad spectrum of symptoms despite apparently comparable "tuber burdens." The biological bases for this range remain controversial. In fact, there is increasing evidence that non-tuberous TSC brain regions can also have dysregulated synaptic functions and play a critical role in the generation of abnormal electrical activity and epilepsy. For example, neuroimaging data indicate that cortical excitability can originate in regions near but not within cortical tubers in some TSC patients (Asano et al., 2000, 2004). Further, a recent postmortem study in humans examined non-tuber cortical areas and identified "dyslamination" characterized by an altered radial orientation of pyramidal cells, blurring of laminar boundaries, and disruption of cortical columnar architecture, isolated balloon cells and heterotopic neurons inside subcortical white matter (Marcotte et al., 2012). Taken, together, cortical tubers alone may not be sufficient to explain neurological symptoms, and microscopic abnormalities outside a tuber result in impaired circuit formation.

Two *Nestin*-promoter driven conditional *Tsc1* knockout mice targeting pyramidal cells, interneurons and glial cells successfully recapitulated pathological features such as subependymal nodule-like lesion (Zhou et al., 2011) or cortical tuber giant cells (Goto et al., 2011). On the other hand, *Emx1-Cre* x $Tsc1^{loxp/loxp}$ mice, which show *Tsc1*-deletion in forebrain pyramidal neurons starting from an early embryonic age, appear to lose cortical lamination without tubers or other obvious focal lesions (Magri et al., 2011; Carson et al., 2012).

Using *in utero* electroporation and live cell imaging, we find a scattered distribution of *Tsc1*-suppressed neurons. Dyslamination results from the delayed departure of the mutant progenitors from the ventricular zone and is correctable with rapamycin treatment during neurogenesis. However, postnatal administration of rapamycin did not correct malpositioning of *Tsc1*-suppressed neurons. Our results indicate that mTOR inhibitor can correct the cortical lamination defect if it is given during corticogenesis. While the result suggests cortical tubers and dyslamination are potentially treatable with a mTOR inhibitor, the treatment is currently not feasible in a human embryo due to a concern for teratogenicity.

Dendritic Branching in TSC

mTOR signaling pathway plays a critical role in dendritogenesis. Inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt pathway, which is upstream of mTOR, reduces dendritic arborization (Jaworski, 2005). In contrast, phosphatase and tensin homolog (PTEN) is a negative regulator of the PI3K-Akt-mTOR pathway by increasing phosphatidyl 3- inositol. Thus, loss-of-function PTEN mutations cause upregulation of mTOR and are associated with autism, macrocephaly, and epilepsy (Butler et al., 2005; Herman et al., 2007; Hoeffer and Klann, 2010). Hippocampal CA1 pyramidal neurons of the *Pten* KO mouse exhibits exuberant arborization (Kwon et al., 2006). *Pten*-deleted pyramidal neurons in the layer 2/3 visual cortex of adult mouse showed an extension of apical dendrite length (Chow et al., 2009). We have limited Sholl analysis to proximal segments of apical dendrites since distal segments were often cut off in the 100 μm sections. However, the difference of apical vs. basal dendrites may also reflect the distinct roles between PTEN and TSC1 proteins. Aberrant dendritic branching has also been reported in *Tsc-1* and *-2* RNA interference model in hippocampal neurons although they show tortuous but not exuberant dendritic arbors (Tavazoie et al., 2005). Neurons in different brain regions and ages have different local connectivity, which is also likely to contribute to the interpretation. Nevertheless, abnormally elaborate dendritic

arborization occur in response to up-regulation of mTOR signaling pathway.

Recent evidence suggests that each dendritic arbor process distinct information. For example, in a pyramidal neuron of the mouse visual cortex, each dendritic branch is tuned to a different orientation, and they are summated in the soma (Hausser, 2000; Jia et al., 2010; Grienberger et al., 2015). Further study is needed to address whether dendritic integration and sensory processing are dysregulated in *Tsc1*-suppressed neurons.

In mice, the final morphology of the dendritic tree is formed in the first 2 weeks of postnatal development during a period of maximum afferent innervation and synapse formation (Cline, 2001; Wong and Ghosh, 2002), then the large-scale dendritic structures become markedly stable (Trachtenberg et al., 2002; Holtmaat et al., 2006; Lee et al., 2006). We observe that rapamycin treatment is effective in correcting exuberant branching of proximal basal dendrites when it is administered between P1 to P13 but not between P15 and P27 (**Figure 5**). Our results indicate that there is a critical period for rapamycin response to normalize exuberant dendritic branching of *Tsc-1*-suppressed neurons that correspond to the first 2 weeks of postnatal life. Collectively, mTOR pathway is responsible for dendritic arborization in an early postnatal period before spine maturation occurs.

Dendritic Spine Defect in TSC

In the rodent central visual system, experience-dependent synapse formation starts after eye-opening at P13, the onset of patterned vision (Yoshii et al., 2003, 2011; Yoshii and Constantine-Paton, 2007). Spine formation and pruning are maximal during the critical period, which starts P16 peaks at P28 and decline from P33 (Hensch, 2005). We find that *Tsc-1* suppressed neurons in the visual cortex have an increase in dendric spine density. Our observation is in line with a previous study showing pruning defect in TSC (Tang et al., 2014). We also find that the rapamycin effect on aberrant spine formation is optimal between P15 and P27 when activity-dependent synapse formation is at its peak.

Important questions that need to be addressed in the future are whether disorganized lamination and neuronal morphology also disrupt local and long-range connections, and whether mTOR inhibitor treatment can correct them. The balance between excitation and inhibition undergoes complex regulation within the local cortical circuitry. A recent study in WT mouse somatosensory cortex using optogenetics and electrophysiology showed that horizontal projections originating from layer 2/3 pyramidal cells suppress activities of adjacent cortical regions within the same layer by lateral inhibition while facilitating layer 5 neuron activity (Adesnik and Scanziani, 2010). It is likely that an excitatory-inhibitory balance in the horizontal and vertical circuits is altered in TSC. Another critical question is whether the dyslaminated cortex can still establish normal long-range connectivity (Normand and Rasband, 2015).

CONCLUSION

Our study suggests that *Tsc-1* suppressed cortical neurons show alterations in cellular organization and differentiation and that each process has a distinct critical period in which rapamycin corrects the abnormal cellular process. Clinical studies have documented encouraging observations that rapamycin derivative such as everolimus is effective for not only controlling SEGA growth but also improves the overall outcome of seizure frequency (Krueger et al., 2010; French et al., 2016). Ideally, mTOR inhibitor should be started as soon as the diagnosis is made. However, even if the treatment is initiated shortly after birth, migration and dendritogenesis defects that have already occurred may be irreversible, and they may lead to a secondary effect during circuit formation. For example, proper lamination is essential for sensory processing (Adesnik et al., 2012). While rapamycin treatment is effective in correcting dendritic spine formation, our results suggest that there may be a limit in compensating for the structural changes preceding mTOR inhibition. Therefore, further studies are needed to understand whether there is an additional therapeutic target to further improve neuronal connectivity.

AUTHOR CONTRIBUTIONS

AY designed, performed, supervised all experiments, and wrote the manuscript. RC and FCdA performed experiments on migration and contributed to writing the manuscript. TM performed Sholl analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00409/full#supplementary-material

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BRIEF RESEARCH REPORT
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Differential Genetic Effect of the Norepinephrine Transporter Promoter Polymorphisms on Attention Problems in Clinical and Non-clinical Samples

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Among the monoaminergic modulatory neurotransmitters, norepinephrine is involved in task orienting, hence noradrenergic genetic variants have been studied in connection to attentional processes. The role of this catecholamine system is also highlighted by the selective norepinephrine transporter blocking atomoxetine, which has proved to be effective in the pharmacological treatment of Attention Deficit Hyperactivity Disorder (ADHD). In the present genetic association study three single nucleotide polymorphisms (rs28386840, rs2242446, rs3785143 SNPs) were analyzed from the 5′ region of the norepinephrine transporter (*NET, SLC6A2*) gene, which have been linked to ADHD previously. Attention problems scores of the mother-rated Child Behavior Checklist (CBCL) were used in separate analyses of 88 preschoolers (59.1% male, 6 years of age) recruited from the general population and 120 child psychiatry patients with ADHD diagnosis (85.8% male, age: 9.8 ± 2.9). The *NET* SNPs showed associations with attention problems, but the direction was different in the two groups. Regarding the promoter variant rs28386840, which showed the most consistent association, the T-allele-carrier patients with ADHD had lower CBCL attention problems scores compared to patients with AA genotype (p = 0.023), whereas T-allele-carriers in the community sample had more attention problems (p = 0.042). Based on previous reports of lower NE levels in ADHD children and the inverted-U shape effect of NE on cognitive functions, we propose that rs28386840 (-3081) T-allele, which is associated with lower NET expression (and potentially higher synaptic NE level) would support attention processes among ADHD patients (similarly as atomoxetine increases NE levels), whereas it would hinder cortical functions in healthy children.

Keywords: catecholamine, noradrenaline, SLC6A2 (solute carrier family 6, member 2), ADHD (attention deficit hyperactivity disorder), inattention

INTRODUCTION

Attention problems have gained increasing interest during the last decades, as the proportion of children with attention deficit hyperactivity disorder (ADHD) diagnosis has risen dramatically in many countries, creating social and scientific debates (Singh, 2008). Although the prevalence of ADHD increased in Western societies, the worldwide prevalence seems to be a stable 5–6% among school-age children (Polanczyk et al., 2014). Therefore, identifying potential risk and protective factors at an early age could help developing preventive strategies. Since both ADHD diagnosis and attention problems show substantial genetic background with complex inheritance, searching for genetic markers has been in the center of many studies.

Importantly, heritability estimates of complex traits vary widely from childhood to adulthood (Polderman et al., 2015). Twin studies of children using parent or teacher ratings reported high heritability estimates ($h^2 \sim 0.7$) for attention problems (Chang et al., 2013; Kan et al., 2013). Heritability estimates of attention problems based on self-report questionnaires decrease in adolescents and adults ($h^2 \sim 0.4$–0.5, Kan et al., 2013). Clearly, there is a substantial effect of the assessment method (see examples listed by Faraone and Larsson, 2018), but the underlying mechanisms may also change during development (Chang et al., 2013). Therefore, our aim was to identify genetic factor(s) of attention problems using a mother-rated symptom scale in a community sample of children in addition to child psychiatry patients, because childhood is potentially the most sensitive period to detect genetic effects.

Attention is often modeled as separate networks responsible for alerting, orienting, and executive control, which are linked to specific neurotransmitter systems (Raz and Buhle, 2006). The norepinephrine (NE) system projects to various cortical areas and functions mostly in alerting, whereas the mesocortical dopamine system is involved in executive control. For optimal cognitive functioning appropriate levels of catecholamine (dopamine and NE) transmitters were proposed, since both lower and higher tone of catecholamines in the prefrontal cortex (PFC) can worsen performance (Berridge and Arnsten, 2013), resulting in inverted-U shaped modulatory effects of these catecholamines. Therefore, association studies trying to identify genetic variations of attention phenotypes have been focusing on catecholamine neurotransmitter systems.

Recently, we have reported genetic associations of single nucleotide polymorphisms (SNPs) of the norepinephrine transporter gene (*NET, SLC6A2*) with ADHD symptom severity but not with ADHD diagnosis *per se* (Angyal et al., 2018). In the present study, we wanted to test if this genetic association could be extended to a non-clinical range of inattention. Therefore, we assessed attention problems with a widely used parent-rated symptom list in both clinical and community samples. Polymorphisms from the 5′ end of the *NET* gene were chosen based on their previous associations with ADHD-related phenotypes in different ethnic groups (Joung et al., 2010; Sengupta et al., 2012; Hohmann et al., 2015). Importantly, these SNPs were in high (but not complete) linkage in the previously studied Hungarian population (Angyal et al., 2018). The promoter SNPs rs28386840 (-3081 A/T) and rs2242446 (-182 T/C) can potentially influence gene expression (Zill et al., 2002; Kim et al., 2006; Sigurdardottir et al., 2016), hence can have functional consequences. A recent brain imaging study showed differential genetic effects of these *NET* promoter variants on transporter density in ADHD patients and controls (Sigurdardottir et al., 2016). Therefore, we conducted the symptom-scale based genetic association analyses separately in the clinical and community samples. Case-control analyses were not run for these samples, because larger ADHD and control groups were compared earlier in our meta-analysis of *NET* polymorphisms (Angyal et al., 2018).

METHODS

The study was designed in compliance with the Helsinki Declaration and was approved by the Local Scientific and Research Ethics Committee of the Hungarian Medical Research Council. The participating parents (mostly mothers) provided written informed consent. The two samples and genotyping methods are described in details by Angyal et al. (2018) and Birkas et al. (2006). Briefly, DNA was isolated from buccal swabs, and *NET* SNPs were genotyped with pre-designed TaqMan probes (rs28386840: C__60398891_10, rs2242446: C__26354911_10, rs3785143: C__27481932_10) on 7300 Real-Time PCR System (Applied BioSystem). No significant deviations from Hardy–Weinberg equilibrium ($p > 0.1$) were detected for the *NET* polymorphisms in any of the tested samples. Both the clinical and the community samples were ethnically homogeneous Caucasian origin and consisted of unrelated individuals.

For psychiatric symptom assessment, the Hungarian version of the Child Behavior Checklist (CBCL, Achenbach, 1991; Gadoros, 1996) was used, applying the standardized T-scores, as these were corrected for sex and age differences. CBCL was available for 88 children in the community sample (mean age: 6.3 ± 0.2 years, 59.1% boys). In the clinical sample, 120 patients (mean age: 9.8 ± 2.9 years, 85.8% boys) had ADHD according to DSM-IV criteria (American Psychiatric Association, 1994) either as primary or secondary diagnosis. Additional 72 patients diagnosed with tic-disorders (but not with ADHD) had CBCL data, yielding a total number of 192 patients comprising an extended child psychiatry patient sample (mean age: 10.0 ± 3.2 years, 81.3% boys). Comorbid conditions were assessed by the Hungarian child version of the Mini-International Neuropsychiatric Interview (MINI-Kid; Balazs et al., 2004). Among the 120 patients with ADHD, 30% had Tourette syndrome, 14.2% obsessive compulsive disorder, 27.5% learning disorder, 23.3% conduct disorder, and 14.2% anxiety disorder. In the extended child psychiatry sample, 62.5% had ADHD, 35.4% Tourette syndrome, 34.4% obsessive compulsive disorder, 19.3% learning disorder, 16.1% conduct disorder, and 26.0% anxiety disorder.

Statistical analyses were carried out with SPSS 20 for Windows, using the T-score of the CBCL attention problems scale as dependent variable and the genotype categories (main

allele homozygotes vs. minor allele carriers) as independent variable, with sex and age covariates in univariate analysis of variance in the clinical samples. Whereas CBCL T-scores were compared between the two genotype groups by Mann–Whitney *U*-tests in the community sample. Quantitative analyses of estimated haplotypes were performed with the THESIAS program (Tregouet and Garelle, 2007).

RESULTS

Genetic associations of the CBCL attention problems were tested separately in the community and the patient samples (**Table 1**). In these quantitative analyses the rare homozygote and heterozygote genotypes were grouped together to increase statistical power. In the community sample, the promoter rs28386840 and the intronic rs3785143 showed nominally significant associations with attention problems ($Z = -2.03$, $p = 0.042$, and $Z = -1.97$, $p = 0.049$, respectively). Among patients with ADHD, the two promoter SNPs showed associations with attention problems (rs28386840: $F(1,116) = 5.33$, $p = 0.023$, $\eta^2 = 0.04$, observed power: 0.63; rs2242446: $F(1,116) = 5.53$, $p = 0.020$, $\eta^2 = 0.05$, observed power: 0.64). Similar associations (with higher power) were detected in the extended child psychiatry patient sample: rs28386840: $F(1,188) = 11.55$, $p = 0.001$, $\eta^2 = 0.06$, observed power: 0.92; rs2242446: $F(1,188) = 9.40$, $p = 0.002$, $\eta^2 = 0.05$, observed power: 0.86). Importantly, the means of the genotype groups showed different patterns in the clinical and community samples (**Table 1**).

Using all three *NET* SNPs in the estimation of haplotype effect, the rs28386840-T∼rs2242446-C∼rs3785143-T (abbreviated as T-C-T) haplotype group showed significantly higher attention problems scores in the community sample than the most frequent A-T-C haplotype group ($p = 0.031$, see 95% CI error bars above the baseline on **Figure 1A**). For patients with ADHD, there were no significant differences between the three most frequent haplotype groups, however, in the extended child psychiatry patient sample, both the T-C-C and the T-C-T haplotype groups showed significantly lower attention problems scores compared to the A-T-C haplotype group (T-C-C: $p = 0.031$, T-C-T: $p = 0.005$), indicating the importance of the promoter polymorphisms.

DISCUSSION

The involvement of the NE system in attentional networks and in ADHD pathogenesis has long been demonstrated (Ehlers and Todd, 2017; Faraone and Larsson, 2018). For example, the effectiveness of the selective norepinephrine transporter inhibitor atomoxetine was shown in ADHD treatment (Hazell et al., 2011). Furthermore, since the availability of dopamine transporter is low in the cortex, but NET is relatively abundant and can take up extracellular dopamine (Moron et al., 2002), imbalances in NET expression may contribute to attention problems due to suboptimal cortical catecholamine (both dopamine and NE) functioning.

Previously, we reported genetic associations between *NET* gene polymorphisms and inattention symptom severity on the ADHD-Rating Scale among ADHD patients (intronic rs3785143-T and promoter rs2242446-C allele carriers showed lower inattention scores, Angyal et al., 2018). Our haplotype analyses indicated that a combination of three SNPs from the 5′ end of the *NET* gene, namely the rs28386840-T∼rs2242446-C∼rs3785143-T haplotype group had significantly different score compared to the most common A-T-C haplotype group. These associations were now supported in the same group of ADHD patients using different, mother-reported questionnaire data (**Table 1**). In order to test genetic markers in the full range of attention (dis)functioning, we extended our analyses to healthy preschoolers recruited from the general population. The associations observed in this community sample, however, were in the opposite direction (**Table 1**), indicating that the underlying mechanisms may be more complex. Since other quantitative analyses of *NET* polymorphisms and attention problem scores reported mostly non-significant differences among ADHD patients (Joung et al., 2010; Park et al., 2012; Sengupta et al., 2012) and in a community sample (Hohmann et al., 2015), it remains to be seen if our genetic findings could be supported by replication studies.

Based on the inverted-U shape effect of NE (first described by Gold and van Buskirk, 1978, for more details see Arnsten, 2009), we propose that the *NET* rs28386840-T∼rs2242446-C∼rs3785143-T haplotype and/or the functional rs28386840 (−3081) T-allele have differential effects on attentional performance (**Figure 1B**). The −3081 T-allele showed reduced transcriptional efficiency *in vitro* (Kim et al., 2006), potentially resulting in relatively higher catecholamine levels in cortical areas. However, we have to note that an *in vivo* study using positron emission tomography to measure subcortical NET levels in adult ADHD patients and controls showed opposite effect of the -3081 T-allele in the thalamus of control subjects (no difference in NET density was observed among ADHD patients by the *NET* promoter genotypes, Sigurdardottir et al., 2016). Unfortunately, cortical areas could not be measured in this study, leaving the question open if either SNP could affect *NET* expression in the cortex.

We acknowledge that attention problems have multiple components, and genetic variants contributing to cortical *NET* expression would represent only a small part in attention processes. Since genetic factors potentially interact with each other and with environmental factors, the differential susceptibility model was tested (for more details on the model, see Belsky and Pluess, 2009). According to this model, the *NET* promoter polymorphism(s) could act as plasticity allele(s), resulting in opposite effects in positive and negative environments (reflecting that psychiatry patients have more stressful life events). In order to test this hypothesis, interaction analyses were performed in the community sample, where (mother-reported) stressful life events data was available. Since no significant interaction of life stress and genotype was observed, we rejected this model.

In conclusion, our results showed opposite genetic effects of *NET* promoter polymorphisms on attentional problems in a

TABLE 1 | CBCL attention problems scores according to the NET genotypes in the community and clinical samples.

	Community sample *N* = 88		Patients with ADHD *N* = 120		Child psychiatry patients *N* = 192	
	N	**Mean ± SD**	***N***	**Mean ± SD**	***N***	**Mean ± SD**
rs28386840 (−3081 A/T)						
AA	**40**	**55.80 ± 6.32**	**71**	**72.72 ± 8.38**	**106**	**70.61 ± 9.31**
AT	39	59.10 ± 8.31	43	69.49 ± 7.78	75	66.16 ± 9.16
TT	9	59.00 ± 7.43	6	67.33 ± 9.61	11	66.0 ± 10.95
AT + TT	**48**	**59.08 ± 8.08**	**49**	**69.22 ± 7.94**	**86**	**66.14 ± 9.33**
p-value		0.042		0.023		0.001
rs2242446 (−182 T/C)						
TT	**40**	**56.05 ± 6.75**	**68**	**72.74 ± 8.55**	**100**	**70.45 ± 9.46**
CT	39	58.85 ± 8.05	43	70.19 ± 7.50	77	66.90 ± 9.28
CC	9	59.00 ± 7.43	9	65.67 ± 8.38	15	65.13 ± 9.64
CT + CC	**48**	**58.88 ± 7.86**	**52**	**69.40 ± 7.77**	**92**	**66.61 ± 9.31**
p-value		0.064		0.020		0.002
rs3785143 (intronic C/T)*						
CC	66	56.71 ± 7.02	102	71.65 ± 8.18	158	69.27 ± 9.10
CT	22	60.23 ± 8.30	18	69.28 ± 9.26	34	65.53 ± 11.11
p-value		0.049		0.209		0.030

*In the clinical sample 120 patients had ADHD as primary or secondary diagnosis. With further 72 patients diagnosed with tic-disorders (but not with ADHD) an extended sample of 192 child psychiatry patients was also tested in a separate analysis of variance (with sex and age covariates). *At the intronic SNP only 2 children had TT genotype in the larger patient group (N = 192), who were grouped together with CT heterozygotes. Where three genotype groups were present, the minor allele carrier group was compared to the main allele homozygote group (shown in bold).*

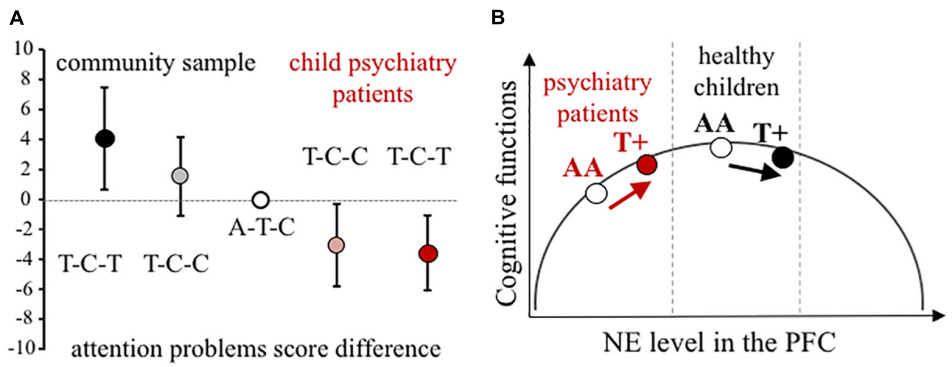

FIGURE 1 | Effect of *NET* gene variants on attention processes. **(A)** Difference scores of CBCL attention problems at the *NET* haplotype groups in the community and the extended child psychiatry patient samples. Haplotypes are constructed from rs28386840, rs2242446, rs3785143 alleles. The differences in attention problems scores are presented with 95% CI of the estimated rs28386840-T ~ rs2242446-C ~ rs3785143-T haplotype (dark symbols, T-C-T) and T-C-C haplotype (shaded symbols) compared to the most frequent A-T-C haplotype (open circle), based on THESIAS calculations. **(B)** Proposed mechanism of the differential genetic effect of the rs28386840 (-3081) A/T SNP. The T+ group represents both AT and TT genotypes. Groups of child psychiatry patients are indicated in red.

community sample of children compared to patients recruited at a child psychiatry clinic. The inverted-U shape modulatory effect can explain the observed contradictions if lower baseline cortical catecholamine levels are assumed in ADHD patients (see **Figure 1B**). According to earlier reports, disturbance of both dopamine and NE can be hypothesized in the background of ADHD (Oades, 2002). For example, measures of blood and urinary NE metabolite 3-methoxy-4-hydroxyphenylglycol indicated lower NE functioning in ADHD children (Hanna et al., 1996; Halperin et al., 1997; Llorente et al., 2006), although comorbid conditions can change the ratio of dopamine/NE (Halperin et al., 1997; Oades and Müller, 1997). Therefore, further studies are required to reveal the exact nature of neurotransmitter imbalances in ADHD in order to draw a more precise model for our *NET* genetic findings.

Limitations of our study include the relatively small sample size, which did not allow testing gene-gene interactions, and the high comorbidity rates in the clinical sample, thus it cannot be regarded as a purely ADHD patient sample. In addition, due to potential rater bias and cultural effects on the attention problems

scale of the CBCL (Crijnen et al., 1999), our findings should be replicated in other cultural settings and/or with teacher- or self-report data. Future studies should also test adult patients and control subjects to see if this differential noradrenergic genetic effect is stable over time.

AUTHOR CONTRIBUTIONS

ZN conceived and managed the genetic association study and drafted the manuscript. NA carried out the genotyping and helped in manuscript preparation. ZT and EBo collected the questionnaire data at the child psychiatry clinic. EBi and KL collected the questionnaire data in the longitudinal study of healthy children. KL was responsible for data management and analyses of the community sample. MS-S and JG designed the data collection and acquired the financial support for it. All authors have reviewed and approved the manuscript.

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HYPOTHESIS AND THEORY
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The Protracted Maturation of Associative Layer IIIC Pyramidal Neurons in the Human Prefrontal Cortex During Childhood: A Major Role in Cognitive Development and Selective Alteration in Autism

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The human specific cognitive shift starts around the age of 2 years with the onset of self-awareness, and continues with extraordinary increase in cognitive capacities during early childhood. Diffuse changes in functional connectivity in children aged 2–6 years indicate an increase in the capacity of cortical network. Interestingly, structural network complexity does not increase during this time and, thus, it is likely to be induced by selective maturation of a specific neuronal subclass. Here, we provide an overview of a subclass of cortico-cortical neurons, the associative layer IIIC pyramids of the human prefrontal cortex. Their local axonal collaterals are in control of the prefrontal cortico-cortical output, while their long projections modulate inter-areal processing. In this way, layer IIIC pyramids are the major integrative element of cortical processing, and changes in their connectivity patterns will affect global cortical functioning. Layer IIIC neurons have a unique pattern of dendritic maturation. In contrast to other classes of principal neurons, they undergo an additional phase of extensive dendritic growth during early childhood, and show characteristic molecular changes. Taken together, circuits associated with layer IIIC neurons have the most protracted period of developmental plasticity. This unique feature is advanced but also provides a window of opportunity for pathological events to disrupt normal formation of cognitive circuits involving layer IIIC neurons. In this manuscript, we discuss how disrupted dendritic and axonal maturation of layer IIIC neurons may lead into global cortical disconnectivity, affecting development of complex communication and social abilities. We also propose a model that developmentally dictated incorporation of layer IIIC neurons into maturing cortico-cortical circuits between 2 to 6 years will reveal a previous (perinatal) lesion affecting other classes of principal neurons. This "disclosure" of pre-existing functionally silent lesions of other neuronal classes induced by development of layer IIIC associative neurons, or

their direct alteration, could be found in different forms of autism spectrum disorders. Understanding the gene-environment interaction in shaping cognitive microcircuitries may be fundamental for developing rehabilitation and prevention strategies in autism spectrum and other cognitive disorders.

Keywords: cerebral cortex, theory of mind, cortico-cortical neurons, dendritic development, schizophrenia, excitatory transmission, glutamate

QUANTITATIVE EXPANSION OF THE CEREBRAL CORTEX AND MICROCIRCUITRY CHANGES: ROLE IN THE APPEARANCE OF COMPLEX HUMAN-SPECIFIC COGNITION

Increase in brain size, particularly an increase in the number of neuronal columns of the cerebral cortex, is the prerequisite enabling humans to achieve tremendous mental capabilities such as self-awareness, consciousness, language, abstract thinking, cognitive flexibility, mathematical abilities, as well as representational memory and complex social cognition (1–6). These abilities are not only species-specific features; the cognitive state achieved by humans represents a new qualitative level in mental functioning (7–9). It is correct that some animal species, in particular apes, are able to achieve a rudimentary level of some of these mental abilities (10–12). However, complex neuropsychiatric disorders as autism, schizophrenia or psychopathy are not present in any other species (13–17), which sets humans apart regarding cognitive and emotional features and capacities.

One of the most important human-specific abilities is complex social cognition, which includes processing, storing, and applying information about other people and social situations (18). Social cognition is the base for complex personal competencies, which are altered in the above mentioned diseases. From a neurobiological point of view, it is interesting that fundamental cognitive shift, which sets up human-specific cognitive abilities, the ability to understand the mental state of oneself or others (mentalization, i.e., "theory of mind"), appears in the period of transition from infancy to childhood (19–21). Humans and great apes (as our closest relatives) share roughly the same course of psychological development during the first 18 months of life (22). Around this age in both species, the brain nearly achieves adult neuronal composition, and even overall size (23–25). Nevertheless, in humans, intensive and diffuse changes in functional connectivity continue throughout the rest of childhood (26–31), while apes do not exhibit further important progress in cognitive capacities after the second postnatal year (22).

How did this unmatched shift in mental functioning between apes and humans appear without a robust quantitative increase in overall brain structure, i.e., overall increase in complexity of dendrites, or formation of new pathways and connections on most of the neurons? It should be noted, that a tremendous increase in the number of cortical neurons and connections, is a biological prerequisite to enabling high cognitive functioning (32–37). But at a certain point further quantitative expansion is not enough to initiate a more complex functional outcome, since the present pattern of organization does not allow proper integration inside numerically expanded circuitries. To make such an expanding system function properly, new microcircuitries that provide novel integrative properties are needed (31, 38–42). As such, the enhanced integration across cortical areas, along with an increase in network processing capacities, could come as a result of structural changes inside few selective microcircuitries (43–48).

It is possible that such changes are focused onto specific cortical areas. For example, the prefrontal cortex has abundant connections to most of the remaining cortical areas (49–53). Therefore, changes in functional properties of prefrontal cortex output (**Figure 1**) change the information processing throughout the whole brain (54). To produce considerable functional changes to the output, structural changes within the prefrontal cortex do not need to be "robust," i.e., they do not need to include dendritic growth of a wide range of neurons. Even fine changes, e.g., growth focused on selective neuronal populations that have rich local connectivity, may cause dramatic changes in functional properties of the prefrontal cortex. Based on previous work by our and other research groups, we suggest that deep located large layer III(C) pyramidal neurons (L3N) of the human prefrontal cortex could perform such a role.

The L3N have a unique developmental pattern during early childhood (55, 56) that correlates with the appearance and boost in the maturation of cognitive abilities, such as self-awareness and complex social cognition. With a detailed overview of mature connectivity patterns, here we present a possible integrative role of L3N in cortico-cortical network processing. Their selective alteration in pathological conditions could produce immense changes in mental capacities, due to the failure of proper integration (57, 58). Therefore, we suggest that alterations in mentalization and communication abilities found in the autism spectrum and social communication disorder are a result of disrupted development of circuitries established by L3N (59).

MOLECULAR FEATURES AND CYTOARCHITECTURE OF HUMAN PREFRONTAL CORTEX: THE ROLE OF ASSOCIATIVE NEURONS IN INTER-AREAL AND INTER-COLUMNAR CONNECTIVITY

Evolution of the human brain (**Figures 2A,B**) is characterized by a sharp linear increase in the number of cortical neurons

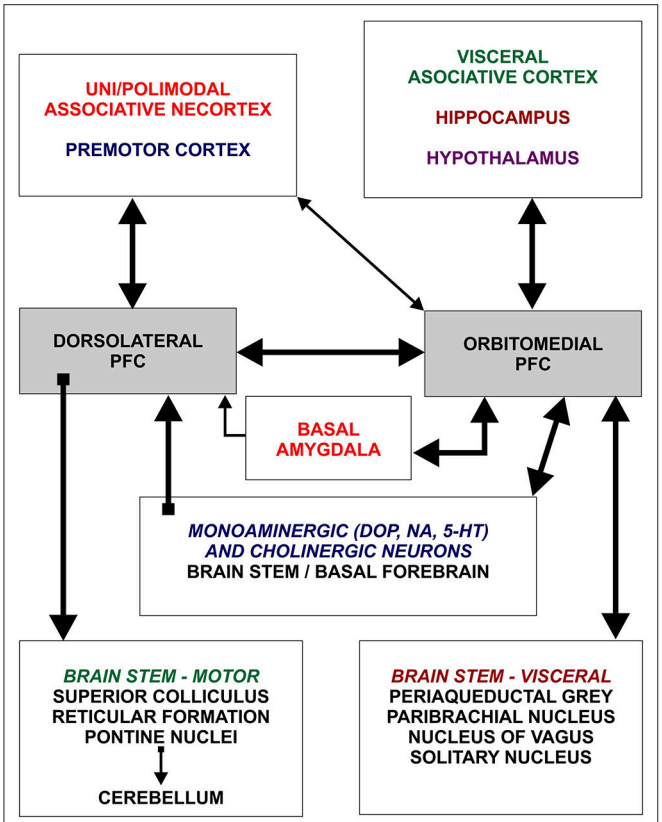

FIGURE 1 | Schematic representation of two main subdivisions of the prefrontal cortex and general organization of their afferent and efferent connections. Dorsolateral prefrontal cortex establishes rich connections with all neocortical areas, except primary regions, whereas orbitomedial prefrontal cortex is mainly connected with hippocampus and cortical regions processing visceral information. Note that dorsolateral and orbitomedial prefrontal cortex are densely interconnected. Figure is based on Groenewegen and Uylings (49). DOP, dopamine; NA, noradrenaline; 5HT, serotonin.

(67–69), but also with an exponential increase in the number of cortico-cortical projecting neurons (50, 67, 68). This leads to increased thickness of upper cortical layers (**Figures 2C–E**) which contain more neurons than lower layers populated by subcortically projecting neurons (3, 4, 39, 65, 66, 70, 71). So, the primate neocortex is characterized by a tremendous increase in the number of columns and changes to their internal neuronal composition (**Figure 2F**). Last but not least is the increase in width inside and between columns. The increase in width of columns is a result of increase in dendritic complexity of principal cells (72), i.e., more "space" between columns is a result of increased interconnectivity (73).

These changes are present in a majority of cortical areas, but are most prominent in several prefrontal cortex areas and related with their important integrative function inside cortico-cortical network (52, 53, 74, 75). The connectivity pattern of human prefrontal cortex (**Figure 1**), particularly its highly expanded dorso-lateral part, is characterized by massive reciprocal projections to both, multimodal and unimodal-parasensory associative areas (44, 76). This allows the prefrontal cortex to have a major role in regulating synchronous and coordinated activity between cortical areas. Experimental studies in monkeys and functional studies in humans have confirmed that the prefrontal cortex is functionally the highest associative region of the primate brain (38, 49, 51).

In humans, some areas of the prefrontal cortex show a specific cytoarchitecture, the magnopyramidality of layer III (77), i.e., deep located large layer III neurons exceed the size of large layer V pyramids. When compared to other populations of cortico-cortical projecting neurons, the L3N show strong acetylcholinesterase (AChE) (78–80) and SMI32 (antibody against phosphorylated protein H) reactivity (81–83) as a result of higher metabolic rate and prominent axonal tree. The density of SMI32/AChE reactive L3N, as well as their size and intensity of staining, is higher in human associative areas than in monkeys. In lower mammals, SMI32/AChE reactivity in deep layer III was not found (84).

The L3N are a subset of cortico-cortical neurons with long ipsi- and contra-lateral projections (**Figure 2G**) (40, 60, 61, 85). Individual L3N establish projections to several different areas suggesting a major role in inter-areal integration that grants them the title "associative" neurons (86). They are characterized by an astonishing number of intracortical axonal collaterals (**Figure 2H**), that extend around the cell, having dense columnar distribution of their terminal ramification through layers II and III (62–64). Thus, L3N are playing the major role in intercolumnar connectivity within a particular cortical area. In monkeys, L3N are indeed the key element for processing working memory and other prefrontal cortex-dependent high cognitive functions (87–90).

Above mentioned features of connectivity, functional properties and evolutionary expansion support the idea that L3N underlie the highly efficient network integration throughout the human cerebral cortex. We propose that selective structural and molecular changes of associative L3N in the human prefrontal cortex around the age of 2 and several upcoming years (55, 56), change the properties of the whole prefrontal cortex output, and have a pivotal role in cognitive maturation characterizing the preschool period. Developmental changes selectively related to this neuron class may be crucial for the appearance of cognitive abilities needed for the understanding of higher levels of inter-personal interaction, and to lay foundation for a further increase in cognitive capacity observed later throughout the childhood and adolescence, that ultimately leads to socio-emotional maturity. Selective alterations of the L3N were described in neuropathological states characterized by intense and global changes in the efficiency of the cortical network (91–96). We propose that selective alterations of associative L3N have a pivotal role in the "dis-connectivity" of prefrontal cortex found in autism spectrum disorder (97, 98), but also in other prefrontal cortex-associated disorders, like schizophrenia (90, 99, 100). These two disorders share similarities in cognitive pathology, and are characterized by

global cortical dysfunction, without concurrent structural alterations and specific structural pathology identified so far (16, 17).

UNDERSTANDING THE MENTAL STATES OF ONESELF OR OTHERS: NEURONAL DEVELOPMENT OF PREFRONTAL CORTEX DURING EARLY CHILDHOOD AND FOCAL DISCONNECTIVITY IN AUTISM

The capacity to attribute mental states (mentalization) remains one of the quintessential abilities that makes us human and is defined as the "theory-of-mind" (ToM) (20, 21, 101, 102). This ability appears during the second year of life and subsequently sophisticates through childhood with cognitive spurts at specific time points (103). Following temporal pattern at which various levels of ToM have been achieved is important since it reflects changes inside cortical circuitries which allow shifts in mental capacities.

Most data marks the infant to child transition as a point when first ToM abilities appear, i.e., during the second year of life. Infants around 12–15 months of age display behaviors that are prerequisites to ToM development (104–106). Nevertheless, it is difficult to talk about internalization of mental abilities before 18 months of age (107, 108). By the age of 2, children are clearly aware that there is a difference between thoughts in their mind, and things in their surroundings (109). An average 3-year-old knows that the brain has a set of mental functions, such as dreaming, wanting, thinking, and that different persons may want, like and feel different things. Further important cognitive twists occur around the age of 4, when children realize that thoughts might not be true (110). Also, a 4-year-old can remember that their own belief has changed which is not the case with a 3-year-old (111). By the end of early childhood (ages 5 to 6), children realize that people talk and act on the basis how they think the world is, even when it does not reflect the reality of the situation. They can keep secrets and understand that sometimes a person may believe something that is not true, and that what a person does or says, can be based on a false belief (112, 113).

The cortical areas related to ToM tasks typically activate the frontal lobe. In particular, neuroimaging studies of ToM showed activations in the dorsal prefrontal cortex (Brodmann area 9). However, other frontal regions were also involved in understanding and controlling oneself, as well as in interaction of thoughts about oneself and others (112, 114–117). Therefore, the prefrontal cortex can be considered as a region with a key role in social cognition, and it is assumed that pathological substrates in states characterized by disrupted social cognition, such as psychopathy personality (14) and autism spectrum disorder (59, 118), must be located within.

In autism spectrum disorder (ASD) and related social (pragmatic) communication disorders, social interactions are affected (119) due to difficulties in the aptitude for inferring other people's states of mind, such as intentions, beliefs, desires and wishes (120). When a false-belief test is applied to children with ASD, most of them fail even at the age of 11, in contrast to typically developed children who pass the test by the age of 4 (121). In a modified (122) and simplified (123) form of the false belief task, typically developing children show ToM abilities latest by the age of 2.5, while many of them show it already at the age of 1.5 years (107, 124). These abilities are lacking in ASD subjects, showing that deficiency in the ability to reflect on the contents of one's own and other's minds (101, 125) is a core cognitive feature of ASD. This lack is a consequence of a disturbed cognitive development at some point during the period of early childhood (1.5–6 years) (126–128). In search for neurobiological correlations, it is important to recognize that cognitive impairment in ASD is specific and different from learning difficulties of blind or deaf people (129–131).

Neuroimaging data show that dorsolateral and medial prefrontal cortex in ASD are hyperconnected during the second year of life (132–137). In parallel with hyperconnectivity, many of ASD individuals undergo brain overgrowth that is particularly pronounced in the frontal lobes (138–141). The hyperconnectivity later on changes to hypoconnectivity (142–144). Therefore, it is still debated if ASD should be considered as a disorder characterized by hyper- or hypoconnectivity (145, 146).

Nevertheless, the concept of hypo- or hyperconnectivity seems to be oversimplified. Neuroimaging data are in line with the view that ASD symptomatology is the result of disconnection in areas involved in the processing of language, executive and socioemotional reaction as well as in abstract and conceptual thinking. Furthermore, there is a disconnection in cortical regions that are highly evolved in humans and involve higher-order associative processing along with the prefrontal cortex (147–151). Alteration in ASD was also found in many regions of the temporal lobe and in adjoining parts of the occipital and parietal lobes, including the insula and regions important for ToM processing (117, 125, 152, 153). However, the majority of functional and structural connectivity studies in ASD suggest that the key disconnection must be between the frontal lobe and other higher order association cortices (154–160), and that the frontotemporal, frontolimbic, frontoparietal, and interhemispheric connections are altered. In addition, alterations in synaptic organization related to specific deficits were found (161–166). The type and range of cognitive pathology in ASD suggests that structural alterations are focused, as well as delicate, but present even among highly-functional adults with ASD (122, 167–169). Preservation (or even enhancement) of other mental functions (170, 171) suggests that development of certain circuitries is spared, supporting the model of "focal disconnections" which appear during development. Therefore, ASD should be considered as a form of "developmental disconnection syndrome" (172–174).

In conclusion, structural and functional data in ASD suggest that development of specific micro-circuitries is disrupted during the ToM acquiring stage of infant to child transition (second year of life), or in milder ASD forms during the upcoming

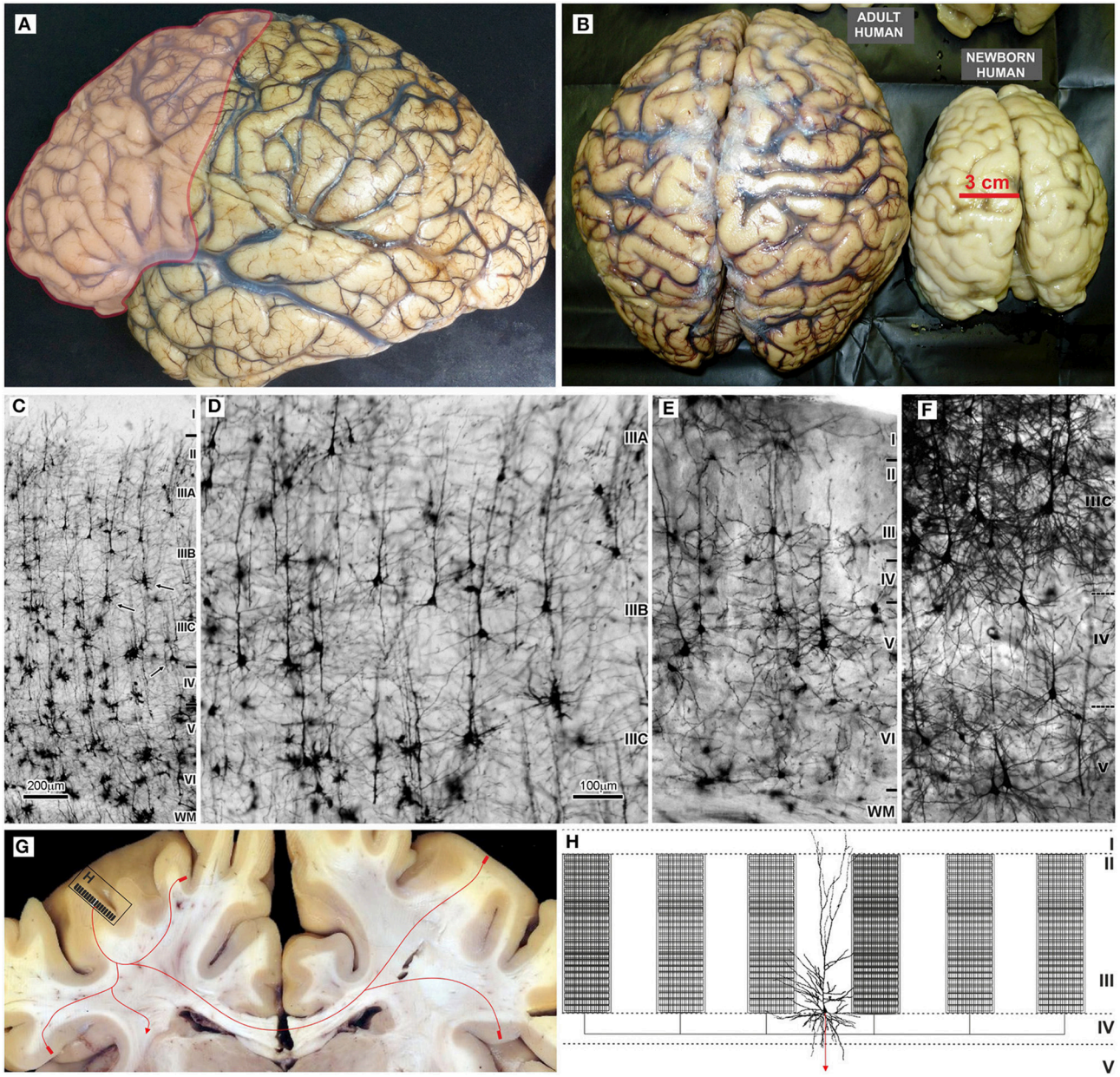

FIGURE 2 | Macroscopic and microscopic features of the adult human brain, including schematic organization of extrinsic and intrinsic cortical projections of associative layer IIIC neurons. **(A)** In the adult human brain frontal granular cortex occupies 80% of the frontal lobe, and almost one third (red) of the total cortical surface. **(B)** Around the age of two, the brain is very close to its adult size (23). Microphotography of Golgi Cox **(C–E)** and rapid Golgi **(F)** impregnated sections of the associative areas in the human **(C,D,F)**, and mice neocortex **(E)**. **(G)** Large layer IIIC neurons are considered to be associative neurons, connecting several higher order areas in the ipsi- and contralateral hemisphere, with the columnar pattern of axon ramification (60, 61). Ipsilateral collaterals are much more numerous **(H)**, and around 80% of synapses are established within the area of origin. Local axon branches are forming numerous terminal ramifications which have columnar distribution through layers II and III, and extend several millimeters around the cell body (62–64). The figure is a compilation of figures published by Hladnik et al. (65) **(A,B)** and Džaja et al. (66) **(C–E)**. Scale bar 200 μm **(C)** and 100 μm **(D–F)**.

years (118, 120, 175, 176). The role of distinct neuron classes in the prefrontal cortex for processing ToM and complex social cognition is not yet defined, and therefore the neuronal correlates of ASD pathology remain unknown (177).

Based on connectivity patterns, as well as the pattern of their development and maturation, selective changes of associative L3N microcircuits in the prefrontal cortex could represent one of the major biological substrates for normal cognitive development during early childhood. Consequently, abnormal L3N development could be associated with appearance of ASD symptomatology.

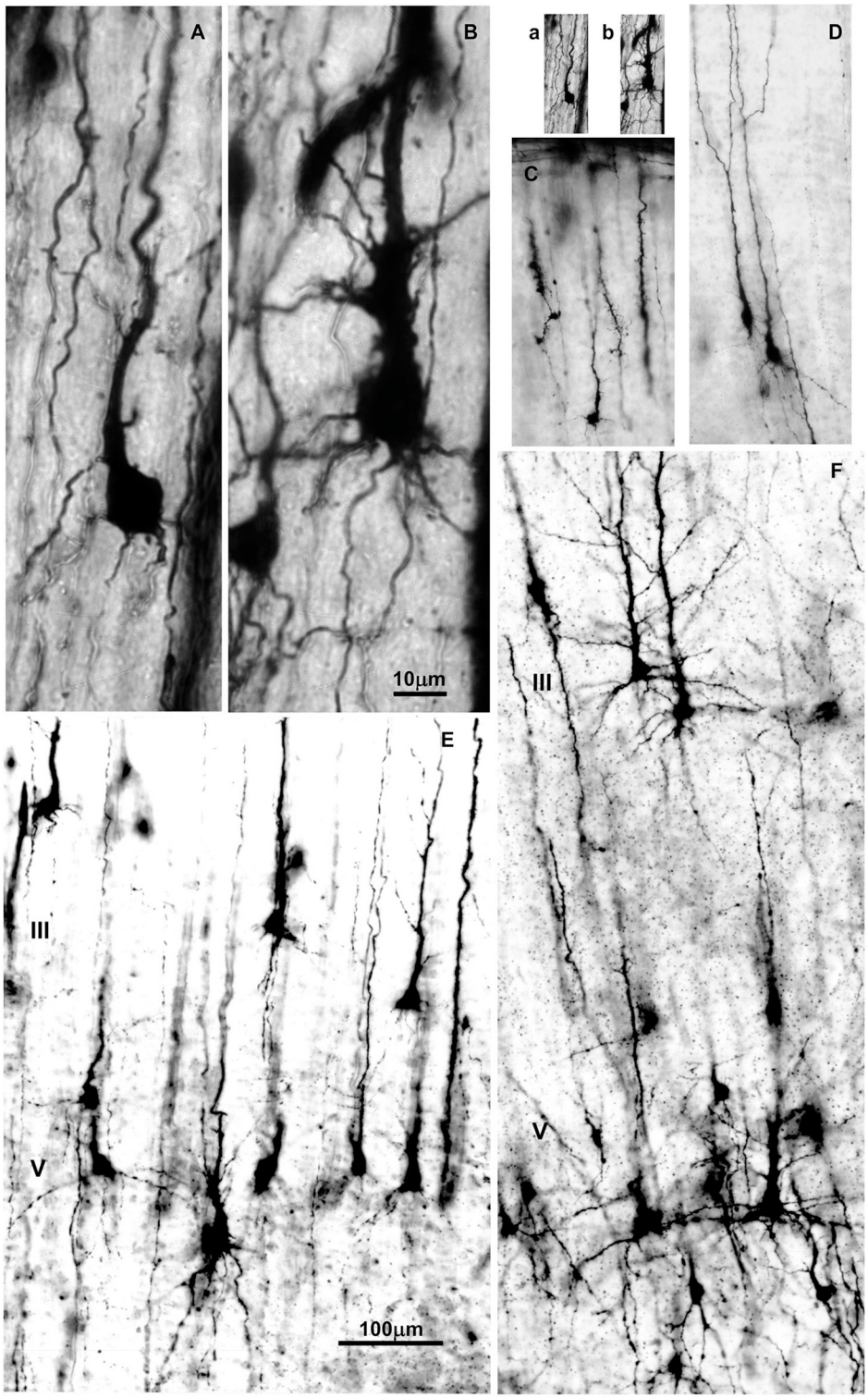

FIGURE 3 | Changes in dendritic morphology of rapid-Golgi impregnated pyramidal neurons in the dorsolateral part of the prefrontal cortex during the second half of gestation **(A–D)** and first postnatal month **(E,F)**. Microphotography of rapid Golgi impregnated sections in the human fetal prospective dorsolateral prefrontal cortex at 21 **(A,B)**, and 32 postconceptional weeks **(C,D)**, newborn **(E)** and one month old infant **(F)**. Scale bar 10 μm **(A,B)** and 100 μm **(C–F)**. **(a,b)** are **(A,B)** shown at the same magnification as **(C–F)**.

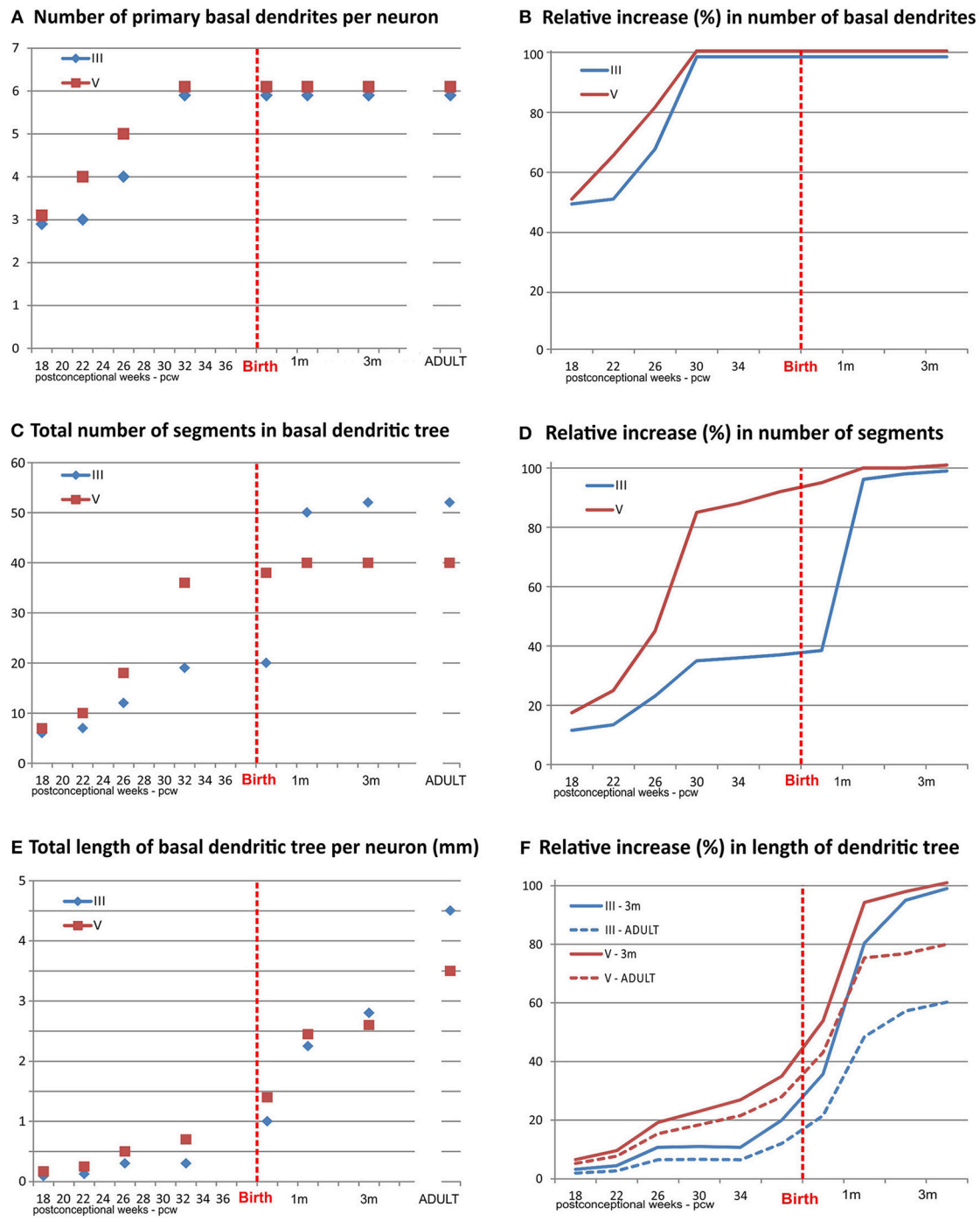

FIGURE 4 | Graphical presentation of quantitative data from the basal dendritic tree of large layer III and V pyramidal neurons in the human dorsolateral prefrontal cortex, impregnated by a rapid Golgi method, in a period of rapid growth between 18 postconceptional weeks, and third postnatal month, compared to adult values. An outgrowth of primary basal dendrites **(A)** started earlier on layer V pyramidal neurons, during the middle trimester of gestation prior to layer III, but not later on **(B)**. The number of basal dendritic segments (indicating frequency of bifurcation) shows a clear inside-out gradient until birth **(C)**. A constant, slow outgrowth of new segments is present, for both classes, during the middle trimester of gestation, followed by rapid increase in period 26–32 PCW for layer V pyramidal neurons **(D)**. The major outgrowth of new segments occurred for layer III pyramids during the first postnatal month. No additional segment outgrowth is observed after first postnatal month. Despite rapid segment outgrowth up to the 32 postconceptional weeks, the increase in total length for layer V pyramids **(E)** was rather slow. Most of the elongation occurred later, during the last 2 months of gestation and first postnatal month **(F)**. At the same time, opposite to layer V, a considerable increase in length occurred for layer III during the period of rapid segment outgrowth. At the 3rd postnatal month, layer III rapid Golgi impregnated neurons have just exceeded 50%, whereas layer V pyramidal neurons exceed 80% of their adult length (dashed lines). Data shown here were extrapolated from the studies of Mrzljak et al. (190) and Petanjek et al. (55).

SEQUENTIAL DIFFERENTIATION OF PRINCIPAL NEURONS IN THE PREFRONTAL CORTEX AND EARLY DEVELOPMENT OF CORTICO-CORTICAL MICROCIRCUITRY

Mechanism of Dendritic Growth

Development of dendrites is one of the essential processes in differentiation and maturation of neuronal circuitry (178–182). Developmental changes in dendritic size and complexity will define the total neuronal receptive field. Since dendritic development occurs in parallel with rapid synaptogenesis (183) and axon growth (184, 185), it will affect both the neuronal functional response to the input and the neuronal output (186). Dendritic development typically undergoes three phases:

The first phase of dendritic growth starts after the neuron arrives to its final position within the cortical plate (37, 187, 188). This phase is characterized by the protrusion of primary basal dendrites and apical dendrite, which arise from the cell body, including appearance of oblique dendrites which grow out on the proximal site of the apical dendrite (189). No significant outgrowth of additional branches on primary oblique and basal dendrites occurred during this phase (**Figure 3**).

The second phase is characterized by extensive and rapid growth of the dendritic tree. Initially, new segments grow out on the, during first phase formed primary basal and oblique dendrites (**Figures 4A,B**). This is followed by an increase in the size of the dendritic tree achieved mainly through elongation of present branches. Importantly, the appearance of functional glutamate receptors is crucial for inducement of rapid dendritic growth (183, 191–195). This strongly supports the view that ingrowth of glutamatergic thalamo-cortical and cortico-cortical afferents during the fetal and perinatal period, triggers the rapid dendritic growth of principal neurons (196).

The final stage of dendritic growth (once up to 20% of total dendritic length is established) is characterized by significant, but much slower elongation of dendrites than during the second phase. Many connections established on developing dendrites are functional at the beginning of this stage, making dendritic development more sensitive to environmental influences ("nurture"). In contrast to the second phase, glutamatergic activity during the third phase stabilizes the dendritic tree instead of promoting its growth (199–201). As such, the last stage of dendritic development is the longest and is characterized by a slighter increase in the length of the dendritic tree (202, 203).

Intensive Perinatal Dendritic Growth of Associative Neurons Results in Early Functional Microcircuitries

Dendritic development and synaptic rearrangement of principal neurons have been extensively studied in the monkey and human prefrontal cortex (189, 190, 197, 199, 204–221). In the human fetal prefrontal cortex, intensive dendritic growth (second phase) of both deep layer III and V principal neurons, starts 12–15 weeks after they arrived into the cortical plate. However, beginning of the second phase differs between these two subpopulations of principal neurons. For the large layer V pyramidal neurons it coincides with the ingrowth of thalamo-cortical fibers into the cortical plate by the end of the middle trimester of gestation (188, 196, 222, 223). In contrast, intensive growth of the L3N begins with ingrowth of cortico-cortical fibers by the end of the last trimester of gestation (224, 225). Thus, for the two main classes of large pyramidal neurons in the human prefrontal cortex there is an inside-out gradient of differentiation during the prenatal period, and intensive growth seems to be induced by the arrival of specific glutamatergic afferents.

At birth, large layer V principal neurons have already attained their adult dendritic complexity (branching pattern), while L3N are not well developed (190), suggesting that the cortico-cortical network is not highly functional at that time (226–228). Indeed, the most intensive dendritic development of the L3N is the first postnatal month, when around 60% of basal dendritic segments appear (**Figures 4C,D**), and

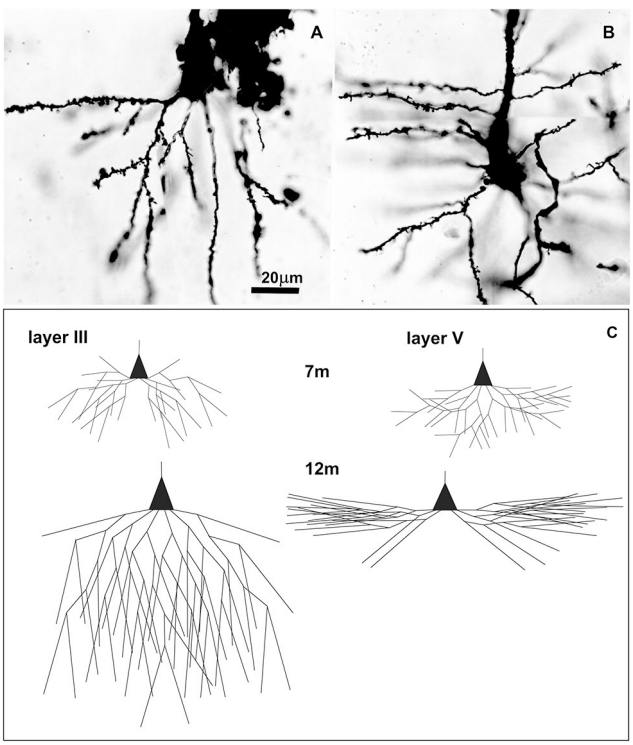

FIGURE 5 | Golgi Cox impregnated pyramidal neurons in human Brodmann area 9 at 7 postnatal months and changes in basal dendritic tree between 7 and 12 months. Microphotography of Golgi Cox impregnated large pyramidal neurons in deep layer III **(A)** and layer V **(B)** at 7 postnatal months at higher magnification. Schematic drawings **(C)** indicating changes in length and complexity of dendritic tree of Golgi-Cox impregnated neurons between 7 and 12 months, showing that major dendritic growth for those two populations of neurons, occurred during the second half of the first postnatal year. A total number of segments is approximated on the basis of neuronal reconstructions (197), and percentage of segments cut at a particular dendritic order (198).

almost half of the total size is achieved (**Figures 4E,F**). Thus, the L3N dendritic tree "catch-up" large layer V pyramidal neurons in absolute values of their length and complexity soon after birth. By the third postnatal month both classes are equal in total dendritic tree length, and have achieved not only adult complexity, but also, adult-like overall dendritic three shape.

The maturity level of layer V principal cells reached soon after birth is not surprising, as these cells are key neuronal elements for processing early executive functions of the prefrontal cortex (43, 229–231). However, the maturity level of L3N reached between first and third postnatal month is somewhat surprising (55, 56), as these cells are believed to be the key elements involved in sophisticated, evolutionarily recent, and human-specific cognitive functions that develop later on (232, 233). Such an early functioning cortico-cortical neuronal network centered on L3N may represent a neurobiological basis for cognitive functions present already in the first months after birth (234–238). Behavioral and functional studies found that the perinatal period (32 week of gestation−3 months postnatal) is characterized by rapid transformation and disappearance of fetal patterns of behavior, but also with concomitant appearance of certain aspects of cognitive functions, which will intensively develop through infancy (124, 239–246).

Sequential Development of Microcircuitries in the Human Prefrontal Cortex During the First Postnatal Year

Not all classes of principal neurons in the prefrontal cortex undergo intensive dendritic growth during the prenatal and perinatal period as observed for large pyramidal neurons impregnated with the rapid Golgi method. Subpopulations of pyramidal neurons impregnated by the Golgi Cox method (197, 207) undergo a major dendritic growth after birth, mainly during the second half of the first postnatal year (**Figure 5**). Different modification of Golgi methods have a selective affinity to stain different neuronal subpopulations, i.e., the rapid Golgi method is more prone to impregnate large pyramidal cells. These differences in timing of intensive dendritic growth between different subclasses of principal neurons (247, 248) suggest that there is a different gradient of maturation for different subclasses of neurons, even within the same layer.

In our recent work, by using rapid Golgi method and encompassing broader population of the layer III impregnated principal neurons (**Figure 6**), we showed significant differences in the level of dendritic differentiation during the first postnatal month within frontal lobe, that includes dorsolateral part and Broca's region as well as primary motor and premotor cortices (249). The populations of L3N attained a highly developed dendritic tree in all analyzed areas, whereas dendrites of other principal neurons in layer III were less differentiated. Such findings show an asynchronous maturation of different microcircuitries throughout the cortico-cortical network: some of them reach functional level soon after birth, while others are still very immature (47, 250–252). This is in contrast to the traditional view of hierarchical neuronal development across the cerebral cortex, which suggests a sequential gradient of maturation from lower to higher order areas. We propose that there is a sequential maturation of distinct elements, forming cortico-cortical circuitries across all frontal areas (88). Such a pattern of development may represent a neurobiological basis for the sequential development of cognitive functions during the first and second postnatal year (124, 235, 253–256). Also, rapid dendritic growth is related with maturity of glutamatergic and GABA-ergic receptors (257, 258), making dendritic differentiation more prone to environmental influences. As such, for the development of early maturing neurons, as is the case for L3N, already during the perinatal and early postnatal period, the environment has an important role in regulating their morphological differentiation (259–263).

SELECTIVE MATURATION OF THE ASSOCIATIVE LARGE LAYER IIIC NEURONS DURING EARLY CHILDHOOD AND PROTRACTED DEVELOPMENT OF "COGNITIVE" MICROCIRCUITRY THROUGH ADOLESCENCE

Unique Pattern of Dendritic Growth of Associative Neurons in Human Prefrontal Cortex

For most subpopulations of principal neurons in the prefrontal (197, 207, 208, 218, 219, 221, 264, 265), and other regions of the human cerebral cortex (266–276) major postnatal dendritic growth occurs during the first year in parallel with massive synaptogenesis (185, 205, 206, 226, 277–284).

An important exception from the typical temporal pattern of dendritic growth (see previous chapter) are associative L3N in the prefrontal cortex (**Figures 7**, **8**). The L3N do not undergo the typical third stage of dendritic development like large layer V pyramidal neurons (55, 56). The layer V neurons attain more than 80% of their adult dendritic length by the third postnatal month. They then continue with further elongation during the third stage of dendritic development for roughly 1 year, and reach adult values around 15 months of age. In contrast, by the third postnatal month dendritic size of the L3N has reached only half of their adult values (**Figures 7A–C**, **8A,C,D**). In addition, basal and oblique dendrites of the L3N have no significant growth ("dormant" period) until the middle of the second year (**Figures 7A,C,D**, **8A,D,E**). Between 16 months and 2.5 years, length of L3N basal and oblique dendrites almost doubled, with growth rate higher than expected for the third stage (**Figures 7A,D,E**, **8A,E,F**). To the best of our knowledge, this second L3N dendritic growth spurt represents an undescribed developmental feature for any class of cortical neurons. In the following period from 2 to 5 years, there is a further increase in synaptic spine density (285) at L3N dendrites, accompanied with molecular changes of this class of neurons. The L3N start to

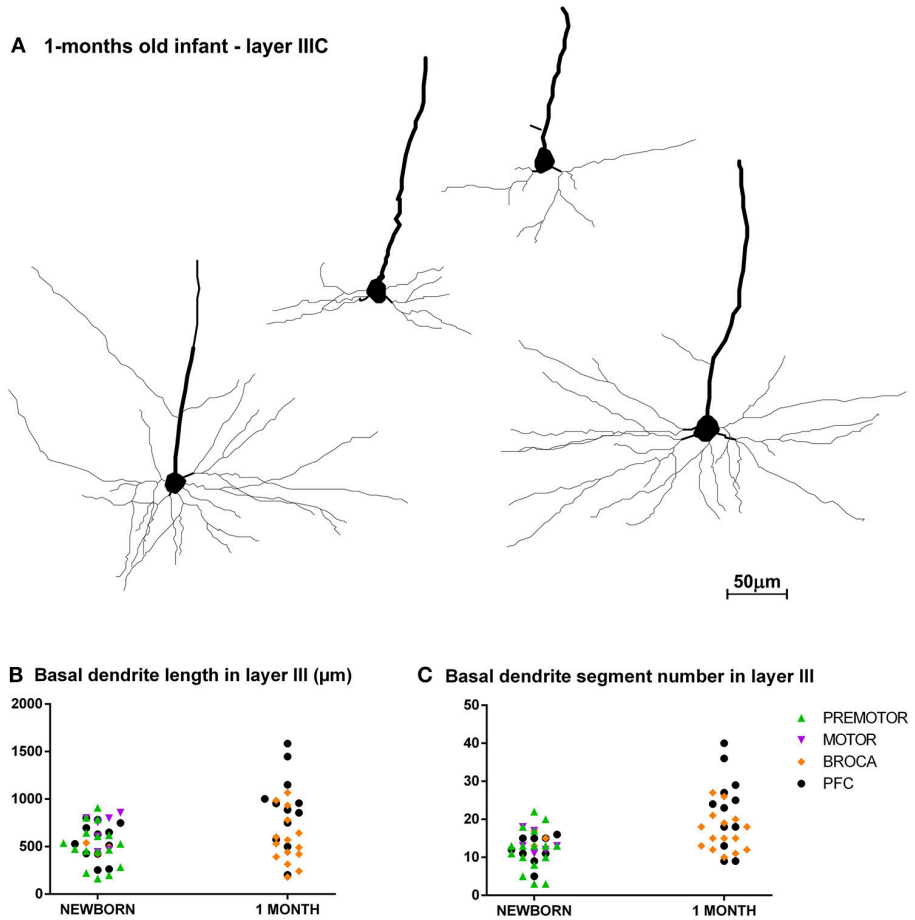

FIGURE 6 | Asynchronous maturation of pyramidal neurons in layer IIIC during first postnatal month. **(A)** Rapid Golgi impregnated deep layer III pyramidal neurons in Brodmann area 9 of a 1-month-old infant in the prefrontal cortex show different stages of differentiation. Quantitative analysis of newborn and 1-month-old infant reveals large differences in total length **(B)**, and number of segments **(C)** in basal dendritic tree of deep layer III pyramidal neurons, across different areas of the human frontal cortex. Each symbol represents mean values per individual neurons.

express strong AChE (78, 79) and SMI-32 (82, 265, 286) reactivity in their bodies and proximal apical and basal dendrites. This unique expression sets them apart from other classes of cortical neurons (**Figure 9**). Additionally, by the age of 5, the L3N show intensive Nissl staining paralleling transient somatic overgrowth (**Figure 8B**) (287–290).

Thus, the L3N acquire a significant portion of their maturity after infancy. We suggest that morphological and molecular changes on dendrites of the L3N in the period from 2 to 6 years are related to the growth and synaptogenesis of their own local intracortical projections, which establish very dense innervation on all cortico-cortical projecting neurons (62–64). Thus, changes in L3N intracortical projections will affect function of all prefrontal cortex neurons that project to other cortical areas. Consequently, L3N changes will be reflected on network processing throughout the whole cerebral cortex. Intracortical projections in experimental studies on rhesus monkey were found to be the last maturing part of the cortical excitatory network (209), whereas basic architecture for cortico-cortical projections is established earlier during infancy (41, 124, 243, 291–294). This leads us to conclude that large scale functional changes in the cerebral cortex, starting around the age of 2, are related with maturation of excitatory intracortical connections. Still, further maturation of cortico-cortical projections could not be excluded (116).

Protracted and Environmentally Driven Synaptic Pruning of Associative Microcircuitries

Structural changes through the cortical network are not finished by the age of 5–6 years, while the circuitry reorganization continues throughout the rest of childhood and adolescence (28, 33, 36, 76, 90, 205, 216, 283, 295–317). Molecular tuning of synaptic strength during development, when synaptic numbers exceed adult values, is proposed to be a major mechanism for the environmental effect on circuitry reorganization. The period of overproduction and elimination of supernumerary

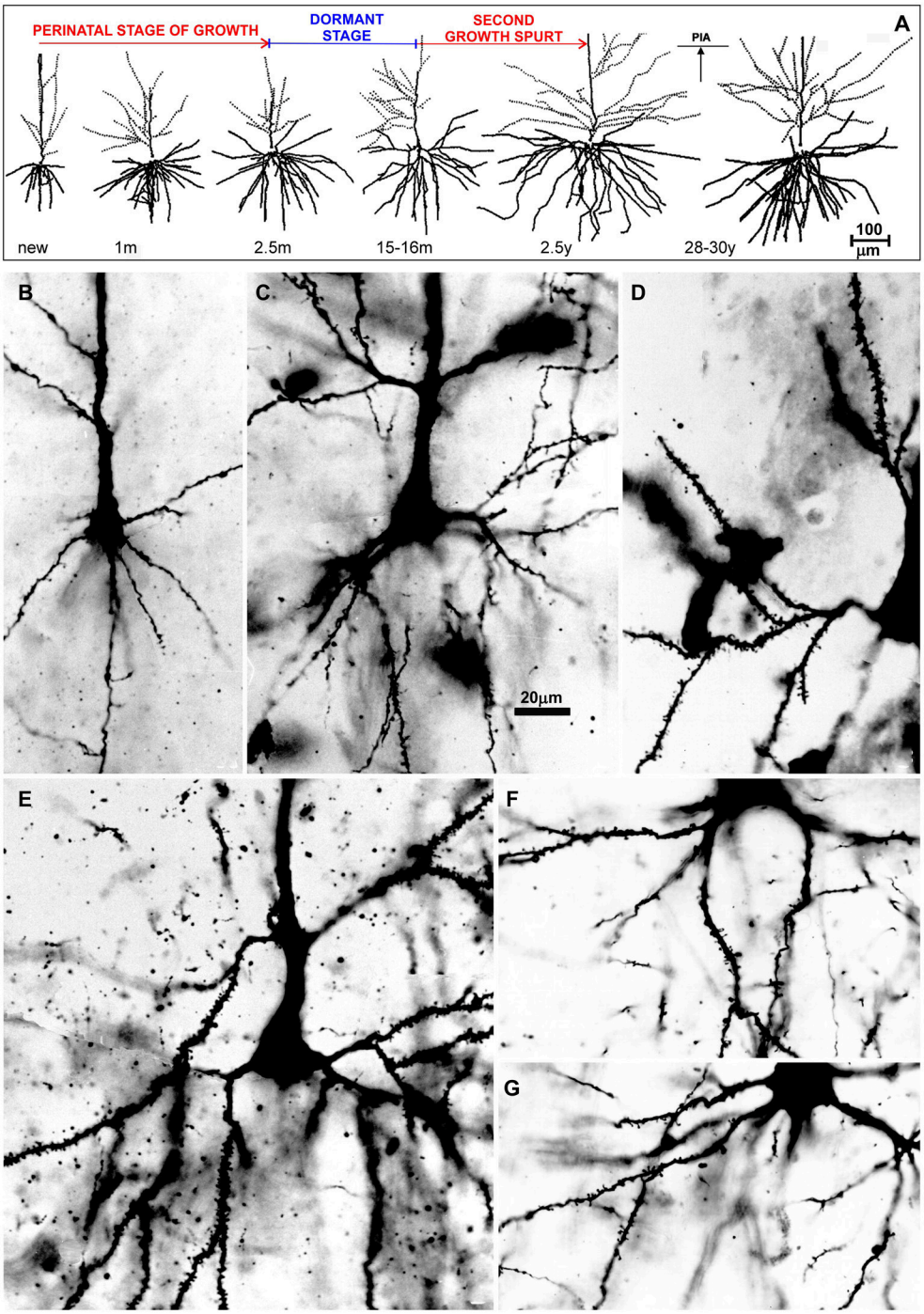

FIGURE 7 | Postnatal development of rapid Golgi impregnated large layer IIIC pyramidal neurons in the magnopyramidal area 9 of the human prefrontal cortex. Three-dimensional reconstructions of basal and apical dendritic trees of rapid Golgi impregnated pyramidal cells in layer IIIC, projected onto the coronal plane **(A)**. Orientation toward the pia is indicated by the arrow. Oblique dendrites originate from the apical dendrite, and are represented by dashed lines. All layer IIIC pyramidal cells are represented at the same magnification (scale bar 100 μm) and at the following ages: newborn, 1-month-old, 2.5-months-old, 15-months-old infants, 2.5-year-old child, and 28-year-old adult. Dendritic trees of layer IIIC pyramidal cells increased between 16 months and 2.5 years of age. Note that there are no obvious differences between layer IIIC pyramidal cells of 2.5-month-old and 16-month-old infants (dormant stage), as well as 2.5-year-old and 28-year-old subjects. Microphotographs showing changes in morphology of rapid Golgi impregnated layer IIIC pyramidal cells of the Brodmann area 9 between: newborn **(B)**, infants aged 1 **(C)** and 16 **(D)** months, 2.5-year-old child **(E)**, 19-year-old **(F)**, and 73-year-old **(G)** adults (the magnification is same for all microphotographs; scale bar –20 μm). Even in these high-power microphotographs, the increase in dendritic complexity (an outgrowth of new segments) between newborn **(B)**, and 1-month-old infant **(C)** is obvious. The figure is taken from Petanjek et al. (55) with permission.

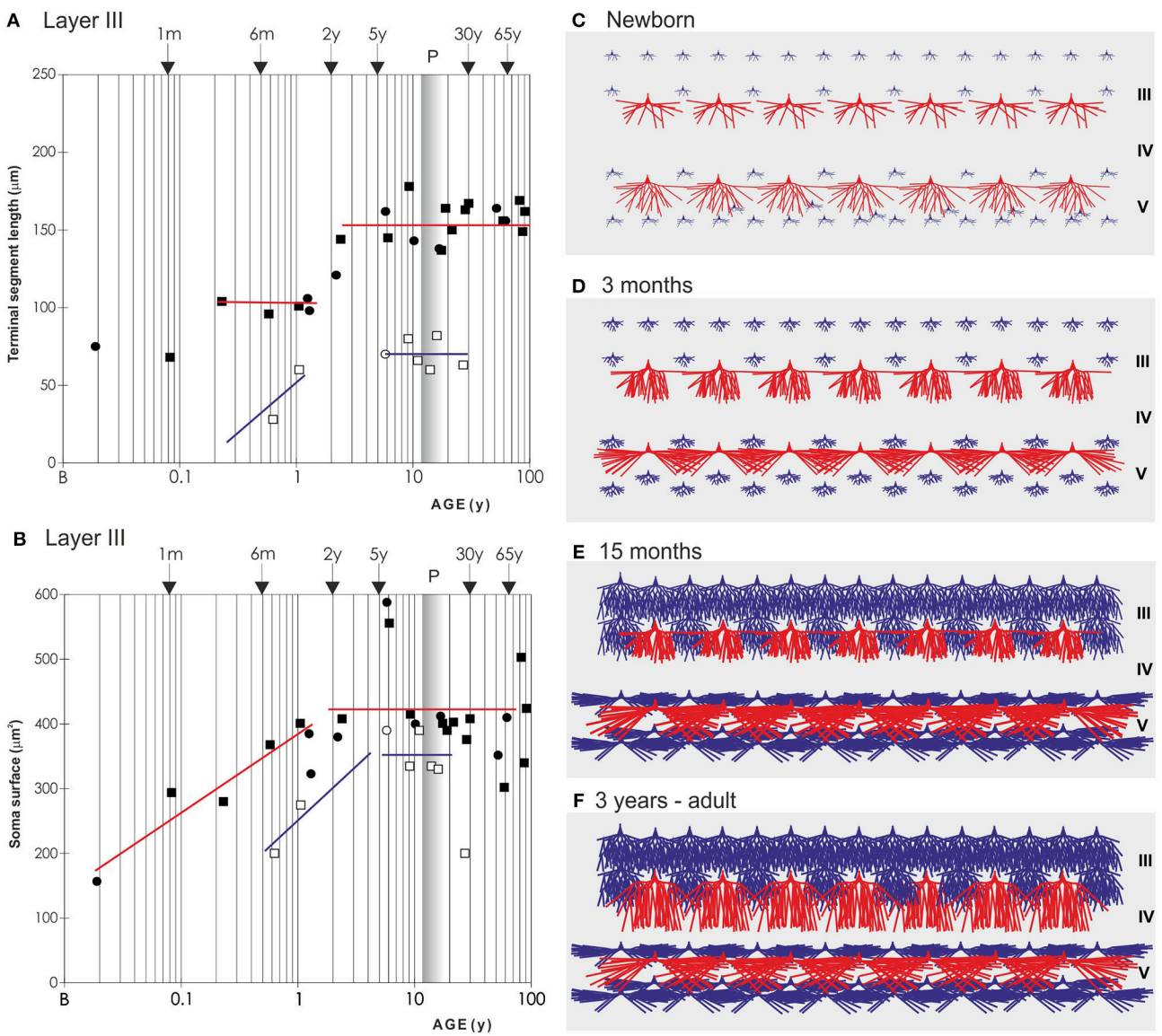

FIGURE 8 | Graphical and schematic presentation of quantitative morphological data from basal dendritic tree of deep layer III and layer V pyramidal neurons, in magnopyramidal area 9 of the human dorsolateral prefrontal cortex impregnated by the Golgi method during postnatal development. Changes in terminal segment length **(A)** and soma size **(B)**, from birth until 91 years of age on layer III pyramidal neurons impregnated with rapid Golgi (full marks—red line), and Golgi Cox method (open marks—blue line). Data about terminal segment length **(A)** are shown here as they participate with 90% in the total length of dendrites. Data from rapid Golgi stained slides, regarding terminal segment length confirm, that the period between 3 and 16 months, is a dormant stage in basal dendritic growth for layer III pyramidal neurons, and that further growth occurs between 16 months and 2.5 years (at least 1/3 of total length). Large temporal overgrowth in cell body size was present in subjects 5 to 6 years old **(B)**. Data shown here were extrapolated from Koenderink et al. (197) and Petanjek et al. (55) studies. Squares represent males and circles represent females. The age is shown in postnatal years on a logarithmic scale. Puberty is marked by a shaded bar. Schematic drawings **(C–F)** of changes in length and complexity of dendritic tree of deep layer III and layer V pyramidal neurons, illustrate changes occurring in the cytoarchitecture and overall neuronal morphology. Total number of segments is estimated on neuronal reconstructions (based on real data) (55, 197) and values of missing dendrites were calculated by formula (198). Red represents rapid-Golgi impregnated neurons, and blue Golgi Cox impregnated pyramidal neurons. Illustrations for Golgi Cox neurons at newborn **(C)** and 3-months-old infant **(D)** stage are prediction based on dendrite growth pattern in the period 7–15 months. By the 15 months **(E)** most of the neurons have achieved an adult level of dendritic size, except large layer IIIC pyramidal cells impregnated by the rapid Golgi method **(F)**.

synaptic spines corresponds to the developmental stage when principal neurons have the highest magnitude of plasticity (185, 201, 260, 277, 304, 318–326). In the prefrontal cortex, the stage of developmental plasticity is highly prolonged and extends even up to the third decade of life (**Figure 10**). Concomitantly, there is a prolonged peak in expression of genes regulating neuronal development, including those associated with schizophrenia (298, 307, 327, 328). The comparative

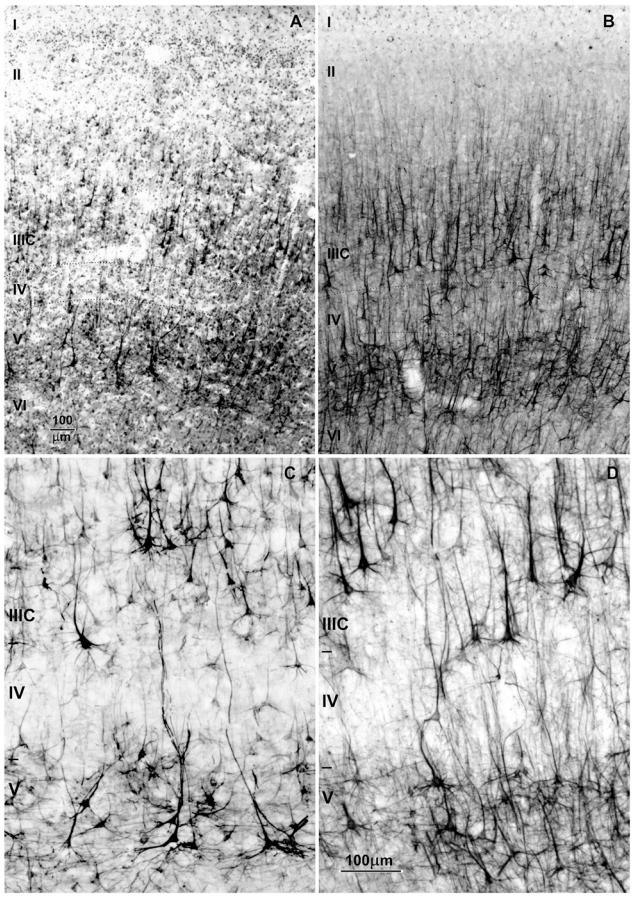

FIGURE 9 | Neurofilament staining (SMI32) in Brodmann area 9 of child and adult human cortex. Microphotography of SMI32 (non-phosphorylated filament H) stained sections in Brodmann area 9 of the human prefrontal cortex at the age of 3 **(A,C)** and 38 years **(B,D)**. The deep part of layer III and layer V are sub-laminas most densely populated with reactive neurons. There are no differences in staining intensity of layer V neurons at both ages, however in the adult subject layer III, the amount of intensely stained pyramidal neurons with clearly visible dendrites has increased. Accumulation of neurofilaments in dendrites is corresponding to the length and complexity of axon, suggesting that pyramidal neurons located deeper in layer III continue with axon growth after the age of 3. Scale bar at 100 µm **(A–D)**.

analysis of mRNA expression in the prefrontal cortex shows that in the human brain, relative to non-human primates, the dramatic changes in transcriptome profiles are delayed (283, 304, 307, 329, 330). So, extraordinary protracted circuitry reorganization is a specific feature of human higher-order associative areas.

It is possible however, that distinct types of microcircuitries may have different rates of synaptic formation and elimination. The pruning of supernumerary dendritic spines during the third decade of life is most pronounced and protracted on the L3N. It starts earlier on segments that are targeted by thalamo-cortical, rather than on those targeted by cortico-cortical projections (285). Studies obtained in monkeys and humans show somewhat higher synaptic overproduction in supragranular (including L3N and cortico-cortical projecting neurons) vs. infragranular (including layer V and subcortical projecting neurons) layers (205, 226). Regional differences in the number of grown and pruned spines on the layer III neurons have been described in the monkey and human cortex, with highest spine overproduction in the prefrontal cortex and lowest in the primary sensory regions (208, 221, 250, 279, 282, 331). The level and duration of synaptic overproduction, and consequently the level and duration of developmental plasticity (332), increases within increasing functional hierarchy of distinct microcircuits. Thus, microcircuits that are processing the highest cognitive functions, such as social abilities, are subject to the highest developmental remodeling induced by psycho-social and emotional environment (32, 39, 250, 252, 260, 305, 333, 334).

THE DEVELOPMENT OF PREFRONTAL CORTEX ASSOCIATIVE NEURONS IN AUTISM: A MODEL OF SELECTIVE NEURONAL VULNERABILITY IN GLOBAL CORTICAL NETWORK DISCONNECTIVITY

Based on the above, we suggest that selective disruption of L3N could cause global cortical network disconnectivity, underlying ASD cognitive symptomatology. The protracted and biphasic pattern of L3N dendritic growth, coupled to the intensive molecular maturation after infancy, is not described for any other population of principal neurons. This developmental timing overlaps with the period when specific ASD symptomatology becomes evident.

Thus, alteration of the specific neuronal population with "strategic" position inside cortical circuitry, like L3N, could lead to global cortical network dysfunction. The timing of appearance and severity of symptoms in ASD might depend on the affected level of structural and molecular maturation of associative neurons during early childhood (2–6 years). For example, if development of the L3N is affected during the second year of life, it is most likely that the elongation of dendrites would be altered. This possible reduction in dendritic size would result in abnormal input on the L3N, and would change their functional properties. Consequently, as the L3N densely innervate all layer II/III cortico-cortical neurons, a robust alteration in dendritic morphology and consequently possible out-growth of intracortical projections of L3N, would lead to disrupted inter-columnar processing (335). We would expect such changes in subjects with a more serious form of ASD, where cognitive pathology already emerged during the second year of life.

In some ASD cases, specific cognitive symptoms are not apparent during the second, and even third year of life. Those subjects only show a subtle deflection of ToM, with no signs of any other neurological or psychiatric comorbidity, like in Asperger's syndrome (122, 167, 168). We hypothesize that in these forms of disorder the L3N alterations are not as robust. Here, the development of the L3N is probably affected later, after the age of 2.5 years, when further molecular maturation of

the L3N occurs. Cognitive pathology is in such cases related to synaptic network architecture or/and fine molecular deflection at the level of individual synapse, without any dendritic and axonal changes that can be defined as pathological.

However, complex cognitive processing is disturbed in most cases of ASD at earlier stages of development (336, 337). Neurological and cognitive pathology is frequently present during the first year, sometimes even at birth (338, 339). In the most serious cases, there is ASD comorbidity with the intellectual developmental disorder (340) or/and epilepsy (341). In cases with absent comorbidity, many parents report a concern about socio-emotional interactions during the first year of life (342–345). Neuroimaging further revealed network inefficiencies during the first year of life (337, 346, 347). However, although specific ASD symptoms are not seen before the age of two, epidemiological and genetic studies support the idea, that alterations of neuronal development occur during the prenatal or perinatal time (348–355).

Thus, the "uniquely biphasic" pattern of dendritic growth of the L3N, with delayed structural and molecular maturation during the post-infant period (after 1.5 years), indicates that this neuronal class is particularly vulnerable during the perinatal period, when harmful events related to ASD are expected to occur. Pathological alterations induced at that stage may not be severe enough to affect functional demands of the L3N at the early postnatal period. However, the perinatal lesion could manifest after the second year, since the role of L3N, in now much complex microcircuitries, becomes more demanding at this time. In this view, the earlier "perinatal lesion" becomes visible as notable functional impairments after the second year (327, 356).

Most ASD cases will be diagnosed earliest by the age of 18 months (103). However, social cognition is often altered earlier, even in infants that do not show neither neurological, nor intellectual impairment. Retrospective studies have shown that ASD affected children clearly have different behavior related to social responses by 12 months, like gazing in a single direction and the way how they respond to their name (357). On functional magnetic resonance imaging (fMRI) altered connectivity in ASD was found by the 6 months (127, 346) and EEG signal was found to be altered by the 3 months (358). Above mentioned behavioral and functional aberrations present at early stages of infancy (337), further suggest that ASD symptoms are related with structural alterations from the early postnatal period. Interestingly, EEG pathology of ASD is first observed in the left temporal electrode (T7), and the frontal lobe starts to differ between 15 and 18 months of age, which corresponds to the beginning of the second L3N growth spurt. The developmental ("biphasic") pattern of L3N, with intensive perinatal dendritic growth (suggesting high vulnerability), and second growth spurt around the age of 2, corresponds to the timing of alterations in cortical functioning and appearance of symptoms in infants with ASD. Understanding relations between L3N development and functional changes of cortical activity is important in early detection of ASD and might help to develop algorithms as combination of functional imaging methods and focused behavioral testing.

GENE-ENVIRONMENT INTERACTION DURING POSTNATAL DEVELOPMENT AFFECTS ASSOCIATIVE CIRCUITRY ARCHITECTURE AND MAY CONTRIBUTE TO AUTISTIC TRAITS

Architecture of the mature cortical network is determined through complex gene-environment interactions during intricate developmental steps (1, 260, 330, 359–365). Given the unimaginable number of possible interactions between genes and environment, there are large interindividual differences in the size of particular areas, and even in internal cytoarchiteconics, particularly within the prefrontal cortex (77, 366–368). Large interindividual differences were found among the dendritic structure of cortico-cortical projecting neurons in high associative areas of the human brain, correlating with the level of education (369). All mentioned interindividual differences point to a strong environmental impact on cortical development.

The mechanism of developmental plasticity leading to interindividual differences in cytoarchitectonics and neuron morphology is related to synaptic overproduction. By activity-dependent molecular tuning of synaptic spines, it is determined which synapse will remain and which will be removed from the network during the pruning process (370, 371). This model is defined as selective stabilization hypothesis (318) and proposes that through synaptic tuning the environment is shaping the architecture of the neural network. The highest degree and longest period of synaptic overproduction are so far described for the L3N in the prefrontal cortex, particularly at dendritic compartments (distal side branches of apical dendrite) targeted by cortico-cortical and intra-cortical projections (285). Altogether, psychological, educational, social and emotional milieu has a predominant influence in reshaping circuitries which are involved in processing the most complex cognitive functions (29, 76, 226, 309, 332, 372–379).

Taken together, these findings suggest that human-specific cognitive functions and circuitry specializations (1) have foundation in interactions between genes (25, 380–382) and environment (242, 363, 383, 384) during the period of synaptic overproduction and pruning. In humans, the period of synaptic overproduction and pruning is the period of the highest magnitude of acquisitions of new knowledge. It might look paradoxical that this occurs with a decrease in the number of synapses, but the final functional outcome of pruning leads to increased connectivity of the cortical network. Therefore, this process allows humans to acquire the highest level of cognition, but it also prolongs the vulnerability period, increasing the chance for the formation of abnormal circuitry (296, 306, 327, 364, 385–387).

Such developmental reshaping could be an important factor in the development of ASD, particularly in subjects with a mild form of the autism spectrum phenotype. Recognition that ToM deficit is a core psycho-pathological substrate of ASD, has allowed better diagnosing of both patients with mild impairment and individuals with atypical symptoms or personality traits associated with ASD, which fall under the broad

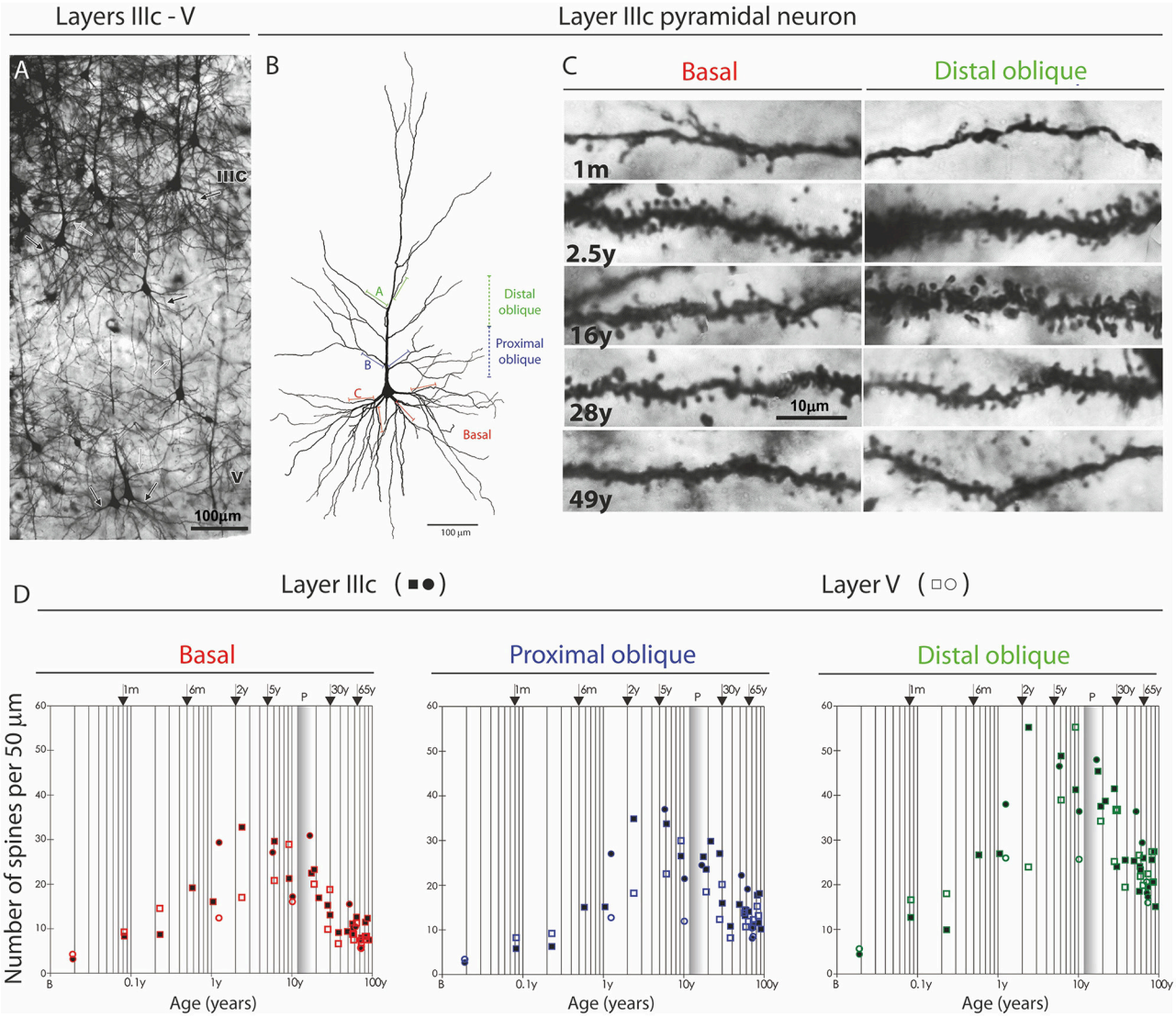

FIGURE 10 | Changes in dendritic synaptic spine density on rapid Golgi impregnated large layer IIIC and layer V pyramidal neurons in magnopyramidal area 9 of the human prefrontal cortex from birth until 86 years of age. **(A)** Representative low magnification photographs of the rapid Golgi-impregnated layer IIIC and V pyramidal cells in the dorsolateral prefrontal cortex of a 16-year-old subject. **(B)** Neurolucida reconstruction of layer IIIC pyramidal neuron of a 49-year-old subject, illustrating dendrites selected for counting spines. **(C)** Representative high power magnification images of layer IIIC pyramidal neuron dendrites. **(D)** Graphs represent changes in spine numbers per 50 µm of dendritic length. The age is presented in postnatal years on a logarithmic scale. Puberty is marked by a shaded bar. Squares represent males, circles females. P, puberty; B, birth (fourth postnatal day); m, months; y, years. Figure published by Petanjek et al. (285).

autism phenotype (388–390). It is possible that the "pathological" substrate can be found only in the circuitry architecture, without any structural or molecular impairment of neurons and their pathways. In line with this possibility is a higher incidence of ASD in school rather than preschool children (391), suggesting that in some cases, "autistic" circuitry architecture is established through late childhood, or even adolescence (103, 392, 393). So, the broad or subthreshold autism spectrum conditions, could be used as a model to understand trajectories of "nature-nurture" interactions, guiding neurodevelopment toward, or away from ASD.

We propose that in such individuals, the emotional and psycho-social environment during infancy and childhood is crucial for the appearance of ASD or autistic traits (387, 394, 395). Vice versa, in individuals with genetic backgrounds to develop ASD or autistic traits, this opens a huge window of opportunity for cognitive rehabilitation, particularly considering the highly extended period of circuitry reorganization in the human prefrontal cortex. At an epidemiological level, mild and atypical cases should be far more numerous then serious cases of ASD, and are therefore of much higher societal impact. Second, enhanced emotional and psychological interaction in infants

and children, have been shown to have a stimulating effect on the development of ToM (396, 397), suggesting that those with mild symptomatology and subtle alterations in circuitry organization have a greater chance for a positive outcome of early intervention (398).

OVERVIEW OF REPORTED CORTICAL NEURONAL PATHOLOGY IN AUTISM

Despite pathological changes observed in the cerebellum, amygdala and brainstem (399–402), imaging data and studies on post-mortem material are implying that cerebral cortex circuitries are the most plausible candidate to produce core deficits of autism. However, specific cortical neuronal alterations are yet to be described and present data are often contradictory. Some ASD cases have reduced neuronal cell body size but increased number of minicolumns and increased neuronal density (403, 404). These findings suggest an increased number of neurons in frontal, temporal and parietal regions of ASD cortices (405, 406). In other ASD cases, neuron numbers and density were unchanged (407), while some have a reduction in the number of neurons (408). Cellular patches were found in prefrontal and temporal cortices of ASD patients, while again no change in in neuron density was described (409).

Cell body size was unaltered in the dorsolateral medial prefrontal cortex (405), superior temporal gyrus (407), anterior cingulate cortex (410), hippocampus (411), and amygdala (412). Smaller cell size was found in cortical regions with identified minicolumnar pathologies in ASD, i.e., frontal regions (403, 406, 413), as well as primary motor, sensory and visual cortices (414). In the fusiform gyrus (415), hippocampus (416) and portions of the anterior cingulate cortex (417, 418), smaller neuron cell bodies of varying types have also been reported. These changes in cell body size are considered to be present in preadolescent stages between ASD and controls, and this effect becomes diminished later on (400, 410). Importantly, changes in cell body size are usually related with changes in dendritic morphology. However, Golgi studies on ASD neocortices, showed that there is no dendritic pathology in neocortical pyramidal and non-pyramidal neurons (411, 419), but increased density of dendritic spines was found in layer II of temporal, parietal and frontal region (420). Higher spine density suggests impaired synaptic pruning, and is correlated to decreased brain weight and lower levels of cognitive functioning in ASD (164, 421–424).

Reduction in neuronal size and loss of neurons in ASD suggests a bias in connectional abnormalities present in multiple areas of the association cortex, specifically within layers that are involved in long-range connectivity (406, 414). The alteration of neuronal classes essential to these circuitries is expected to be the main correlate of altered cognitive processing. In line with that, it was suggested that the total number of a special neuronal subtypes found only in species with highly developed social cognition, von Economo spindle cells, is decreased in autism, but stereological analysis in the frontal part of the insula could not confirm that assumption (425).

Based on the level of gene expression, a reduced number of distinct cell subtypes in layers IV and V, the calbindin and parvalbumin neurons, was suggested (426, 427). So far the only neuron-specific pathology documented histologically in ASD is a decreased number of parvalbumin interneurons in medial prefrontal cortex (428). Parvalbumin expressing cortical neurons provide inhibitory input to cortico-cortical projecting principal cells (429–431). The temporal pattern of change in axon terminals of parvalbumin interneurons parallels the changes in dendritic spine density on layer III principal cells (206, 432). The chandelier subpopulation of parvalbumin neurons, which is projecting to axon initial segment of principal neurons, is found to be affected in prefrontal cortex of ASD subjects (433). Therefore, decreased number of parvalbumin neurons in ASD may be related to alterations of postnatal refinements in cortical circuitry related with associative pyramidal neurons.

In conclusion, despite no direct evidence of L3N pathology in ASD being found, already mentioned findings that in ASD there is a higher spine density in layer II (420), could suggest an altered synaptic pruning of projections arising from associative L3N.

DISCLOSURE OF PRE-EXISTING LESION THROUGH LATE MATURATION OF ASSOCIATIVE NEURONS IN AUTISM

In this manuscript we present an interesting observation about neuron pathology of an ASD case from Zagreb's Neuroembryological Collection (434–436), and evaluate the possibility that appearance of ASD symptoms is correlated with maturation of associative L3N during early childhood, but without disruption of their development.

We performed an in depth analysis of brain tissue from a 23-year-old female with ASD (based on DSM-III-R criteria) (437), with mild intellectual disability and epilepsy. We did not observe changes in the brain's gross morphology, cytoarchitectonic structure, nor expression of non-phosphorylated-neurofilament H (SMI32) which is highly expressed in L3N (**Figure 11**). Unchanged intensity of neurofilament staining (81, 438) suggests normal axonal development of associative neurons. On rapid Golgi impregnated sections of prefrontal cortex (Brodmann area 9), primary motor cortex (Brodmann area 4) and primary visual cortex (Brodmann area 17), we did not observe changes in dendritic size or in spine density of L3N or other classes of principal neurons. However, a small fraction of neurons in the layer II and upper part of layer III in the analyzed areas exhibited abnormalities of dendritic morphology (**Figure 12**).

Alongside well developed and regularly oriented principal neurons, a subset of neurons in layers II/IIIA had spiny dendrites, whose morphology resembled those of immature principal cells (**Figure 13A**). Such dendritic morphology with low spine density is characteristic for developing principal neurons at initial stages of their dendritic differentiation. Developing neurons with similar morphology are found in the neocortex of healthy neonates (**Figure 13B**), but

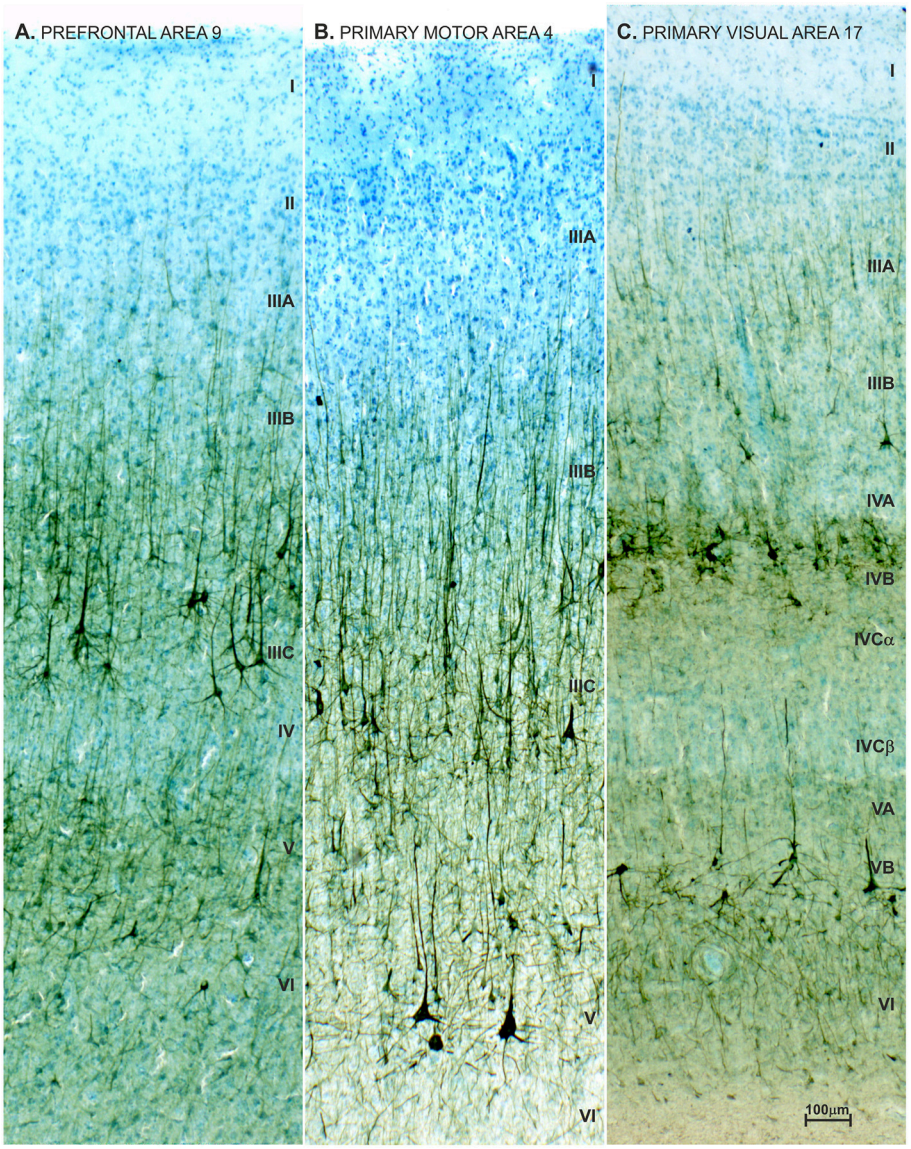

FIGURE 11 | Neurofilament staining (SMI32) in neocortex of adult female with autism. Pattern of SMI32 immunostaining counterstained with Giemsa in the prefrontal area 9 **(A)**, primary motor area 4 **(B)** and primary visual area 17 **(C)**, in a 21-year-old autistic female with a comorbidity in the form of epilepsy and intellectual disability (according to DSMIII classification). The analyzed material is a part of Zagreb's Neuroembryiological Collection (434–436). The distribution, density and level of neurofilament (SMI32) expression did not differ from normative control (81, 438), and no obvious disruption of cytoarchitecture was observed (68, 266). SMI32 stained sections were compared to normative control specimens obtained from the Zagreb Neuroembryological Collection, which includes 29 specimens, with an age span of 19 to 57 years.

not later on. Since only part of the neurons had the immature dendritic morphology (**Figures 13C,D**) (204, 439, 440), we concluded that in the analyzed subject a selective fraction of cortico-cortically projecting neurons is affected. In particular, layer II and upper part of layer III contain cortico-cortical neurons that have relatively short axons, and participated in local microcircuits between neighboring areas (44, 74, 441–445).

Thus, their abnormal development in ASD may be a result of harmful events (including those induced genetically) occurring during the perinatal period. In this manner, development of microcircuitries established by short cortico-cortical neurons would be stalled at the neonatal stage. Importantly, this neuron class is not expected to go through distinct structural complexification after the first year. Therefore, we hypothesed that they will achieve adult structure by the age of 1, but full functional capacity will be achieved through maturation of associative neurons and related circuitries during early childhood (2–6 years). By having subtle alterations of the short cortico-cortical network, first symptoms, in general,

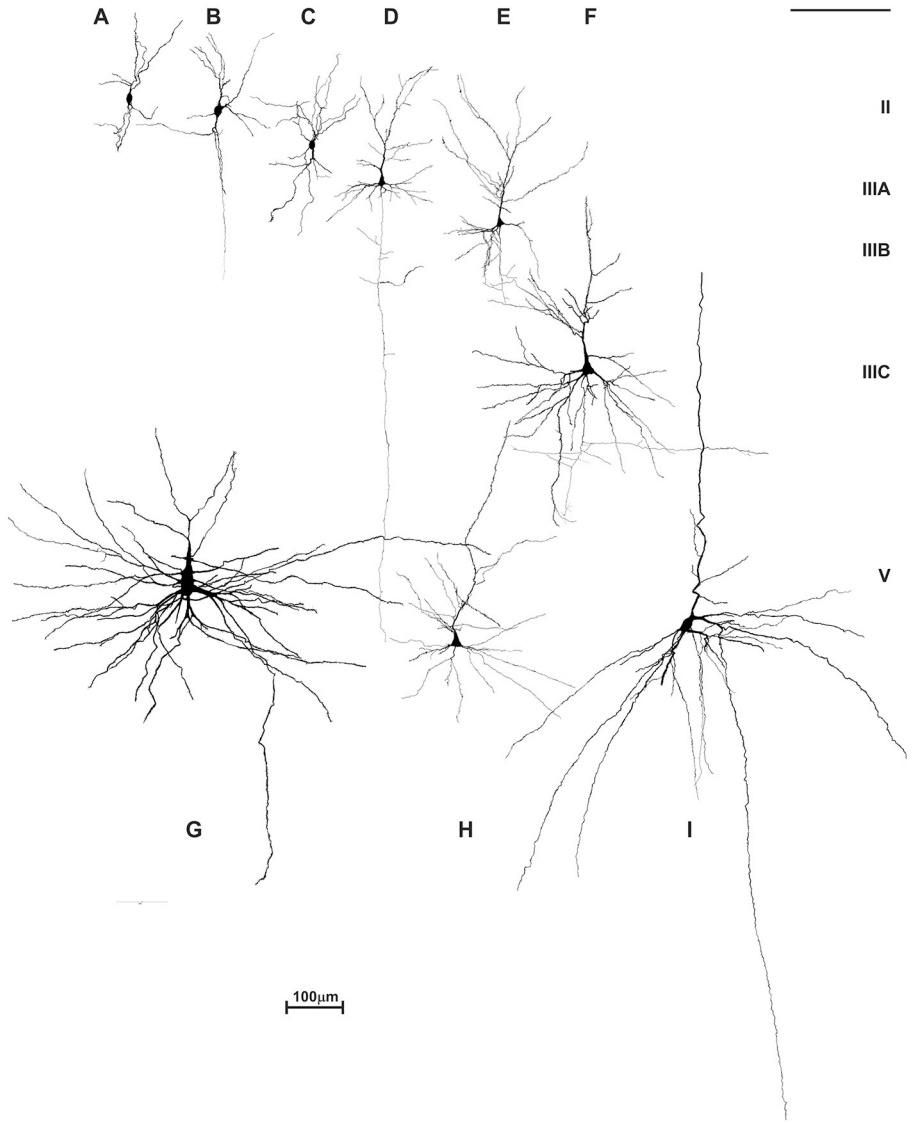

FIGURE 12 | Reconstructions of rapid Golgi impregnated principal neurons in the neocortex of an adult female with autism. Neurons are marked in order of their laminar position: atypical pyramid-like cells **(A–C)** and typical pyramidal cells in analyzed neocortices were shown: layer II **(D)**, layer IIIA/B pyramidal cells **(E)**, layer IIIC pyramidal cells in prefrontal cortex **(F)**, giant Betz pyramidal cells in layer V of motor cortex **(G)**, small pyramidal cells of layer V **(H)** and large Meynert pyramidal cells of layer V/VI in area striata **(I)**. Atypical pyramid-like cells **(A–C)** were mainly located in the layer II, and some in the layer IIIA, but not in any other layers. They have oval cell bodies with bipolar orientation of dendrites, low spine density, and axons directed toward white matter. Such cell types were not observed in Golgi sections of a normal, adult human cerebral cortex. Golgi sections from this case were compared to 29 rapid Golgi stained specimens, with an age span of 19 to 57 years. No other qualitative signs of dendritic, nor spine pathology and density could be found on rapid Golgi slices, and morphology, cell body size, dendritic extent, and complexity **(D–I)** corresponded to pyramidal neurons of the same laminar position in an aged-matched controls.

would not appear before final maturation of local intracortical connections which occur later during childhood. It means that development of L3N and their projections in ASD could take a fully regular course, but may trigger appearance of symptoms.

Neurodevelopmental model with early structural lesions and a delayed appearance of symptoms is already established for schizophrenia (385, 446). Typical schizophrenia symptoms occur predominantly during late adolescence or early adulthood. Such timing is linked to massive synaptic pruning in the prefrontal cortex that occurs as part of normal development. So, in schizophrenia the appearance of symptoms is not a result of disrupted development at that time (90, 99, 447, 448). In fact, events occurring through the course of regular development are a trigger which may cause an already present, but for a long time asymptomatic impairment, to become eminent. Direct evidence for such a hypothesis comes from patients with metachromatic leukodystrophy,

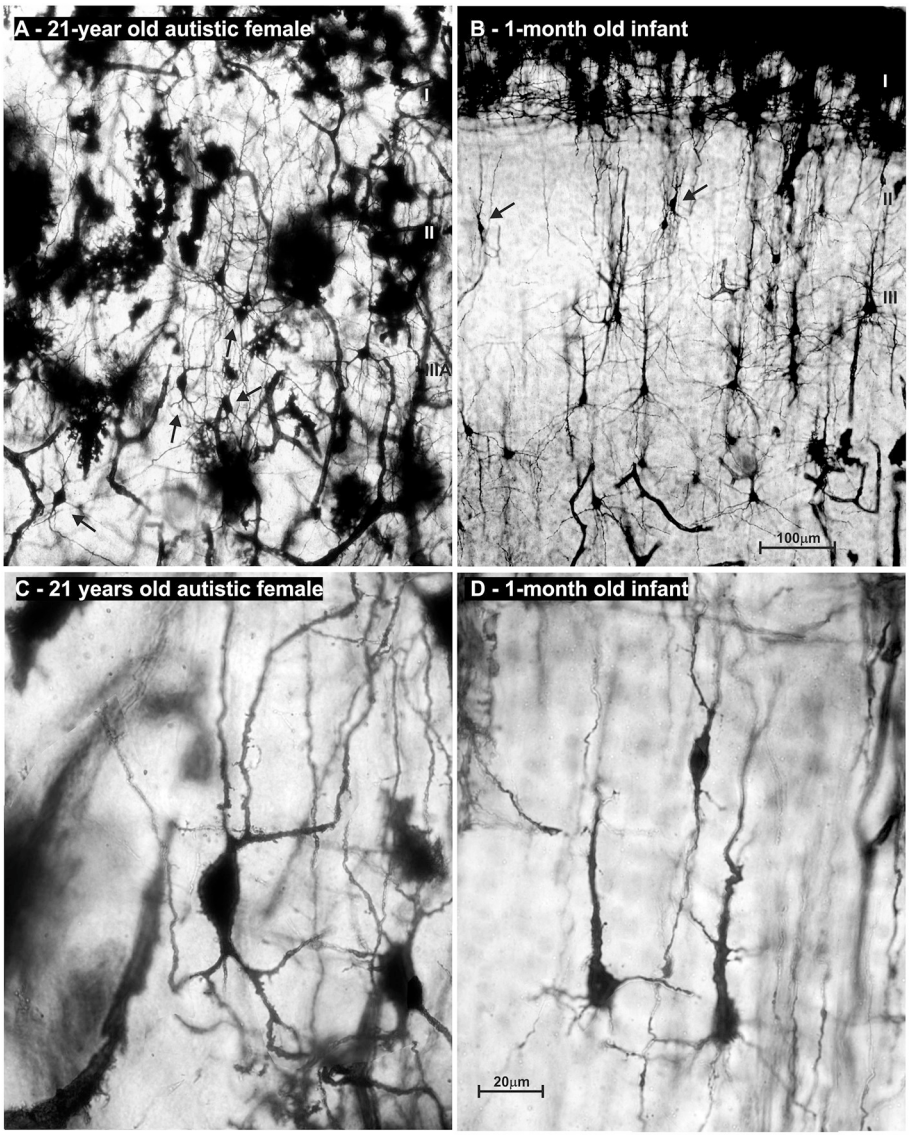

FIGURE 13 | Microphotography of rapid Golgi sections in the prefrontal cortex of an adult female with autism, compared to a normal 1-month-old infant. On low power microphotography of layer II/IIIA in the prefrontal cortex of a 21-year-old autistic female, numerous bipolar—pyramid-like cells were found **(A)**. In the prefrontal cortex of a normal 1-month old infant **(B)**, numerous neurons with similar morphology were found in the same position (arrows). High power microphotography shows layer II bipolar-pyramid like cells in the analyzed subject **(C)**. Note that dendrites had spines, but their density was low. Upper dendrites were directed to the layer I, resembling apical dendrite morphology. On the other pole, two dendrites resembled basal dendrite morphology. An axon arose from one of those dendrites and was directed to the white matter. In the prefrontal cortex of a newborn infant, immature pyramidal neurons with similar morphology are found **(D)**. In the adult autistic subject neuron morphology of spiny bipolar neurons regarding cell body shape, and dendritic complexity is similar only larger when compared to healthy neonate. The presented material is a part of Zagreb's Neuroembryological Collection, which also includes normative control specimens of 31 rapid Golgi specimens, lifespan from infant to adolescent. Scale bar indicates 100 μm **(A,B)** and 20 μm **(C,D)**.

a disorder characterized by demyelination present at birth. The lesion remains without exacerbation up until late adolescence, when a schizophrenia-like psychosis will emerge (449, 450). So, a fixed "lesion" from earlier in development has been silent for decades, and interacts with normal brain maturational events that manifest much later in life. Despite the causative process not being obvious, it is still present long before the symptoms appear and any diagnosis is made (327, 360, 451–454).

DEVELOPMENT OF ASSOCIATIVE NEURONS DURING CHILDHOOD AND RELATION TO ASD SYMPTOMS: ALTERED DEVELOPMENT OR TRIGGER FOR PRE-EXISTING LESION?

In this manuscript, we hypothesize that selective alteration of a specific subset of principal neurons could lead to global changes

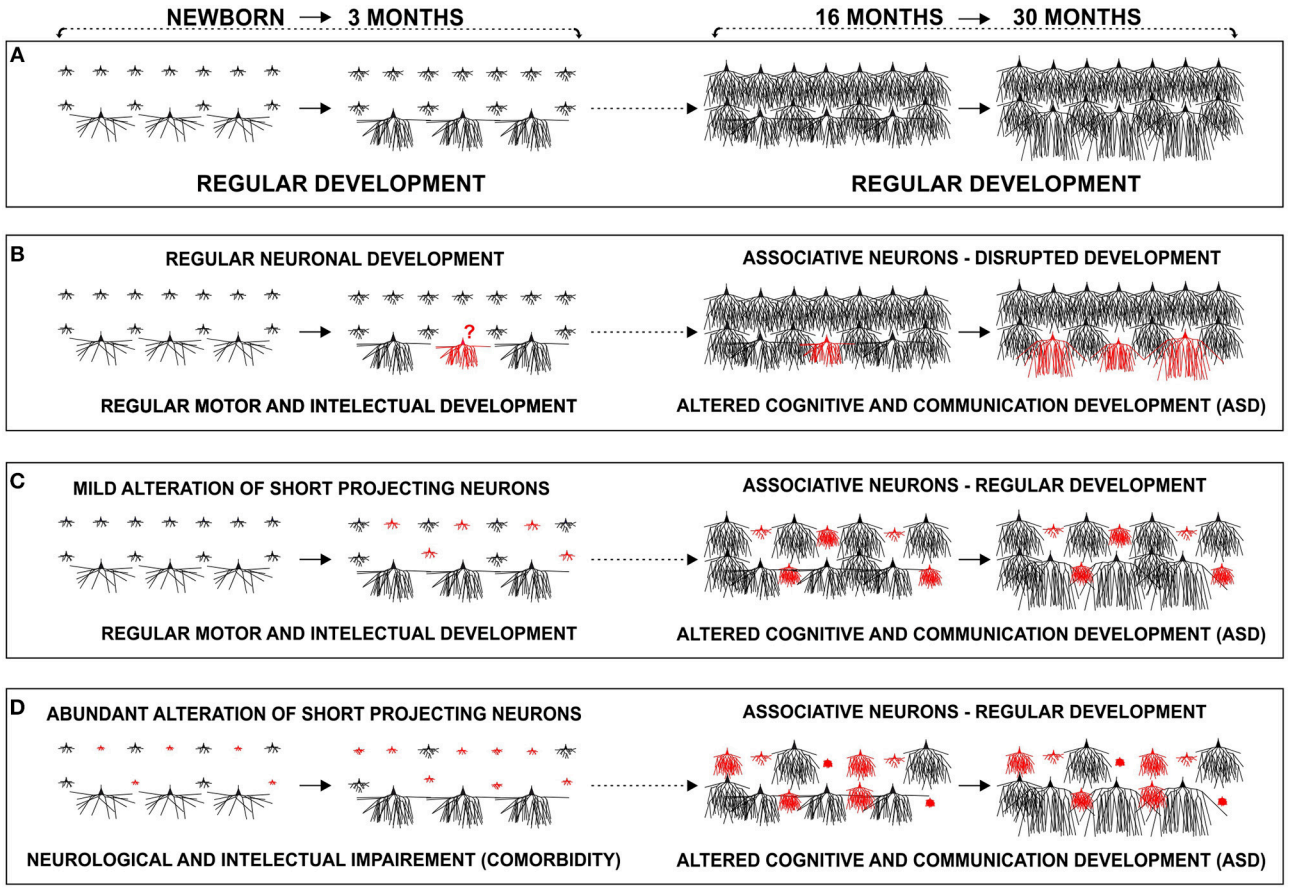

FIGURE 14 | Proposed developmental models of cortico-cortical neuron alterations in autism: schematic representation of changes in dendritic morphology of layer III in the human prefrontal cortex during the early postnatal period (newborn–3 months) and around the second year (16–30 months). **(A)** In a typically developing infant, associative neurons undergo intensive dendritic differentiation during the first 3 postnatal months, whereas other classes of cortico-cortical projecting neurons, undergo major dendritic growth later during their first postnatal year. A late, second growth spurt, with large dendritic elongation in the period from 18–30 months, is so far described only in associative neurons. We propose that maturation of associative neurons during early childhood (1.5–6 years), plays a major role in global, functional changes of the cortical network, related with tremendous cognitive development. **(B)** Disrupted development of associative neurons (red), around age of 2, does not allow enhancement in inter-columnar and inter-areal connectivity, leading to cortical network impairment. Development of associative neurons does not have to be disrupted **(C,D)**. There might be a preexisting (perinatal) alteration, in a subset of short cortico-cortical neurons (red) that would become evident around the age of 2, during typical maturation of intra-cortical connections established by associative neurons. **(C)** In the case of mild perinatal disruption of short cortico-cortical neurons, the lesion would remain functionally silent until late differentiation of associative neurons, whereas in **(D)** cases where alterations are more robust, comorbidities in form of neurological, and intellectual impairment, manifest earlier. We suggest that alteration can be present in various forms thus creating many phenotypes of ASD.

in cortical network connectivity. We applied this model to the ASD and social (pragmatic) communication disorders, which include disrupted social and communication functioning, with more or less severe global disconnectivity.

We propose that contrary to normally developing children (**Figure 14A**), there might be a disrupted development of inter-collumnar connectivity within the prefrontal cortex of ASD patients, as these microciruitries undergo intensive maturation in the period between 2 and 6 years when ASD manifests. Associative L3N, which are the main source of local excitatory cortico-cortical connections, and are thus responsible for inter-columnar connectivity, undergo intensive structural and molecular changes during the same time (**Figure 14B**). Disrupted maturation of intracortical connectivity may then consequently alter outputs from the prefrontal cortex. The severity of this pathology would depend on the extent and timing of disruption within those microcircuitries.

Lacking evidence for structural pathology of the L3N in ASD both in our reported case and overall in the literature, opens the possibility that differentiation of this neuron class takes a regular course. Thus, their developmental incorporation into maturing circuits during childhood will reveal a pre-existing (perinatal) lesion in other neuronal classes and microciruitries. Despite regular development of intracortical connections during the second year, and throughout the rest of early childhood, the cortical network will not be able to reach a new/higher level of information processing, as there is a pre-existing alteration in other classes of projection neurons, e.g., a subset of cortico-cortical neurons with short

projections. We propose that in the case of a more subtle disruption of neurons, which are a source of short cortico-cortical circuitries, the lesion remains fully silent until the age of 2 (**Figure 14C**), whereas in the case of more robust alteration, neurological, and intellectual comorbidity may appear earlier (**Figure 14D**).

These two proposed models are also not mutually exclusive. Direct alteration of L3N or "disclosure" of pre-existing lesions on other neuronal classes during differentiation of associative neurons around the age of 2, could be present in different phenotypes of ASD, or even act at the same time (455, 456).

The important concept in understanding the mechanism of ASD is gene-environment interaction in shaping the architecture of the developing neuronal network (457, 458). The environmental factor may induce or prevent appearance of the ASD pathological functioning, like infection, malnutrition, toxins, or vascular insult (227, 354, 356, 459–462).

While not specifically recognized yet, structural and molecular alteration of mirocircuitry is clearly related with ASD, but in the subthreshold autism phenotype there might be "autistic architecture" of the cortical network, without evident structural or molecular pathology. It is intriguing that in such conditions, psychosocial ambience is exclusively related with appearance of autistic traits, particularly taking into consideration that associative and intracortical circuitries have the highest rate, and most protracted period of synaptic overproduction. Finally, the protracted period of highly plastic circuits involved in ASD pathology opens a new potential in rehabilitation strategies, particularly if early clinical detection approaches are applied (348, 351, 358, 463–472).

ETHICS STATEMENT

This study was carried out in accordance with Croatian legislation and the approval of the Ethical Committee School of Medicine, University of Zagreb (number: 380-59-10106-14-55/152; class 641-01/14-02/01; 1 July 2014).

AUTHOR CONTRIBUTIONS

ZP, DS, and DD contributed equally to the work. ZP designed concept of research, performed Golgi experiments, analyzed Golgi and SMI32 sections, wrote the manuscript, prepared figures. DS analyzed Golgi and SMI32 sections, performed quantitative analysis of neuron morphology in the neonate, prepared figures, wrote and structured the manuscript, and the literature. DD analyzed Golgi and SMI32 sections, wrote and revised the manuscript, and the literature. AH interpretated the data, revised the manuscript. MRR reconstructed Golgi impregnated neurons, revised the manuscript. NJ-M analyzed Golgi and SMI32 sections, performed immunohistochemical experiments, revised manuscript. All authors read and approved the final version.

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Childhood Trauma in Schizophrenia: Current Findings and Research Perspectives

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Schizophrenia is a severe neuropsychiatric disorder with persistence of symptoms throughout adult life in most of the affected patients. This unfavorable course is associated with multiple episodes and residual symptoms, mainly negative symptoms and cognitive deficits. The neural diathesis-stress model proposes that psychosocial stress acts on a pre-existing vulnerability and thus triggers the symptoms of schizophrenia. Childhood trauma is a severe form of stress that renders individuals more vulnerable to developing schizophrenia; neurobiological effects of such trauma on the endocrine system and epigenetic mechanisms are discussed. Childhood trauma is associated with impaired working memory, executive function, verbal learning, and attention in schizophrenia patients, including those at ultra-high risk to develop psychosis. In these patients, higher levels of childhood trauma were correlated with higher levels of attenuated positive symptoms, general symptoms, and depressive symptoms; lower levels of global functioning; and poorer cognitive performance in visual episodic memory end executive functions. In this review, we discuss effects of specific gene variants that interact with childhood trauma in patients with schizophrenia and describe new findings on the brain structural and functional level. Additive effects between childhood trauma and brain-derived neurotrophic factor methionine carriers on volume loss of the hippocampal subregions cornu ammonis (CA)4/dentate gyrus and CA2/3 have been reported in schizophrenia patients. A functional magnetic resonance imaging study showed that childhood trauma exposure resulted in aberrant function of parietal areas involved in working memory and of visual cortical areas involved in attention. In a theory of mind task reflecting social cognition, childhood trauma was associated with activation of the posterior cingulate gyrus, precuneus, and dorsomedial prefrontal cortex in patients with schizophrenia. In addition, decreased connectivity was shown between the posterior cingulate/precuneus region and the amygdala in patients

with high levels of physical neglect and sexual abuse during childhood, suggesting that disturbances in specific brain networks underlie cognitive abilities. Finally, we discuss some of the questionnaires that are commonly used to assess childhood trauma and outline possibilities to use recent biostatistical methods, such as machine learning, to analyze the resulting datasets.

Keywords: neurodevelopment, childhood trauma, diagnostic tools, schizophrenia, machine learning

INTRODUCTION

Schizophrenia is a severe neuropsychiatric disorder that affects about 1% of the population (Jablensky, 1995). It is particularly prevalent in young adults between 20 and 30 years of age and leads to disability in about half of the patients (Murray and Lopez, 1996). The disorder is among the leading cause of years lived with disability worldwide (Whiteford et al., 2013), and, among all mental illnesses, schizophrenia is associated with the highest socioeconomic costs (Gustavsson et al., 2011). This high disorder burden is due to the early onset of schizophrenia in late adolescence and early adulthood and the persistence of symptoms throughout adult life in over 90% of affected patients despite meeting remission criteria (Häfner and an der Heiden, 2007; Schennach et al., 2015). Symptom improvement has been measured as "therapeutic response," which was defined by, e.g., a 20% symptom reduction after 4 weeks of treatment (Kane et al., 1984). Subsequently, the term "remission" was introduced, requiring a simultaneous reduction of all diagnosis-specific core symptoms (positive and negative symptoms) to a level of "mild or less" on established questionnaires (Positive and Negative Syndrome Scale, Brief Psychiatric Rating Scale, Scale for the Assessment of Positive Symptoms, Scale for the Assessment of Negative Symptoms) for a minimum of 6 months (Andreasen et al., 2005). However, since only a small portion of schizophrenia patients can achieve this, the new definition of "recovery" was conceived, which takes into account not only a reduction of clinical symptoms, but also an improvement in occupational, social and adaptive functioning (Chan et al., 2018). However, only 20% of people with schizophrenia are able to work in the primary labor market, and only about 30% are able to maintain a stable relationship (Häfner and an der Heiden, 2007). The unfavorable disorder course is associated with multiple episodes and residual symptoms, mainly negative symptoms and cognitive deficits (McGrath et al., 2008). Cognitive deficits as a core feature of the disorder are present in domains such as episodic memory, executive function, social cognition, and attention (Green, 1996; Hoff et al., 2005). These deficits may lead to memory decline, social withdrawal, and ultimately impaired social and role functioning as measured by the Global Assessment of Functioning (GAF) scale (Green et al., 2015a). Several studies have operationalized the term "recovery" by using the GAF scale to investigate the long-term outcome and its relevant influencing factors in psychosis patients (Scott et al., 2013; Amminger et al., 2015; Koutsouleris et al., 2016; Jagannath et al., 2018; Lho et al., 2019). While childhood trauma has been repeatedly shown to negatively impact "recovery" among schizophrenia patients (Alameda et al., 2015, 2017; Trauelsen et al., 2016), some of these findings were only partially replicated (Trotta et al., 2016; Ajnakina et al., 2018), hereby leading to a rather heterogeneous body of evidence and consequently emphasizing the need for further research into the neurobiological underpinnings of this association.

RISK FACTORS FOR SCHIZOPHRENIA AND THE NEURODEVELOPMENTAL HYPOTHESIS

Twin studies found a heritability of about 60–80% for schizophrenia (Sullivan et al., 2003), and new genome-wide association studies (GWASs) revealed a total of 145 genetic risk loci, the single nucleotide polymorphisms (SNPs), each with only a weak effect (Pardinas et al., 2018). GWAS-based schizophrenia polygenic risk scores showed associations with social and cognitive impairments during early childhood, which were interpreted as being possible early manifestations of genetic liability (Riglin et al., 2017). In schizophrenia, however, about 8,300 SNPs have been estimated to contribute to a common risk of only 32% (Ripke et al., 2013), suggesting that—in addition to the genetic background—environmental factors may be the basis of pathophysiological processes (Manolio et al., 2009).

Schizophrenia has been regarded as a neurodevelopmental disorder in which defective genes and environmental factors interact and induce symptoms of the disorder. The neurodevelopmental hypothesis proposes that schizophrenia is related to adverse conditions, such as genetic background and environmental factors, which lead to abnormal brain development. Disorder onset and first symptoms occur in early adulthood, after synaptic pruning (Weinberger, 1996; Fatemi and Folsom, 2009). In the two-hit model, a neurodevelopmental disturbance during the perinatal period may lead to dysfunction of neuronal circuits and vulnerability to stress during vulnerable brain periods, and later psychosocial stress or drug abuse, for example, may then trigger the disorder (Schmitt et al., 2014). Today, researchers propose that several hits in the form of genetic and environmental risk factors may interact in a complex way during key periods of neurodevelopment and cumulate in the expression of the disorder state (**Figure 1**); these risk factors are hypothesized to be common across neuropsychiatric disorders such as schizophrenia, bipolar disorder, and major depression (Davis et al., 2016). The neural diathesis-stress model proposes that psychosocial stress acts upon a pre-existing vulnerability and triggers the symptoms of schizophrenia (Walker and Diforio, 1997). Specific stress factors have been

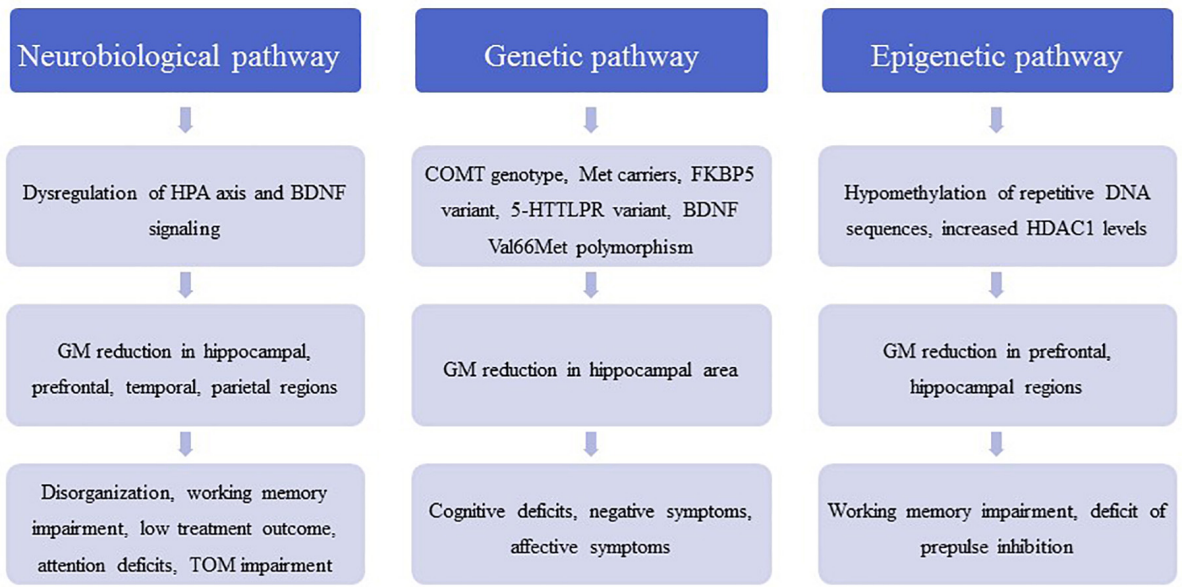

FIGURE 1 | The figure contains a general outline of the three main pathophysiological pathways between childhood trauma and schizophrenic symptoms. These pathways are meant to illustrate the pathological cascade ranging from molecular and (epi-) genetic abnormalities to neuroanatomical changes and eventually to the development of disorder-related symptoms. HPA, hypothalamic–pituitary–adrenal axis; BDNF, brain-derived neurotrophic factor; COMT, catechol-*O*-methyltransferase; FKBP5, FK506 binding protein 5; 5-HTTLPR, serotonin-transporter-linked polymorphic region; HDAC1, histone deacetylase 1; TOM, theory of mind.

identified that trigger or worsen symptoms of the disorder, such as perceived uncontrollable threats to important goals and socio-evaluative threats (Jones and Fernyhough, 2007). In addition, schizophrenia patients are more emotionally reactive than non-psychiatric controls to stressors such as higher arousal and anxiety (Docherty et al., 2009).

NEUROBIOLOGICAL EFFECTS OF STRESS

Stress sensitization may play a role in schizophrenia by lowering the vulnerability threshold for the disorder. The neurobiological consequence of stress sensitization involves dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis, which is the major stress neuroendocrine system of the body and is involved in the production of the stress hormone cortisol by the adrenal glands (Holtzman et al., 2013; Schmitt et al., 2014). A stress-induced activation of the HPA axis contributes to dopamine sensitization in mesolimbic areas and increases stress-induced striatal dopamine release (van Winkel et al., 2008). These effects are important because increased dopamine neurotransmission with overstimulation of the D_2 receptors in several brain regions has been hypothesized in the pathophysiology of schizophrenia, a hypothesis that is supported by the antipsychotic effects of dopamine receptor antagonists (Falkai et al., 2011).

In animal models, acute or chronic stress decreased brain-derived neurotrophic factor (BDNF) levels in the hippocampus, which is involved in synaptogenesis (Neto et al., 2011). In accordance with these findings, stress was found to reduce hippocampal dendrites (Magarinos et al., 2011). Additionally, application of glucocorticoids reduced hippocampal BDNF levels, mimicking the stress reaction (Neto et al., 2011). Animal models have shown that chronic stress or repeated administration of glucocorticoids results in degeneration of hippocampal neurons, with decreased soma size and atrophy of dendrites (Sapolsky et al., 1990; Watanabe et al., 1992). This stress-induced glucocorticoid neurotoxicity (Arango et al., 2001; Frodl and O'Keane, 2013) may contribute to a volume loss in vulnerable brain regions such as the hippocampus; hippocampal volume reduction has been reported for schizophrenia even in early disorder stages (Adriano et al., 2012). Moreover, elevated glucocorticoids can suppress myelination and may affect calcium channels (Damsted et al., 2011). Both mechanisms are related to the pathophysiology of schizophrenia and result in impaired macro- and microconnectivity (Berger and Bartsch, 2014; Cassoli et al., 2015).

In rodents, juvenile social isolation and maternal separation are models of childhood stress, and these animal models have shown lasting effects on the HPA axis along with deficits in hippocampus-dependent learning and memory (Bremne and Vermetten, 2001). The mouse model of juvenile social isolation builds on social isolation immediately after weaning: social isolation leads to schizophrenia-related behavior, such as deficits in prepulse inhibition of the acoustic startle response (PPI) and working memory and decreased social exploration. Furthermore, deficits in oligodendrocyte morphology, reduced myelin thickness,

and decreased myelin basic protein and myelin-associated glycoprotein expression have been detected in brain regions (Varty et al., 2006; Makinodan et al., 2012) and resemble the deficit of myelination and oligodendrocytes in schizophrenia (Cassoli et al., 2015). Importantly, in contrast with the effects of adult social isolation this early-induced phenotype cannot be rescued by later social re-integration (Makinodan et al., 2012), implicating impaired recovery, such as in schizophrenia (**Table 1**).

STRESS RESPONSE IN PATIENTS WITH SCHIZOPHRENIA AND INDIVIDUALS AT ULTRA-HIGH RISK OF DEVELOPING PSYCHOSIS

An increased release of glucocorticoids has been proposed to play a role in the pathophysiology of schizophrenia (Corcoran et al., 2003), and the stress-diathesis model proposes that schizophrenia is associated with elevated baseline and challenge-induced HPA activity (Walker et al., 2008). In addition, cortisol treatment can induce psychotic symptoms (Walker et al., 2008). This model is supported by reports of increased levels of blood cortisol (Ryan et al., 2004) and a blunted cortisol response to stress (Mondelli et al., 2010); the latter was suggested to reflect impaired responsiveness of a desensitized system. Indeed, a meta-analysis on stress-moderating effects of baseline cortisol levels revealed that schizophrenia patients have lower cortisol levels than controls during anticipation of social stress and after exposure to it (Ciufolini et al., 2014). A reduced ability of these patients to appropriately contextualize past experiences has been hypothesized to underlie the missing cortisol response in these experiments (Ciufolini et al., 2014). A blunted cortisol stress reactivity in schizophrenia patients was again reported in a more recent meta-analysis by Zorn et al. (2017), who also pointed toward a possible publication bias as well as an overall small number of studies with properly standardized cortisol protocols as limiting factors for the interpretation of these findings. Moreover, treatment with antipsychotics may have influenced the results (Walker et al., 2008). However, the HPA axis response was also impaired in medication-naïve patients with first-episode schizophrenia, i.e., the cortisol response was flattened, indicating impairments in stress processing (van Venrooij et al., 2012).

According to the neurodevelopmental hypothesis, prodromal and psychotic symptoms occur for the first time in adolescence. In adolescents at ultra-high risk of psychosis (UHR), increased resting cortisol levels have been reported and associated with higher rates of critical statements from relatives and negative self-concept (Carol and Mittal, 2015), indicating that a dysfunction of the HPA axis is related to environmental characteristics. The cortisol level after awakening, which reflects HPA regulation, was also increased in this patient group compared with healthy controls (Nordholm et al., 2018). Additionally, in UHR adolescents a reduced stress responsivity of the HPA axis was correlated with smaller gray matter volumes of the hippocampus and prefrontal, temporal, and parietal cortices, which may represent the neural components in the stress vulnerability model (Valli et al., 2016) (**Figure 1**). Interestingly, those individuals who subsequently developed psychosis showed a significant blunting of the HPA stress response (Valli et al., 2016).

TABLE 1 | Major animal models of early life stress corresponding to childhood trauma.

Study	Stress paradigm	Effects on behavior	Effects on brain biology
Bahari-Javan et al., 2017	Maternal separation	Short-term memory↓ Prepulse inhibition↓ Novel object recognition learning↓ HDAC inhibitor MS-274 rescues ELS induced impairment in PPI and improves novel object recognition learning	Hdac1 mRNA↑ HDAC1 protein↑ DNA-methylation of the Hdac1 gene at the glucocorticoid receptor (GR-) binding site↓
Makinodan et al., 2012	Juvenile social isolation	Social interaction↓ Working memory↓ No reversed behavior by reintroduction to a social environment	Oligodendrocytes with simpler morphology Myelin Basic Protein mRNA↓ Myelin Associated Glycoprotein mRNA↓ Myelin thickness↓ Neuregulin1 type III mRNA↓
Varty et al., 2006	Isolation rearing	Prepulse inhibition↓	

EFFECTS OF CHILDHOOD MALTREATMENT ON EPIGENETIC PROCESSES

In addition to effects on the hormone system, environmental factors, such as childhood trauma, may contribute to genome–environment interactions; these interactions are mediated by epigenetic processes, such as DNA methylation and histone modifications (Fischer, 2014). Hypomethylation of DNA repetitive sequences has been detected in first-episode schizophrenia patients with a history of childhood trauma (Misiak et al., 2015). Inhibitors of histone deacetylases (HDAC) have been suggested to improve cognitive function and ameliorate disorder pathogenesis in neuropsychiatric disorders such as schizophrenia (Nestler et al., 2016). In schizophrenia patients, we found that the experience of childhood trauma was related to increased HDAC1 levels in blood samples (Bahari-Javan et al., 2017). This corresponds with recent findings that HDAC1 levels are increased in the prefrontal cortex and hippocampus of patients with schizophrenia (Benes et al., 2007; Sharma et al., 2008; Bahari-Javan et al., 2017) (**Figure 1**). Interestingly, in mice with early life stress as a model of childhood trauma HDAC1 expression is increased in the prefrontal cortex and hippocampus, and these mice display schizophrenia-like behavioral phenotypes, such as deficits in PPI, working memory, and synaptic plasticity (Bahari-Javan et al., 2017) (**Table 1**).

The effects of childhood trauma on epigenetic mechanisms and the relationship with cognition and disorder symptoms should be investigated in more detail in larger studies in schizophrenia patients.

CHILDHOOD TRAUMA IN SCHIZOPHRENIA: EVIDENCE FROM HUMAN STUDIES

Childhood trauma can be assumed to be a severe form of stress that renders individuals more vulnerable to developing schizophrenia. In a meta-analysis of 18 case-control studies (including 2048 patients with psychosis and 1856 non-psychiatric controls), 10 prospective studies (including 41,803 participants), and 8 population-based cross-sectional studies (35,546 participants), Varese et al. (2012) found that adverse experiences in childhood significantly increased the risk to develop psychosis and schizophrenia. The group showed a significant association between childhood adversity, including trauma, and psychosis: the odds ratio was between 2.72 and 2.99, indicating a strong association between childhood adversity and psychosis, including schizophrenia. Epidemiological studies show that exposure to early stress in the form of abuse and neglect in childhood increases the risk to later develop schizophrenia (Bonoldi et al., 2013). In schizophrenia patients, the most frequent subtype of trauma was emotional neglect, but rates of physical abuse and physical neglect were also significantly increased (Larsson et al., 2013). Childhood abuse and neglect are known to have a negative influence on cognition in patients with schizophrenia and bipolar disorder (Shannon et al., 2011). In first-episode schizophrenia patients, exposure to childhood neglect was a predictor for impairment in social cognition and poorer verbal learning, whereas abuse was not (Kilian et al., 2017). A study in Chinese patients with schizophrenia reported that physical neglect was negatively correlated with delayed memory and attention and with the total cognition score (Li et al., 2017). A large study assessed 406 patients with schizophrenia spectrum disorders with the Childhood Trauma Questionnaire and found that physical abuse, sexual abuse, and physical neglect were significantly associated with reduced scores in working memory, executive function, and verbal tasks (Aas et al., 2012b). In another study, metacognitive capacity was lower in patients with childhood emotional abuse (Aydin et al., 2016). Female patients who reported childhood physical abuse had more psychotic and depressive symptoms than both women without this history and men with or without a trauma history (Kelly et al., 2016).

UHR individuals more frequently had a history of childhood trauma, such as emotional and sexual abuse as well as emotional and physical neglect, while emotional neglect in particular was associated with paranoid symptoms (Appiah-Kusi et al., 2017). Even in UHR individuals, a history of childhood maltreatment predicted poorer functioning at follow-up in both those who had transitioned to psychosis and those who had not (Yung et al., 2015). Childhood trauma did not predict transition to psychosis, but after a 2-year follow-up UHR individuals with higher levels of childhood trauma had higher levels of attenuated positive symptoms, general symptoms, and depressive symptoms and lower levels of global functioning (Kraan et al., 2015). In children born to parents with major psychoses, those who were exposed to abuse or neglect had lower IQ and GAF scores and displayed poorer cognitive performance in visual episodic memory end executive functions (Berthelot et al., 2015).

INTERACTION OF CHILDHOOD TRAUMA WITH GENETIC FACTORS

Gene–environment interactions have been suggested to play a role in the pathophysiology of schizophrenia (**Figure 1**). In 429 patients with schizophrenia or schizoaffective disorder, the catechol-*O*-methyltransferase (COMT) genotype moderated the effects of childhood trauma on cognition and symptoms in methionine (met) carriers with a history of childhood physical abuse and more severe positive symptoms; Met carriers with a history of emotional neglect had more severe negative symptoms (Green et al., 2014). In another study, a variant of the FK506 binding protein 5 (FKBP5) gene interacted with childhood trauma and affected attention in both schizophrenia patients and healthy controls (Green et al., 2015b). In patients with schizophrenia and affective disorders, an interaction between a variant in the serotonin transporter gene 5-HTTLPR and childhood trauma was observed in the California Verbal Learning Test (Aas et al., 2012a). A variant of BDNF Val66Met polymorphism was shown to moderate the impact of childhood adversity on later expression of affective symptoms in schizophrenia patients (Sahu et al., 2016). In 249 patients with schizophrenia spectrum disorder, carriers of the met allele of the BDNF gene exposed to high levels of childhood physical and emotional abuse demonstrated poorer cognitive functioning than monozygotic valine carriers. Moreover, Met carriers exposed to childhood sexual abuse showed reduced right hippocampus volume (Aas et al., 2013), suggesting negative effects on neuroplasticity in the brain. On an epigenetic level, a recent review concluded that childhood trauma was associated with global DNA hypomethylation and reduced BDNF gene-expression in first-episode psychosis subjects (Tomassi and Tosato, 2017). However, the literature on gene–environment relationship in the etiology of psychosis is rather heterogeneous as the results from candidate gene studies could quite frequently not be replicated (Zwicker et al., 2018). Thus, epidemiological studies investigating the interplay between familial and environmental factors in the development of psychosis within large cohorts are another valuable resource for further insight. Using these epidemiological approaches, it was found that environmental risk factors, such as childhood adversity, and a family history of affective and psychotic disorders additively impact the psychosis risk across a multidimensional spectrum of positive, negative, cognitive and affective symptoms (Binbay et al., 2012; Pries et al., 2018; Radhakrishnan et al., 2018). Moreover, studies repeatedly showed that childhood adversity and familial liability increased the risk predominantly for positive symptoms of psychosis, such as delusions and hallucinations, as

well as affective symptoms (Jeppesen et al., 2015; Smeets et al., 2015; Veling et al., 2016). Therefore, the connection between childhood trauma, familial liability and the onset of psychosis is increasingly being labeled as one of the key mechanisms of the proposed "affective pathway" to psychosis (Isvoranu et al., 2017).

BRAIN STRUCTURAL AND FUNCTIONAL CORRELATES OF CHILDHOOD TRAUMA

Emotional neglect in patients with schizophrenia was negatively associated with total gray matter volume and specifically with the density and volume of the dorsolateral prefrontal cortex, which in turn predicted disorganization (Cancel et al., 2015). Interestingly, additive effects of childhood trauma and being a BDNF met carrier on volume loss in the hippocampal subregions cornu ammonis (CA)4/dentate gyrus and CA2/3 have been reported in schizophrenia (Aas et al., 2014). Childhood maltreatment has been associated with reduced hippocampal volume as well as amygdala hyperreactivity and was shown to predict poor treatment outcome (Teicher and Samson, 2013). A functional magnetic resonance imaging study showed that childhood trauma exposure resulted in aberrant function of parietal areas involved in working memory and of visual cortical areas involved in attention. On the basis of these data, the authors hypothesized that childhood trauma in psychosis contributes to alterations in attention during performance of working memory tasks (Quide et al., 2017a). During a theory-of-mind task that reflected social cognition, childhood trauma was associated with activation of the posterior cingulate gyrus, precuneus, and dorsomedial prefrontal cortex in patients with schizophrenia (Quide et al., 2017b). In addition, decreased connectivity between the posterior cingulate/precuneus region and the amygdala was shown in patients with high levels of physical neglect and sexual abuse during childhood (Cancel et al., 2017) (**Figure 1**). Finally, an fMRI study showed an increased brain response to emotionally negative faces compared with the response to positive faces in patients with psychosis and high childhood trauma, as assessed by the Childhood Trauma questionnaire (Aas et al., 2017). Overall, findings from MRI, genetic, and large-scale gene expression and epigenetic studies often were not reproducible and need to be replicated in larger samples before final conclusions can be drawn.

LACK OF SPECIFICITY OF FINDINGS FOR SCHIZOPHRENIA

It must be noted that effects of childhood trauma are not specific for schizophrenia. In childhood-maltreatment related post-traumatic stress disorder (PTSD), a recent meta-analysis clearly showed bilateral reduction of hippocampal and amygdala volumes in the PTSD group compared to healthy controls (Ahmed-Leitao et al., 2016). In addition, cognitive deficits in different domains such as general intelligence, language, information processing, learning and memory and executive skills have been observed in trauma-exposed children with PTSD compared to controls. Trauma-exposed children with PTSD had poorer general intelligence and visuospatial skills compared with trauma-exposed children who did not develop PTSD (Malarbi et al., 2017). Dysfunction of the HPA axis in PTSD has been reported, particularly hypersensitivity of the glucocorticoid receptor (GR). Single-nucleotide polymorphisms (SNPs) in the GR and FKBP5 gene were associated with PTSD risk and the FKBP5 gene SNP interacted with childhood adversity to moderate PTSD risk (Binder et al., 2008; Castro-Vale et al., 2016). Other neurochemical markers for PTSD include neurotrophic factors such as BDNF (Bandelow et al., 2017). Regarding epigenetic factors, DNA methylation is so far the best studied in PTSD and could be responsible for long-lasting effects of gene–environmental interactions (Rampp et al., 2014). Furthermore, effects of parental trauma could be transmitted to the next generation by epigenetic marks (Ramo-Fernandez et al., 2015).

A meta-analysis showed that childhood psychological abuse and neglect were strongly associated with depression (Infurna et al., 2016). Other factors of childhood maltreatment related to adult depression were emotional abuse, sexual abuse, domestic violence and physical abuse (Mandelli et al., 2015). Regarding genetic factors, the corticotropin-releasing hormone receptor 1 (CRHR1) gene may moderate the effects of childhood trauma on depression (Heim et al., 2009; Ressler et al., 2010). BDNF gene methylation level was correlated with depression (Chen et al., 2017).

TOOLS TO ASSESS CHILDHOOD TRAUMA

In the field of childhood trauma research, it is not uncommon to investigate early stress by clinically assessing whether some form of maltreatment took place in the individual's childhood without applying standardized trauma or maltreatment questionnaires (Choi and Sikkema, 2016; Green et al., 2017). However, in the context of clinical studies and to further both the validity and the reliability of the observed effects in childhood trauma studies, standardized instruments should be used. Below, we present a representative selection of the most commonly used questionnaires because it would be beyond the scope of this article to include all the available ones.

Overall, questionnaires on childhood trauma can be categorized into instruments to diagnose PTSD and more specialized assessment tools, whose goal is to assess childhood maltreatment in depth rather than to validate a DSM or ICD diagnosis. A vast number of PTSD-specific questionnaires are available, but we will give an overview of three structured interviews and one self-report measure. The Structured Clinical Interview for DSM-IV (SCID) and the Composite International Diagnostic Interview (CIDI) are structured interviews that cover the entire spectrum of mental disorders and can be applied by both trained professionals and trained lay interviewers. Both interviews have a specific section on PTSD, are frequently used in epidemiological studies and can be used to validate a suspected diagnosis of PTSD (Kessler et al., 2007; Stein et al., 2014; Guina et al., 2016). Another instrument that has also been extensively

reviewed and is regarded by some as the gold standard in diagnosing PTSD is the Clinician-Administered PTSD Scale (CAPS). The CAPS is a 30-item structured interview that should ideally be administered by clinicians and clinical researchers with a working knowledge of PTSD (Weathers et al., 2001, 2018). In addition to these structured interviews, the PTSD Checklist for DSM-5 (PCL-5), a 20-item self-report measure that assesses the 20 DSM-5 symptoms of PTSD, can be used to solidify a PTSD diagnosis (Franklin et al., 2018).

Besides these PTSD-specific diagnostic instruments, a large group of questionnaires focuses on distinct types of childhood maltreatment that do not automatically have to fulfill the PTSD criteria. Childhood maltreatment is usually assessed along the domains of abuse (physical, sexual, emotional/psychological) and neglect (emotional/psychological, physical) (Hovdestad et al., 2015). The most commonly used childhood maltreatment self-reports and semi-structured interviews are described here. The Childhood Trauma Questionnaire (CTQ, Bernstein et al., 1997) is one of the most frequently used self-reports in the current literature (Viola et al., 2016). It has a total of 28 items and measures the above mentioned five types of maltreatment, i.e., emotional, physical, and sexual abuse, and emotional and physical neglect. It also includes a three-item minimization/denial scale to assess the potential underreporting of maltreatment. Another common self-report tool is the Personal Safety Questionnaire (PSQ), which is based on the Conflicts Tactics Scales (Straus and Douglas, 2004). The PSQ queries the occurrence of specific incidents and mainly focuses on physical or sexual abuse; it can be used to sequentially assess incidents that occur in childhood, adolescence, or adulthood. This feature allows researchers to examine both the type (physical or sexual) and timing of abuse over life periods (Rich-Edwards et al., 2011). A questionnaire that specifically focuses on sexual abuse in childhood is the Child Sexual Assaults Scale (CSAS, Koss et al., 1987). This instrument assesses sexual abuse along five subscales: demographic variables subscale, PTSD symptom subscale, center for epidemiologic studies depression subscale, traumatic events questionnaire, and childhood sexual experiences subscale (Yampolsky et al., 2010). An advantage of the CSAS is that it not only assesses possible traumatizing sexual events, but it also checks for PTSD and affective symptoms, therefore mirroring the complex nature of this kind of childhood trauma. Because large multi-center studies have become more important in today's psychiatry, the Early Trauma Inventory Self Report (ETI-SR) represents a powerful assessment tool that has the advantage of being validated many languages (German, French,

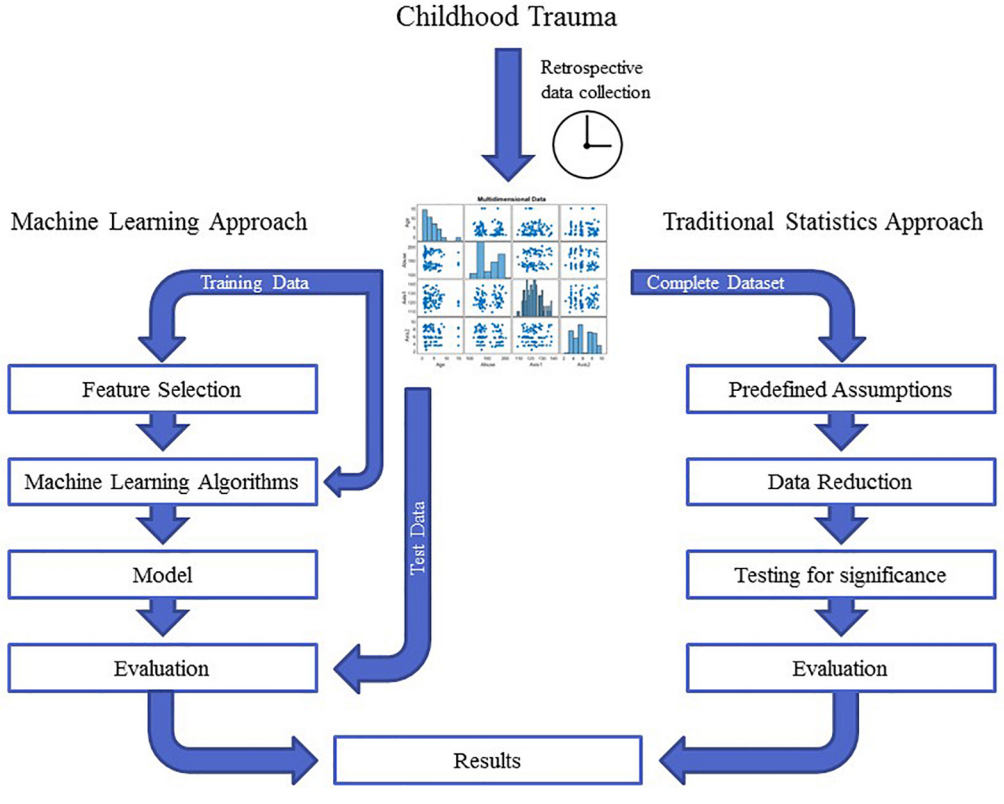

FIGURE 2 | The graph depicts the different workflows in traditional statistics compared with machine learning approaches. In traditional statistics, one approaches a dataset with predefined assumptions, reduces the entire dataset according to those assumptions and then tests a certain hypothesis for significance. In contrast, unbiased machine learning approaches split the dataset into training and test data and let an algorithm learn from the training data in an unbiased and hypothesis-free manner. The evaluation of the analysis then depends on how well the model performs when applied to the test data. These two approaches can yield quite different results.

Chinese, Spanish, Portuguese, Plaza et al., 2012). The ETI-SR is a 56-item inventory that assesses the presence of childhood trauma with a series of "yes or no" questions and includes specific items for physical (9 items), emotional (7 items), and sexual abuse (15 items) and general trauma (31 items). It also assesses the frequency of trauma, age at trauma, perpetrator, and other variables before age 18 (Bremner et al., 2007; Plaza et al., 2011). The Traumatic Life Events Questionnaire (TLEQ) can be a viable alternative if a broader perspective on possible traumatic or adverse life events is desired. This tool assesses exposure to 16 types of potentially traumatic events, including natural disasters, exposure to warfare, unexpected death of a loved one, severe physical assault, different forms of sexual abuse, and experiences of stalking, and also accounts for the frequency and severity of the named traumatic experiences (Kubany et al., 2000). A rather brief self-report questionnaire is the Adverse Childhood Experiences (ACE) questionnaire (Felitti et al., 1998). In a total of 17 questions, this questionnaire assesses childhood abuse within the domains of psychological, physical, and sexual abuse. Additionally, it sets itself apart from many other self-reports because it also includes four categories of childhood exposure to household dysfunction, i.e., substance abuse, mental illness, violent treatment of mother or stepmother, and criminal behavior in the household.

With regards to semi-structured interviews, two more questionnaires are of interest because they both have specific advantages and can be useful in clinical studies: The Early Trauma Inventory and the Children's Life Events Scale (CLES). The former instrument is a semi-structured interview that assesses four domains of traumatic experiences (physical, emotional, and sexual abuse and general traumatic experience) and then addresses the most serious trauma in an additional question (Bremner et al., 2000). This additional question can be very useful in clinical settings because of the potential need for an extended conversation about the most burdensome issue. The CLES, which is an expansion of The Source of Stress Inventory (Chandler, 1981), is a checklist composed of 50 moderate-to-major stressful childhood events that covers categories such as negative emotional feedback, family deaths, maltreatment, failure in school, and family dysfunction (Crossfield et al., 2002; Grandin et al., 2007).

When selecting a questionnaire, equally important to the frequency of use is the analysis of the resulting dataset. Therefore, in the next section we critically discuss current analysis methods and give an outlook on advanced mathematical analysis methods.

NOVEL APPROACHES FOR ASSESSING AND ANALYZING CHILDHOOD MALTREATMENT

Childhood trauma poses several challenges when it comes to data integration and data analysis, mainly with regards to the temporal resolution and the reciprocity and interdependency of the observed phenotypes. The temporal problem arises because most adverse events, which presumably occurred

Supervised learning		
Category	**Algorithms**	**Purpose**
Classification and regression trees	Boosted trees, bootstrap aggregated trees (including random forests), rotation forests	Classification, Regression
Regression analysis	Linear/logistic regression models	Regression
Naive Bayes (NB)	Gaussian NB, multinomial NB, Bernoulli NB	Classification
Artificial Neural Networks (ANN)	Feedforward NN (including Convolutional NN), Regulatory Feedback NN, Radial Basis Function NN, Recurrent NN, Modular NN	Classification, Regression
Vector Machines	Relevance Vector Machines (RVM), Support Vector Machines (SVM)	Classification, Regression

Unsupervised learning		
Category	**Algorithms**	**Purpose**
Clustering	Hierarchical clustering, k-means, (Non-/Bayesian) mixture models, DBSCAN, OPTICS algorithm	Clustering, Classification
ANN	Autoencoders, Deep Belief Networks, Hebbian Learning, Generative Adversarial Networks, self-organizing maps	Clustering, Classification
Latent variable models	Expectation-maximization algorithm, PCA, ICA, Non-negative Matrix Factorization, Singular Value Decomposition (including PLS)	Dimensionality Reduction, Clustering, Classification

FIGURE 3 | The figure depicts the most widely used supervised and unsupervised learning algorithms. NN, neural network; DBSCAN, Density-Based Spatial Clustering of Applications with Noise; OPTICS, ordering points to identify the clustering structure; PCA, principal component analysis; ICA, independent component analysis; PLS, partial least squares.

in childhood, can only be assessed retrospectively and are therefore prone to a certain recollection bias (MacDonald et al., 2015). Furthermore, the sequence in which adverse events in childhood were experienced and psychiatric symptoms developed is often unclear. Another issue lies in the reductionist steps that most studies take during "preprocessing" of the data on adverse experiences or events. In the first step, the data are categorized into specific overarching domains, such as physical or emotional abuse (Morgan and Fisher, 2007), which removes a great amount of the detailed information given by the individual. The next quite common reductionist step is to build sum scores for these domains or, in some cases, a total score for all domains (Hovdestad et al., 2015). In this second step, information given by patients is summarized into nominal or ordinal categories, for example "childhood trauma present" versus "childhood trauma absent" or "high childhood trauma," "medium childhood trauma," and "low childhood trauma" (Daruy-Filho et al., 2011; Agnew-Blais and Danese, 2016). This step removes a great amount of variance and heterogeneity within the dataset that could be important for future analyses. Overall, these preprocessing steps take the interdependency and reciprocity of these adversary factors and their association with the observed psychopathology out of the equation. The possible interactive effects between various kinds of adverse experiences, psychopathological symptoms, and organic features (i.e., structural and functional MRI, DNA variants, gene expression, or epigenetic mechanisms) of the affected individual are largely removed. Thus, most current studies in the field of childhood trauma research are trying to investigate a highly dynamic phenomenon, in which various risk and protective factors interact with each other and produce complex clinical and organic phenotypes, with simplified models that use ordinal and nominal grouping and univariate statistics (**Figure 2**). At the same time, age and sex are mostly controlled for, even though age- and sex-specific effects are found in various psychiatric disorders (Cascio et al., 2012; Gur and Gur, 2016). Based on these methodological issues, the potential advantage of using unbiased and explorative machine learning and multivariate analysis techniques becomes evident (Dwyer et al., 2018b; Jollans and Whelan, 2018). While supervised learning algorithms such as neural networks, tree-based algorithms and vector machines can deliver insights into psychiatric disorders through classification and regression of labeled training data (Bzdok et al., 2018), unsupervised learning algorithms are able to complement this by uncovering latent structures within an unlabeled training dataset (**Figure 3**). Hence, latent variable models based on factor analysis or singular value decomposition (i.e., principal component analysis, non-negative matrix factorization, partial least squares) might be used to explore associative effects between variables of interest (Jessen et al., 2018; Stein-O'Brien et al., 2018). In this context, these associative effects could then be used to further explore causal links between different kinds of childhood adversity, psychopathology, and organic features, e.g., MRI images or DNA expression profiles (Krakauer et al., 2017). Other unsupervised techniques like hierarchical clustering or self-organizing maps could be employed to find mathematically sound clusters of adverse childhood effects or certain phenotypical or organic patterns of childhood trauma, that would be lost if one kept to the overly restrictive approach of using DMS diagnoses or categorical/nominal grouping of childhood trauma loading (Dwyer et al., 2018a). Another interesting topic for analysis with multivariate tools is the timeline of each individual, which is defined by specific childhood trauma experiences and onset and development of certain symptoms.

The effect of childhood trauma on psychopathology and organic variables, such as brain structure and DNA variants (see above), cannot be reduced to a static observation, and we need to consider longitudinal data, the course of disorder, and the biography of each individual. Therefore, mixture models involving (Hidden) Markov Models, Directed Graphical Models, and Bayesian Networks, would help to model, predict and consequently explain the connection and evolution of childhood trauma, psychopathology, and, if desired, its organic correlates (Orphanou et al., 2014; Ryali et al., 2016; Seltman et al., 2016). Some of these approaches have already been undertaken in the field of PTSD research (Galatzer-Levy et al., 2014; Karstoft et al., 2015); however, to our knowledge in the field of childhood trauma and psychosis research no studies have yet been published on machine learning techniques (**Figure 1**). Therefore, this still unexplored field of unbiased, data-driven childhood trauma research has exciting potential and should be one of the priorities for future research.

AUTHOR CONTRIBUTIONS

PF, BM, AS, and DP designed this manuscript. DP, AS, LK, FS, SP, BM, AF, TS, NK, and PF managed the literature searches, interpreted the data, and prepared the manuscript. All authors contributed to and approved the final manuscript and reviewed it critically for important intellectual content.

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REVIEW
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Bridging Basic and Clinical Research in Early Life Adversity, DNA Methylation, and Major Depressive Disorder

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Early life adversity (ELA)- including childhood physical, emotional, and sexual abuse, as well as childhood neglect- is an important predictive factor for negative psychopathology, including Major Depressive Disorder (MDD). ELA can epigenetically regulate key emotional and behavioral systems in ways that can stably persist into adulthood and contribute to the development of MDD and other psychopathology. DNA methylation has been one of the most investigated forms of epigenetic regulation in ELA to MDD pathway. From these studies, genes and sites associated with ELA/MDD have been identified and should be further investigated in order to identify potential avenues for intervention.

Keywords: epigenetics, major depressive disorder, early life adversity, DNA methylation, biomarkers, antidepressant therapies

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INTRODUCTION

Early life adversity (ELA)- which includes forms of child maltreatment such as physical abuse, sexual abuse, psychological and emotional abuse, and childhood neglect- remains a major public health and welfare issue in high income countries (Krugers et al., 2017). It is defined as any act that is either actively conducted, or neglected to be conducted, by a parent or caregiver that either harms or has potential to harm a child, regardless of intent. These acts include physical abuse such as hitting, punching, beating, strangling, and shaking, sexual abuse such as penetration, sexual contact, and exposure to sexual activity, emotional and psychological abuse such as terrorizing, intimidating, and belittling, and forms of neglect such as failure to provide clothing, food, shelter, or neglect in seeking medical care for a child (Leeb et al., 2008). Overall, the various forms of ELA are estimated to affect approximately 10–15% of children (typically categorized as individuals under the age of 18). Specifically, between 4 and 16% of children experience physical abuse, and approximately 15% of children are subjected to sexual abuse annually (Fergusson et al., 2000; Woodman et al., 2008; Gilbert et al., 2009).

In the United States, approximately 80% of substantiated ELA cases are found to be perpetuated by either one or both parents, with 77% of physical abuse, 26% of sexual abuse, 81% of emotional/psychological abuse, and 87% of neglect cases being perpetrated by a

parent (US Department of Health and Human Services, 2008; Jonson-Reid et al., 2012; Nemeroff, 2016). Additionally, 29% of sexual abuse cases are perpetrated by a non-parent relative (Fergusson et al., 2000). It is worth noting that these estimates reflect only officially reported and substantiated cases, and that the actual burden of ELA likely exceeds these estimates.

This substantial phenomenon of ELA is of particular importance as a public health issue considering that repeated exposure to ELA, as well as exposure to multiple types of ELA, has the potential to inflict severe and lasting physical and psychological consequences that represent serious public healthcare and legal costs, as well as increased risk of mortality (Gilbert et al., 2009). ELA has been shown to contribute significantly to the risk of criminal behavior, childhood/adolescent behavioral problems, general physical heath, obesity, promiscuity, prostitution and sex trading, substance use, attempted suicide, and various psychopathologies such as post-traumatic stress disorder (PTSD), and Major Depressive Disorder (MDD), bipolar disorder, and conduct disorder (Thomas et al., 2008; Gilbert et al., 2009; Weder et al., 2014; Nemeroff, 2016; Krugers et al., 2017).

The role of ELA in the development of MDD is of particular interest with regard to public health and welfare, as MDD is the leading cause of global disability, affecting over 300 million people worldwide (World Health Organization [WHO], 2017). It is characterized by persistent depressed mood and loss of interest and pleasure, and may also include symptoms such as weight loss or gain, insomnia or hypersomnia, fatigue, loss of motivation or concentration, recurring thoughts of death, and suicidal ideation (Zajecka et al., 2013).

It is believed that exposure to repeated ELA may increase risk for MDD by cumulating in a hostile and unstable early environment that may trigger adaptive responses in the brain in crucial response systems (such as stress and emotional regulation). There is evidence that these regulatory changes can stably and radically impact aspects of personality development and cognitive functioning in ways that can increase the risk for MDD and associated psychopathologies (Saavedra et al., 2016). The etiology underlying the pathophysiology of MDD is a complex and often varied interplay between genetic, epigenetic, environmental, clinical, and social factors. Research suggests that one of these factors-ELA-can trigger epigenetic alterations (through events such as DNA methylation) in neural systems and genes associated with increased stress response [such as the in the hypothalamic pituitary adrenal axis (HPA)] as a survival mechanism. The persistence of these epigenetic changes often become maladaptive as the early life environment changes, but the altered function of the stress systems does not. While it is not fully understood how these alterations contribute specifically to MDD symptomology, DNA methylation events in these systems in individuals who have experienced ELA have been associated with MDD (Saavedra et al., 2016; Pishva et al., 2017).

It is worth noting that ELA itself is a complex set of phenomena that increases the complexity of MDD pathophysiology, and includes different severities and consequences. There is evidence of a dose-response relationship between ELA and psychopathologies such as MDD, wherein a higher severity of childhood adversity has been linked to increased incidence of psychopathology, higher comorbidity among psychiatric diagnoses, and more severe symptoms within diagnoses (Edwards et al., 2003; Chapman et al., 2004; Heim and Binder, 2012).

With respect to MDD specifically, ELA has been shown to correspond to a more severe course of depression, particularly in terms of increased chronicity, resistance to psychopharmacology and psychotherapy, and greater presentation of atypical symptoms (such as hypersomnia, increased appetite, and increased sensitivity to rejection) (Withers et al., 2013).

There is evidence that certain factors, such as microstructural differences in white matter, as measured by fractional anisotropy, and increased neural fiber connectivity, may be protective against the development of MDD in individuals who experienced ELA. Fractional anisotropy measures the anisotropic movement of water molecules (thought to be important in extrasynaptic communication) associated with fiber bundles (Morgan et al., 2013). One study found that unaffected first degree relatives of individuals with MDD, who also experienced ELA, had significantly increase fractional anisotropy in the right frontal and orbitofrontal lobes, corpus callosum, inferior fronto-occipital fasciculus (IFO), left superior longitudinal fasciculus (SLF) and right fornix (Frodl et al., 2012).

However, resilience is a complex topic in ELA and MDD research, and personal coping mechanisms, behavioral factors, and the presence of positive interpersonal influences concurrent with the ELA were not examined in this study. Epigenetic regulation related to the growth factor brain-derived neurotrophic factor (BDNF) may also play a role in conferring resilience to MDD in these circumstances, and deserves further investigation (Krishnan et al., 2007). Due to all of these intersecting factors, resilience from MDD in the presence of ELA is therefore difficult to study and adequately quantify. Regardless, while certain biological and environmental factors may act as protective factors against MDD development in individuals who have experienced ELA in ways that are not yet fully understood, it remains true that ELA is one of the greatest risk factors for the development of MDD (Saavedra et al., 2016).

Considering the prominent role that severe ELA plays in the development of MDD, and the overall prevalence of MDD, these figures indicate that ELA imposes a substantial social, economic, and public health cost upon society. Due to this, research identifying the mechanisms of action in the ELA to MDD pathway could prove to be a great asset in the development of clinical treatments of MDD. One mechanism by which ELA has been shown to influence emotional and stress regulation systems is epigenetic regulation, such as DNA methylation (Cecil et al., 2016). Although other forms of ELA have been examined with respect to the ELA to MDD pathway, DNA methylation remains one of the most predominately studied, and will serve as the focus of this review. Here, we will first explore how DNA methylation has been implicated in ELA and MDD, and then examine how DNA methylation also has the potential to impact clinical research into MDD diagnosis and treatment.

DNA METHYLATION IN ELA/MDD

Epigenetic regulation describes any regulatory event which causes alterations in gene expression without changes to the original DNA sequence. Various forms of epigenetic regulation have been extensively studied as causal factors in the depression endophenotype in individuals who have experienced ELA, including DNA methylation, histone modifications, and microRNA.

DNA methylation is one of the most well-investigated forms of epigenetic regulation and has been studied as a causal factor in the development of MDD in both candidate gene and genome wide studies (Lutz et al., 2015). During DNA methylation, the DNA is covalently modified via addition of a methyl group to a cytosine nucleotide. This phenomenon is particularly common in cytosine-guanine dinucleotides (CpGs) (Jin et al., 2011). The methylation status of the nucleotide is read by methyl CpG-binding domain (MBD) proteins- such as MeCP2 and MBD1-4- which interact with histone deactylases and DNA-methyltransferases that induce chromatin condensation (Chen et al., 2003; Chahrour et al., 2008; Guy et al., 2011). When methylation is found in promoter regions, it can interfere with transcription factor binding and lead to gene silencing. Conversely, site specific dissociation of the MBDs – specifically MeCP2- have been associated with demethylation, and have been implicated to play a major role in maintaining DNA methylation status (Martinowich et al., 2003).

During prenatal development, DNA methylation patterns are crucial to the process of cell differentiation. However, changes in DNA methylation occur beyond this period, as a mechanism that helps the genome adapt to external signals in the environment. This form of DNA methylation allows the genome to adjust its function through diverse -yet stable- changes. This is one possible mechanism by which environmental stressors such as ELA may trigger epigenetic changes that could ultimately contribute to psychopathology such as MDD (Turecki, 2014).

GENOME-WIDE AND MULTIPLE LOCI STUDIES

Human Post Mortem Brain Tissue Studies

In addition to approaches based on the investigation of candidate genes, the role of DNA Methylation in ELA/MDD has also been examined using larger-scale, genome-wide studies. One of the earliest studies examined hippocampal tissue from French Canadian men with a history of severe childhood abuse (who also died by suicide) using meDIP, an antibody targeting methylated cytosines, coupled with an tiled array containing gene promoters. Differential DNA methylation indicated that 248 sites were hypermethylated and 114 were hypomethylated (Labonté et al., 2012). Significant differences involved genes implicated in cellular or neural plasticity, and some of these findings included histone cluster 2 H2ab (*HIST2H2AB*), nuclear receptor subfamily 1, group D, member 1 (*NR1D1*); and amyotrophic lateral sclerosis 2 (*ALS2*). Specifically, *in vitro* analysis of *ALS2* methylation levels indicated functional effects on gene expression- supporting the hypothesis that ELA contributes to cell-type specific reprogramming of the epigenome in ways that may influence emotional and behavioral dysregulation (Lutz et al., 2015).

In another region of the brain that is hypothesized to be implicated in ELA/MDD- the anterior cingulate cortex (ACC) – genome-wide DNA methylation was assessed using reduced representation bisulfite sequencing (RRBS) in human post mortem brain tissue of depressed suicides who experienced ELA (**Table 1**) (Lutz et al., 2017b). In this study, the most significantly differentially methylated genes were those related to myelin and oligodendrocytes. Specifically, the three most differentially methylated genes were *LINGO3* (which codes for LINGO proteins that are implicated in myelination), *POU3F1* (a transcription factor that controls myelination), and *ITGB1* (an integrin that mediates oligodendrocyte and axonal interactions). These findings suggested that ELA may lead to myelin alterations, a conclusion also supported by both transcriptomic and morphological changes observed in this study using other techniques (**Table 1**) (Lutz et al., 2017b), and consistent with pre-clinical studies indicating that a critical period in the early social environment regulates myelination that in turn is essential for normal cognitive function (Makinodan et al., 2012).

Peripheral Tissue Studies

Other studies evaluated DNA methylation changes associated with ELA, using peripheral samples. A study investigating methylation in the promoter region of over 20 000 genes, including 489 coding for miRNAs, in the blood DNA of males in the 1958 British Birth Cohort found 997 differentially methylated gene promoters associated with ELA (311 hypermethylated and 686 hypomethylated), which were enriched for genes involved in processes such as transcriptional regulation and development. Additionally, they observed ELA associated methylation in thirty-one miRNA genes, six of which showed hypermethylation consistent with hypomethylation observed in their downstream gene targets (Suderman et al., 2015). Another genome wide study by Weder et al. (2014) used the Illumina 450K BeadChip array to determine potential sites of differential methylation that could predict depression in saliva samples collected from maltreated children compared to non-traumatized. Three genes emerged as predictors of depression in combination with ELA: DNA-Binding Protein Inhibitor ID–3 (*ID3*); and Tubulin Polymerization Promoting Protein (*TPPP*) (Weder et al., 2014); and the neurotransmitter gene glutamate receptor, ionotropic *N*-methyl-D-aspartate 1 (GRIN1) (Weder et al., 2014).

More recently, the Illumina 450K BeadChip array was used by the same group in another study of ELA and MDD that sought to predict differential DNA methylation in maltreated children. In this study (Kaufman et al., 2018), methylation of the Orthodenticle homeobox 2 (*OTX2*) gene significantly predicted depression in saliva samples of maltreated children compared to controls (**Table 1**) (Kaufman et al., 2018).

TABLE 1 | Summary of studies investigating clinical applications of ELA and MDD research involving- or related to – DNA methylation in ELA and MDD.

Reference	Sample Size*	Adversity Group	Sample	Purpose	Methodology	Data
Fuchikami et al., 2011	38 (20+18)	MDD	Blood	Diagnostic biomarker	Bisulphite sequencing	DNA methylation in BDNF
Lopez et al., 2013	25	Treatment naïve MDD	Blood	Antidepressant efficacy biomarker	qRT-PCR	BDNF concentration
Perroud et al., 2013	167 (52+115)	BPD and ELA	Blood	Psychotherpay efficacy biomarker	High-resolution melt assay	DNA methylation in BDNF
Mundorf et al., 2018	60	MDD and ELA	Buccal cells	Diagnostic biomarker	Bisulfite sequencing (with EpiTect Kit)	DNA methylation in MORC1
Covington et al., 2009	Mouse model	Chronic social defeat	Mouse NaC tissue	Antidepressant efficacy biomarker	Gene expression arrays	Expression levels of 12 genes in the NAc
Melas et al., 2012	Mouse model	Flinders sensitive line (FSL) genetic rodent model of depression	Mouse prefrontal cortex tissue	Antidepressant efficacy biomarker	Pyrosequencing	DNA methylation in P11
Lutz et al., 2017b	78 (52+26)	MDD/suicide +ELA and MDD/suicide without ELA	Human post mortem ACC Tissue	Quantifying myelination alterations in MDD	bisulfite sequencing/ RNA sequencing/ stereology and coherent anti-Stokes Raman scattering microscopy.	Methylation and expressionlevels of myelin related genes, and imaging of oligodendrocytes and myelinated axons
Kaufman et al., 2018	157	Children who experienced ELA	Saliva and neuroimaging data	Predicting depression in children who experienced ELA	Illumina 450?K beadchip/fMRI	DNA methylation in OTX2 and functional connectivity between the ventromedial prefrontal cortex and structures of the medial frontal cortex
Sacchet and Gotlib, 2017	80 (40 +40)	MDD	Neuroimaging data	Quantifying myleination alterations in MDD	qMRI	Whole brain, Nac, lateral PFC myelin levels

**sample size: total (adversity group+ control cohort).*

Neuroimaging

Imaging data revealed that methylation of *OTX2* is associated with increased functional connectivity between the ventromedial prefrontal cortex and structures of the medial frontal cortex that have been implicated in MDD- such as the paracingulate gyrus, frontal pole, and subcallosal gyrus (**Table 1**) (Kaufman et al., 2018).

CANDIDATE GENES

HPA Axis

Numerous candidate gene studies have also investigated the relationship between ELA, depressive symptoms, and DNA methylation. The majority of these studies have been performed in peripheral samples such as blood and saliva. However, studies utilizing brain and CNS related tissues and systems have also been widely performed in both animal models and human post mortem tissue studies. One biological system in particular, the hypothalamic-pituitary-adrenal (HPA) axis has been the focus of many of these studies.

The HPA axis is a primary stress response system in mammals that has been extensively studied for its role in various psychopathologies including MDD, bipolar disorder, hypersexual disorder, and behaviors like habitual smoking (Rohleder and Kirschbaum, 2006; Chatzittofis et al., 2016; Murri et al., 2016). The HPA response begins in neurons in the paraventricular nucleus (PVN) of the hypothalamus, where the neurohormones CRF and arginine vasopressin (AVP) are released into blood vessels between the hypothalamus and pituitary gland. The pituitary gland is then stimulated to secrete adrenocorticotropic hormone (ACTH) into the circulation to induce the synthesis and secretion of glucocorticoids like cortisol from the adrenal glands (Pariante and Lightman, 2008; Perroud et al., 2011; Keller et al., 2016).

Studies have demonstrated that ELA can foster HPA axis dysfunction in ways that increase susceptibility to depression. Specifically, this dysfunction may result from disturbances to the negative feedback system of the HPA axis, which is partially controlled by the expression of the glucocorticoid receptor (*GR/NR3C1*) in the hippocampus, expression of the *CRF* gene, or changes in *FKBP5* expression- a gene responsible for stabilizing the conformation of the GR (Pariante and Lightman, 2008; Keller et al., 2016).

Animal Models

Animal models of maternal care have provided a substantial contribution to the understanding of the relationship between

the early environment and functioning of the HPA axis. Rats who receive decreased maternal attention in the form of low licking-grooming (LG) behavior in the first week of life have been found to show increased HPA stress responses and hypothalamic corticotrophin releasing factor (CRF) expression, and decreased GR expression and glucocorticoid feedback sensitivity compared to high LG rats. This is due in large part to differential methylation of the *GR/NR3C1* gene which is downregulated in low LG rats, with increased DNA methylation being observed in the promoter region (exon 17) of *NR3C1* in the hippocampus, which persists into adulthood (Weaver et al., 2007, 2014).

In addition to *NR3C1*, hypothalamic corticotrophin releasing factor (CRF), also plays a key role in this feedback system and exerts control over the HPA axis and other stress responses in the brain. Mice subjected to chronic social defeat display decreased DNA methylation in the *CRF* gene, leading to sustained upregulation of *CRF* in neurons of the paraventricular nucleus (PVN) in the hypothalamus (Elliott et al., 2010). This ELA model leads to stress-induced phosphorylation of MeCP2, which results in MeCP2 being dissociated from the inhibitory complex of methylated CpG sites in the *CRF* promotor region, resulting in increased transcription. This mechanism of *CRF* upregulation is supported by the observation that mice with MeCP2 knockouts in the PVN present with abnormal physiological stress responses (Fyffe et al., 2008). Conversely, a rat model of chronic mild stress actually demonstrated increased DNA methylation of the *CRF* promoter in the PVN, indicating that sex-specific and/or dose-dependent effects may play a role in altered HPA axis activity (Sterrenburg et al., 2011).

Human Post Mortem Brain Tissue Studies

Human post mortem brain tissue studies have also demonstrated ELA-associated epigenetic changes in the HPA axis, as hippocampal tissue obtained from individuals who died by suicide and experienced ELA (particularly severe childhood abuse) displayed an increase in *NR3C1* methylation, and decreased *NR3C1* expression (McGowan et al., 2009; Suderman et al., 2012).

Finally, in the neurons of the hypothalamic paraventricular nucleus, increased arginine vasopressin (AVP) release accompanied by HPA axis hypersensitivity has also been linked to hypomethylation in reduced maternal care animals, in a similar mechanism to *CRF* hypomethylation (Murgatroyd et al., 2009).

Peripheral Tissue Studies

The GR regulating gene, *FKBP5,* has also been the subject of several studies investigating differential methylation in peripheral tissue. Certain polymorphisms of *FKBP5* (rs1360780, rs9296158, rs3800373, and rs9470080) interact with ELA to predict MDD and suicide attempts (Roy et al., 2010; Appel et al., 2011). One study by Klengel et al. (2012) examined *FKBP5* methylation levels in the blood of individuals with a history of ELA and found hypomethylation in intron seven. Another study collected saliva samples from children who had experienced ELA, and assessed them for DNA methylation levels at two CpG sites in intron seven of *FKBP5* (Tyrka et al., 2015). Once again, hypomethylation was found at both sites in intron seven. It was hypothesized that increased cortisol release following ELA in *FKBP5* risk allele carriers would signal differential methylation in *FKBP5* and disrupt the feedback loop responsible for *FKBP5* and GR activity, leading to stress response dysregulation and increased susceptibility to MDD (Tyrka et al., 2015).

NEUROTRANSMITTERS

DNA methylation in neurotransmitter genes has also been explored in CNS and peripheral human samples, as well as animal studies, with respect to the ELA to MDD pathway. In addition to the previous discussed Weder et al. study that implicated the neurotransmitter gene glutamate receptor, ionotropic *N*-methyl-D-aspartate 1 (GRIN1) in ELA/MDD, one gene, *5-HT2A*, has been explored in the prefrontal cortex (PFC) for its potential contribution to ELA related MDD and suicide (Du et al., 2000, 2001; De Luca et al., 2007, 2009). One particular *5-HT2A* polymorphism, the C allele (C102T), has been associated with depression and suicide, and has been found to be more abundant in the PFC of suicides. Although non-significant hypomethylation differences were found in the C allele in suicides, hypermethylation was reported in suicide ideators, indicating potential differential methylation patterns between these two groups (De Luca et al., 2009).

In the anterior insula, downregulation of the kappa opioid receptor (*Kappa*) and decreased DNA methylation in the second intron of the *Kappa* gene has been found in depressed suicides who experienced ELA. This intron serves as a genomic enhancer in GR binding regulated *Kappa* expression; indicating a mechanism by which endogenous opioids act on stress systems (Lutz et al., 2017a).

Numerous studies have investigated the serotonin transporter gene *SLC6A4*, which facilitates neurotransmitter reuptake at serotonergic synapses. Hypermethylation has been demonstrated at various CpG sites in ELA cohorts depending on sex, ELA magnitude, and type of ELA experienced (ex. physical versus sexual abuse) (Vijayendran et al., 2012; Alexander et al., 2014; Booij et al., 2015; Frodl et al., 2015). ELA related *SLC6A4* promoter DNA methylation status was also found to be significantly associated with more severe pre-treatment presentation of depressive symptoms, elevated stress, and higher family history of psychopathology (Menke et al., 2012).

GROWTH FACTORS

Growth factors, such as brain derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF), also show differences in expression and DNA methylation related to ELA and MDD. In particular, BDNF has been the subject of significant basic and clinical research with regards to ELA/MDD, as it has been the subject of candidate gene studies, and shows promise as a biomarker and target for epigenome targeted antidepressant therapy.

In the nucleus accumbens (NAc), glial cell-derived neurotrophic factor (*GDNF)* expression appears to be reduced in chronic early life stressed mice with anxiety- and depressive-like behaviors. This reduction appears to be due to increased DNA methylation related to increased MeCP2 binding (Chahrour et al., 2008; Uchida et al., 2011).

The brain derived neurotrophic factor (*BDNF*) gene is expressed in the adult PFC, and plays a critical role in neural and behavioral plasticity, and the development of psychiatric disorders such as depression, bipolar disorder, and schizophrenia when coupled with ELA (Kundakovic et al., 2014). Hypomethylation of *BDNF* has been observed in both human PFC tissue in individuals who have experienced ELA, as well as in the PFC of rats exposed to early life stress (Roth et al., 2009; Roth and Sweatt, 2011).

With a significant amount of research substantiating the biological relevance of genes such as *BDNF* in the development of ELA related MDD, reliable estimates of methylation at this locus could potentially be used as a diagnostic tool (**Table 1**) (Fuchikami et al., 2011; Kundakovic et al., 2014).

Taken in context with the established ELA related behavioral vulnerabilities that *BDNF* methylation alterations represent, blood *BDNF* expression and methylation levels could potentially be of clinical use as a tool in clinical care of psychopathologies such as MDD, when coupled with the presentation of symptoms and a reported history of ELA (Shonkoff et al., 2009).

One study examined two *BDNF* CpG islands (I and IV) as potential diagnostic biomarkers of MDD. Examining methylation levels at CpG islands I and IV in human cohorts of MDD patients and healthy controls, it was found that CpG 1, but not IV, levels could accurately discriminate controls from the MDD cohort. While this study utilized a relatively small sample size (20 MDD vs. 18 control individuals), it indicated that DNA methylation levels have the potential to be a valuable resource in clinical diagnosis as a biomarker (**Table 1**) (Fuchikami et al., 2011).

Clinical research focused on the epigenetic effects of ELA has the potential to not only create new diagnostic procedures, but also to create novel antidepressant therapies that target the epigenome or use the epigenome as a means to evaluate antidepressant efficacy (Qiu et al., 2017). BDNF in particular appears to be influenced by antidepressant treatment (Fuchikami et al., 2016). Notably, in several preclinical animal models, treatment with the histone deacetylase (HDAC) inhibitor sodium butyrate has yielded reduced depressive and manic-like behaviors, as well as reversed decreased expression in BDNF and other neurotrophic factors in chronically stressed animal models (Moretti et al., 2011; Resende et al., 2013). Similarly, animal models treated with the antidepressant imipramine present with reversed behavioral consequences of early life stress in the *BDNF* promoter region (Tsankova et al., 2006; Hollis et al., 2010, 2011; Covington et al., 2011).

It has been suggested that effective antidepressant treatment should correspond to an increase in peripheral *BDNF*, and that a sustained lack of increase in *BDNF* over the first week of treatment likely predicts treatment resistance (Tadić et al., 2011). This finding is based on *BDNF* exon IV promoter methylation data from an MDD cohort being treated with several antidepressants. This effect was mirrored by findings showing that citalopram treatment in MDD patients increased *BDNF* expression in treatment responders, and significantly reduced H3K27me3 levels at the *BDNF* IV promoter (**Table 1**) (Lopez et al., 2013).

ANIMAL MODELS OF OTX2

In an elegant study, Peña et al. (2017) described that juvenile knockdown of the transcription factor orthodenticle homeobox 2 (*Otx2)* in the ventral tegmental area (VTA) – a brain reward region- increases stress susceptibility similarly to early life stress. This knockdown of *Otx2* was also associated with downregulation of several target genes, many of which play a role in brain development. Animals in this model also exhibited depressive-like behaviors following a second exposure to stress in adulthood. Based on these findings, the researchers proposed that a "two-hit" stress model may be in effect, wherein ELA increases stress susceptibility in the VTA via *Otx2* mediation, contributing to a depression-like state and sustained transcriptional alterations in adults following adult social defeat. As the association between ELA/MDD and OTX2 has been also reported in humans (Kaufman et al., 2018), continued investigation into how *Otx2* mediates sustained stress susceptibility is warranted.

STRUCTURAL STUDIES OF MYELINATION

The role of myelination in ELA/MDD may also be of potential clinical interest. Myelination is essential in developing and maintaining complex cognitive and behavioral functions, as it increases action potential transmission per unit time, thereby increasing the connectivity and information processing capacity of the human brain (Grydeland et al., 2013). Myelination occurs when oligodendrocytes cells generate myelin, which insulates the neuronal axons and facilitates electrical signal propagation, helping maintain the circuitry of neural networks (such as the axon segments within the cortex), which are crucial to optimizing basic cognitive and behavioral functions (Haroutunian et al., 2014; Long and Corfas, 2014). Due to this, reductions in cortical oligodendrocytes and deficits in myelin gene expression have been associated with cognitive and behavioral dysregulations which include- but are not limited to- schizophrenia, bipolar disorder, and MDD (Uranova et al., 2004). Intracortical myelin abnormalities vary across psychopathologies, with deficits in white matter volume and myelination being more prominent in bipolar disorder and MDD (Mosebach et al., 2013). With respect to MDD specifically, quantitative magnetic resonance imaging (qMRI) analysis has revealed reduced myelin levels in the white matter of brain regions such as the nucleus accumbens (NAcc) and lateral prefrontal cortex (LPFC) (**Table 1**) (Sacchet and Gotlib, 2017).

Structural alterations in white matter have also been associated with ELA in MRI studies, with more recent MRI strategies being able to more effectively investigate white matter myelin content

to the level of bundles with hundreds or thousands of axons (Stikov et al., 2015). Although oligodendrocytes and myelin were long thought to be static, these imaging studies, as well as animal studies, have demonstrated that various forms of learning, as well as social and environmental conditions, correlate with structural changes to white matter. This suggests an important role for myelination in brain plasticity (Long and Corfas, 2014).

In the previously summarized recent study by Lutz et al. (2017b), state of the art microscopy methods were utilized to more precisely measure microstructural changes to white matter myelin that are associated with ELA. They discovered that in post mortem brain tissue from adults who experienced ELA and died by suicide, axonal integrity and myelination of individual fibers was disrupted, particularly in small-diameter axons which may be related to cortico-cortical projections. These structural alterations were found in conjunction with cell type-specific differences in DNA methylation of oligodendrocyte genes such as *POU3F1* and *LINGO3* in oligodendrocytes (but not neurons), and global decreased expression of a large collection of myelin related genes in the cingulate cortex. These results suggest that ELA may contribute to a neurobiological vulnerability that increases the risk of psychopathology throughout life in affected individuals (**Table 1**) (Lutz et al., 2017b). These findings may prove of future clinical significance, although further research into the brain region-specific effects of ELA/MDD on myelin is needed before any potential antidepressant therapies or diagnostic procedures related to myelination can be explored further.

ADDITIONAL DNA METHYLATION STUDIES OF CLINICAL INTEREST

BDNF is not the only gene that has potential as a biomarker for MDD in individuals who have experienced ELA. The MORC family CW-type zinc finger 1 (*MORC1)* gene, has been found to be hypermethylated in buccal cells of individuals who experienced ELA and scored high on the Beck Depression Inventory. Researchers have suggested that *MORC1* could reliably be used as a non-invasive diagnostic biomarker for MDD in individuals who have been affected by ELA (**Table 1**) (Mundorf et al., 2018).

Other genes implicated in social defeat models of ELA may also serve as promising targets for the future development of novel antidepressant treatments. One mouse model of ELA that presented with altered gene expression in 12 genes in the NAc, including genes coding for actin cytoskeleton reorganization machinery, transcription factors, signaling molecules, and neurotransmitter receptors, that was reversed by treatment with the antidepressant MS-275 (**Table 1**) (Covington et al., 2009).

In the prefrontal cortex, DNA methylation of the P11 promoter was reduced in MDD patients via administration of escitalopram, increasing P11 and decreasing the presence of DNA methyltransferases (**Table 1**) (Melas et al., 2012). The epigenetic changes detailed in these studies represent both potential targets for antidepressant therapy, as well as potential biomarkers of drug efficacy.

While there has been an ample amount of basic research on the ELA to MDD pipeline examining regions and genes that are differentially methylated in ELA/MDD, clinical research expanding on these findings has been less bountiful. Further clinical studies that examine expression levels of key genes in peripheral tissue (such as buccal swabs or blood) before, during, and after antidepressant treatment in ELA/MDD subjects could feasibly be undertaken to help evaluate the efficacy of many antidepressant treatments, whether they be pharmacological, psychotherapeutic, or through methods such as brain stimulation- the last two of which (to the authors' knowledge) – remain to be explored.

CONCLUSION

The studies described herein have contributed to our understanding of how ELA can epigenetically regulate behavioral and emotional response systems in the brain in ways that contribute to- and increase the vulnerability to- psychopathologies such as MDD. The research indicates that genes that code for the HPA axis, neurotransmitters, growth factors such as BDNF, transcription factors such as OTX2, and myelination/oligodendrocytes may all play a role in this pathway, demonstrating how diverse and complex the epigenetic alterations associated with ELA likely are. Due to this complexity, it is perhaps not surprising that clinical progress-such as the development of biomarkers for ELA/MD and antidepressant therapies that target the epigenome-has thus far been limited and preliminary. This is why further studies will be necessary before the full potential clinical significance of the basic research into the ELA/MDD pathway can be determined.

AUTHOR CONTRIBUTIONS

AB wrote most of the manuscript, including the entire first draft. LF carried out substantial editing, revisions, and rewrites. GT edited and revised the final draft.

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frontiers in Molecular Neuroscience

BRIEF RESEARCH REPORT
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No Association of Variants of the NPY-System With Obsessive-Compulsive Disorder in Children and Adolescents

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Obsessive-compulsive disorder (OCD) causes severe distress and is therefore counted by the World Health Organisation (WHO) as one of the 10 most impairing illnesses. There is evidence for a strong genetic underpinning especially in early onset OCD (eoOCD). Though several genes involved in neurotransmission have been reported as candidates, there is still a need to identify new pathways. In this study, we focussed on genetic variants of the Neuropeptide Y (NPY) system. NPY is one of the most abundant neuropeptides in the human brain with emerging evidence of capacity to modulate stress response, which is of high relevance in OCD. We focussed on tag-SNPs of *NPY* and its receptor gene *NPY1R* in a family-based approach. The sample comprised 86 patients (children and adolescents) with eoOCD with both their biological parents. However, this first study on genetic variants of the NPY-system could not confirm the association between the investigated SNPs and eoOCD. Based on the small sample size results have to be interpreted as preliminary and should be replicated in larger samples. However, also in an additional GWAS analysis in a large sample, we could not observe an associations between NPY and OCD. Overall, these preliminary results point to a minor role of NPY on the stress response of OCD.

Keywords: NPY, obsessive-compulsive, children, anxiety, neuropeptide

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INTRODUCTION

Obsessive-compulsive disorder (OCD) has a life-time prevalence of 2%–3.3%, both in adults and children and has its peaks of onset around 12 years (early onset OCD, eoOCD) and in early adulthood (late onset). The occurring obsessions/compulsions are interfering significantly with the patients' everyday life and cause severe distress and anxiety. Additionally, 75% of the patients have at least one comorbidity. In eoOCD these are in particular attention deficit hyperactivity disorder, major depression and anxiety disorders (Fireman et al., 2001). OCD is the fourth most psychiatric disorder and due to its frequently severe impact on affected patients' lives, the World Health Organisation (WHO) counts OCD to the 10 most impairing illnesses (Karno et al., 1988; Weissman et al., 1994; Lopez and Murray, 1998).

Evidence for a strong genetic component in the development of OCD derives from twin and family genetic studies as well as segregation analyses. Prevalence in first-degree relatives of OCD patients is about four times increased and even about eight times higher in eoOCD patients (Pauls et al., 1995; Alsobrook et al., 1999; Hanna et al., 2005). A higher familial load of eoOCD was observed in general, suggesting a greater importance of genetic factors (Pauls et al., 1995; Nestadt et al., 2000). In the search for genetic underpinnings in OCD, mainly genes of the serotonergic system were focussed, driven by the pharmacological effectivity of selective serotonin reuptake inhibitors (SSRIs). Several studies aimed at the gene encoding the serotonin-transporter (Bengel et al., 1999). Nonetheless, study results are heterogeneous and replications and a recent meta-analysis revealed a rather low effect size for the serotonin transporter (Walitza et al., 2014). Further, evidence emerged that the glutamate system is involved in OCD and might be a potential alternative pharmacological treatment target, especially since the association with the glutamate receptor gene *SLC1A1* has been replicated in several studies (Wendland et al., 2009; Stewart et al., 2013; Grados et al., 2015). However, variance explained by the known candidates is still rather small and given the clinical complexity of OCD there is an understanding that many genes are involved in the disease's pathogenesis.

In the search for further neuronal messengers involved, the Neuropeptide Y (NPY) is a highly interesting candidate (Tatemoto et al., 1982). It is one of the most abundant neuropeptides in the human brain and has multiple regulating effects in the nervous system. NPY, long known as a neuropeptide modulating feeding behavior and energy homeostasis (Morton and Schwartz, 2001), has been reported influencing neuronal processes relevant in psychiatric disorders. For instance, there is a rising evidence that the NPY-system including the NPY-receptors is involved in the development of alcohol and drug dependency (NPY1R), stress coping (NPY1R, NPY2R, NPY5R) and anxiolysis (NPY1R, NPY2R; Gerald et al., 1996; Movafagh et al., 2006; Hirsch and Zukowska, 2012; Pedragosa-Badia et al., 2013). Especially regulation of stress and anxiety levels are crucial elements in the development and maintenance of OCD. Patients affected by OCD suffer fear and simultaneously triggered stress when they experience the feeling "*something is not in order*." NPY is released in many brain areas that participate in stress response. These are, for example, the adrenergic and noradrenergic (NA) neurons of the brainstem, the corticotropin-releasing factor (CRF)-neurons of the nucleus paraventricularis, the amygdala and the hypothalamus as well as an impact on the hypothalamic-pituitary-adrenal (HPA)-axis (Heilig, 2004; Alldredge, 2010). The NPY1R-receptor was shown to modulate anxious behavior and stress which can be reversed by NPY administration (Kormos and Gaszner, 2013). Interestingly, there is also an interaction of NPY and the serotonergic system (Diksic and Young, 2001; Pittenger and Bloch, 2014). SSRI administration leads to a higher NPY release in stressed depressive mice and a mediating role of NPY on SSRI effects is discussed (Caberlotto et al., 1998; Redrobe et al., 2005; Christiansen et al., 2011). This interplay of SSRI and NPY showed for depression might be of relevance in OCD as well, especially due to the high comorbidity rate of the two illnesses (Torres et al., 2016).

Therefore, in this molecular genetic study, we aimed at the NPY-system as a messenger system with potential influence on the pathogenesis of eoOCD. Due to their previously reported functions in the regulation of anxiety and stress, we focussed on *NPY* and its receptor *NPY1R* in a family-based approach. Genetic variants of *NPY* and *NPY1R* were genotyped in a German family-based sample. The present study is the first investigating genes of the NPY-system in eoOCD.

MATERIALS AND METHODS

Subjects

All patients were recruited at the Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital Würzburg, Germany. Patients and parents were all of Caucasian descent and agreed to participate in the study. All participants and, in the case of minors, their parents, gave written informed consent. The study was approved by the Ethics Committees of the University of Würzburg.

Patients were included in the study after they had fulfilled the diagnostic criteria for eoOCD according to DSM-IV (American Psychiatric Association, 2000), which was valid at the time of recruitment, and ICD-10 (Dilling, 2015). Patients and parents were interviewed separately for childrens' psychiatric disorders with the German semi-structured clinical "Diagnostic Interview for Psychiatric Disorders in Children and Adolescents" (DIPS; Schneider et al., 1995). Subsequently, the severity of symptoms was assessed by the Childrens' Yale-Brown Obsessive Compulsive Scale (cY-BOCS; Goodman et al., 1989; Scahill et al., 1997). Subjects with comorbid disorders were only included when OCD was the main psychiatric diagnosis. Senior clinicians or psychologists performed all interviews and ratings.

Exclusion criteria were a lifetime history of psychotic disorders, Tourette's syndrome, autism spectrum disorders, alcohol dependence or mental retardation (IQ $\leq$ 70).

The sample comprised 86 patients (children and adolescents) with eoOCD and their parents. Fifty-one children were female, 35 were male. The patients' mean age was 10.7 years (SD = 2.8) at the onset of disease in a range from 3 to 15 years. In 32 patients onset was earlier than the age of 10 years. Eleven patients had tic-disorders as comorbidities. Further comorbidities as depression, ADHD or anorexia nervosa existed frequently in the patients' medical histories but were not clinically relevant at the time of study inclusion. The sample was part of previous genetic analyses and described further in previous publications (e.g., Walitza et al., 2008).

Based on a power analysis with alpha 0.05 and beta 0.80 (according to Neumann et al., 2014) a sample size of at least $N = 105$ would have been required to unravel significant effects. Therefore, we additionally analyzed associations of NPY and OCD with the recent GWAS by Arnold et al. (2018).

To enlarge the sample size of OCD patients we used the data of the most recent GWAS meta-analysis on OCD

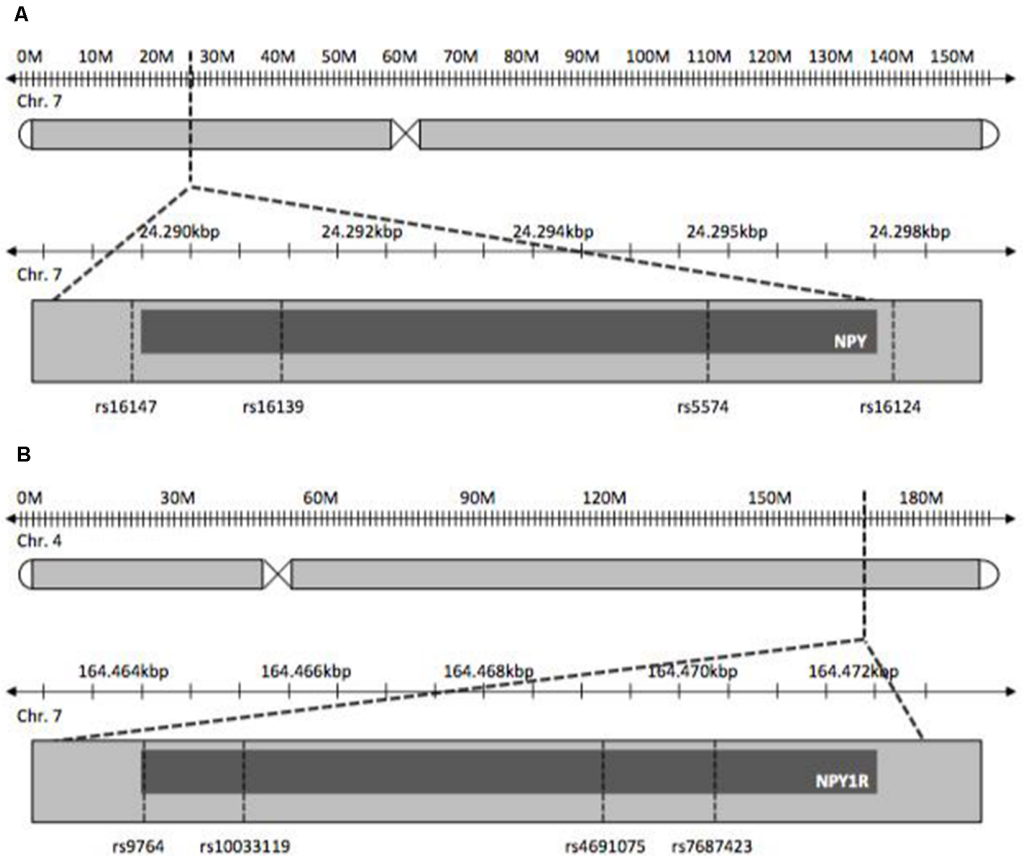

FIGURE 1 | Gene loci of Neuropetide Y (NPY, **A**) and NPY-receptor (NPY1R, **B**).

including 2,688 individuals affected by OCD and 7,037 controls by the Psychiatric Genomics Consortium, and searched the results for each of the SNPs investigated in our study. The GWAS by the Psychiatric Genomics Consortium comprised 2,688 individuals affected by OCD and 7,037 controls. The meta-analysis comprised children as well as adults (Arnold et al., 2018).

Gene Loci

NPY and *NPY1R* SNPs chosen for this study were tag-SNPs and previously published SNPs with reported influence on psychiatric disorders. The latter were studied with regard to stress-related diseases, as OCD is postulated to be, like depression, ADHD and obesity (Peterson et al., 2001; Tiwari et al., 2013). Tag-SNPs were determined using HaploView® to cover both genes completely on the basis of SNP data provided by the International HapMap-Project (International HapMap Consortium, 2003; Barrett et al., 2005; **Figure 1**). Tagger settings included a minor allele frequency >0.1 and $r^2 = 0.9$. Additionally, SNPs known from previously published studies pertaining to other disorders were included in the tagging process. For *NPY*, SNPs rs5574, rs16124, rs16139, rs16147 were determined, and SNPs rs9764, rs4691075, rs7687423 and rs10033119 were found for *NPY1R*.

Genotyping

Genomic DNA was extracted from whole blood following standard protocols. DNA was amplified by standard PCR using specific reverse- and forward-primers for each of the eight SNPs. After amplification genotypes were determined by enzymatic digestion and gel-electrophoresis. Further detailed information on primers and procedures is available upon request.

Statistics

Association between the included markers of *NPY* and *NPY1R* and eoOCD was tested by the Transmission Disequilibrium Test (TDT; Spielman et al., 1993). TDT was performed for all eight SNPs using the software FamHAP® (Herold and Becker, 2008). A p-value < 0.05 was defined as the significance level. Each of the trios was tested for the hypothesis that their tested gene variants of either: (1) *NPY* or (2) *NPY1R* are associated with eoOCD. The examination pattern used for this analysis was a genotype-wise model considering every heterozygous parental genotype separately and corresponds to a test of the global null-hypothesis of transmission equilibrium of both alleles in every parental genotype. All eight SNPs were checked for Mendelian Errors which were exclusion criteria. Moreover, all parental genotype distributions were tested for Hardy-Weinberg equilibrium (HWI).

RESULTS

In one of the *NPY1R*-SNPs, rs4691074, occurred a deviation from the HWI ($p = 0.04$). The remaining seven SNPs were unremarkable ($p > 0.05$). No transmission disequilibrium was observed for the *NPY*-SNPs. The *p*-values exceeded the defined significance level α (**Table 1**). For rs5574, 34 heterogeneous parental couples could be examined ($\chi^2_{(1)} = 1.058$; $p = 0.303$), for rs16124, 85 ($\chi^2_{(1)} = 0.576$; $p = 0.448$), for rs16139, 12 ($\chi^2_{(1)} = 0.333$; $p = 0.564$) and for rs16147, 76 ($\chi^2_{(1)} = 0.842$; $p = 0.359$). Moreover, no transmission disequilibrium could be assessed for the *NPY1R*-SNPs. For rs9764, 72 heterogeneous parental couples could be analyzed ($\chi^2_{(1)} = 0.000$; $p = 1.000$), for rs4691075, 33 ($\chi^2_{(1)} = 1.485$; $p = 0.223$), for rs7687423, 89 ($\chi^2_{(1)} = 0.101$; $p = 0.75$) and for rs10033119, 14 ($\chi^2_{(1)} = 0.000$; $p = 1.000$).

The GWAS meta-analysis also showed no association of any *NPY*- nor *NPY1R*-SNPs with regard to the development of OCD (**Table 2**).

DISCUSSION

This is the first molecular genetic study on potential functional variants of the candidate gene *NPY* and its receptor *NPY1R* in eoOCD. In this study, association was not detected. Thus, we could not confirm a major role of the NPY system in OCD with childhood onset.

As our number of trios was smaller than postulated by the power-analysis, the negative outcome might foremost be due to the sample size. That is why we used the data of the most recent and aforementioned GWAS meta-analysis on OCD by the Psychiatric Genomics Consortium, and searched the results for the investigated SNPs. Though our sample was part of this meta-analysis, a potential effect over a large sample including different ages of onset might have been observable. However, also this meta-analysis showed no association of any *NPY*- nor *NPY1R*-SNPs (Arnold et al., 2018). Our study had been designed as a family-based study to avoid stratification effects, nonetheless, a case-control study, which had not been performed so far with regard to the studied gene loci, would be highly interesting.

However, the NPY system, which is involved in stress response, might still be of relevance in OCD subgroups, although undetected due to the study design. The selected SNPs were either already known from earlier studies to other stress-related psychiatric disorders or tag-SNPs that were selected out of the HapMap data with the help of the programme Haploview® (Barrett et al., 2005) in order to cover *NPY* and *NPY1R*.

TABLE 1 | Transmission Disequilibrium Test (TDT) results.

SNP	Gene	Allele	T	NT	$\chi^2_{(1)}$	*p*TDT
rs5574	NPY	2	20	14	1.059	0.303
		4	14	20		
rs16124	NPY	3	39	46	0.576	0.448
		4	46	39		
rs16139	NPY	2	7	5	0.333	0.564
		4	5	7		
rs16147	NPY	2	34	42	0.842	0.359
		4	42	34		
rs9764	NPY1R	1	36	36	0.000	1.000
		3	36	36		
rs4691075	NPY1R	1	20	13	1.485	0.223
		3	13	20		
rs7687423	NPY1R	2	46	43	0.101	0.750
		4	43	46		
rs10033119	NPY1R	2	7	7	0.000	1.000
		4	7	7		

Note: Column 1 lists the SNP names, column 2 the correspondent gene, column 3 shows the present allele: 1 equal to adenine, 2 equal to cytosine, 3 equal to guanine, 4 equal to thymine. Column 4 shows the number of transmissions (T) for each gene, column 5 shows the number of non-transmitted (NT) alleles. Column 6 represents the chi square value, column 7 the p-value.

TABLE 2 | Meta-analysis results.

Chr.	SNP	bp	A1	A2	Info	OR	SE	*p*
7	rs5574	24329133	T	C	0.9750	0.967152	0.0949	0.7248
7	rs16124	24331799	T	G	0.9735	1.00944	0.0335	0.7802
7	rs16139	24324879	T	C	0.9737	0.983144	0.0335	0.6112
7	rs16147	24323410	T	C	0.9720	1.01572	0.0335	0.6421
4	rs9764	164245405	T	C	1.0030	1.03345	0.0382	0.3881
4	rs4691075	164249485	T	C	0.9948	0.937255	0.0504	0.1991
4	rs7687423	164250797	A	G	0.9800	1.001	0.0347	0.9766
4	rs10033119	164245854	A	G	0.8709	0.962809	0.0851	0.6562

Note: Column 1 lists the respective chromosomes (hg 19), column 2 the correspondent marker name, column 3 shows the base pair location (hg 19), column 4 shows the reference allele for OR (may or may not be minor allele), column 5 the alternative allele. Column 6 represents the imputation information score, column 7 the Odds ratio for the effect of the A1 allele, column 8 the standard error of the log(OR) and column 9 the p-value for the association test in the meta-analysis.

Nonetheless, the selected SNPs are infrequent in the population, thus statistically evaluable transmissions in the sample were rather small.

Reflecting the NPY-system, other effectors like the Y_2-receptor and the Y_5-receptor might also be valuable targets in OCD. The Y_1-receptor was the first choice for this study due to its various anxiolytic and stress-reducing effects and its wide spread dissemination in stress regulating brain regions. Anxious behavior and stress can be reversed with NPY administration (Kormos and Gaszner, 2013). Nevertheless, the influence of the remaining receptors should not be excluded *a priori*. Especially the Y_5-receptor shares a similar effect spectrum with the tested Y_1 (Kormos and Gaszner, 2013). Even though its role is not fully understood yet, an effect on the fear and stress system seems to be obvious and further research worthwhile. The Y_2-receptor, however, could indirectly influence the delicate equilibrium of neurotransmission with its impact on the release of other neurotransmitters (Upadhya et al., 2009; Kormos and Gaszner, 2013). A reduced inhibition of glutamate release, for example, could be a correlate for the increased thalamic and striatal glutamate activity in untreated OCD-children and could explain the upregulation of Y_2-receptors in a state of anxiety in the mouse model (Leckman et al., 1997; Upadhya et al., 2009).

Our sample consists of children and adolescents only, resembling an OCD subgroup with early onset and a significant number of children with an onset before the age of 10 years. A stronger impact of genetic factors is reported for eoOCD and our sample might differ from adult samples regarding genetic underpinnings and impact of life-events (Nestadt et al., 2000; Walitza et al., 2008). The responsivity of the NPY system after stress exposure during the development has been shown in animal models and could be of higher relevance in failed coping and development of OCD in adults (Serova et al., 2017; Yam et al., 2017).

Moreover, comorbidities as e.g., ADHD or depression were accepted in this study. Nonetheless, comorbidities in OCD might indicate distinct neurobiological OCD subgroups with divergent etiologies (Taurines et al., 2010). An analysis reflecting the comorbidities was not applicable due to the sample size. Especially an analysis regarding comorbidity with depression would be of interest (Caberlotto et al., 1998; Redrobe et al., 2005; Christiansen et al., 2011).

Due to the sample size, we were not able to examine an influence of gender, which was reported for other candidate genes in previous studies. In the meta-analyses of the glutamate transporter gene *SLC1A1*, the SNP rs12682807 was found associated only in male probands (Thiele et al., 1998; Arnold et al., 2006; Wendland et al., 2009). Other genetic publications also stated gender differences (Thiele et al., 1998; Dickel et al., 2006; Canals et al., 2012). Gender differences are also found in the clinical perspective with the prevalence of subclinical OCD-symptoms twice as high in boys than in girls (Canals et al., 2012). Furthermore, the two sexes differ in the clinical manifestation which comprises an earlier age of onset as well as a higher prevalence of symptoms belonging to the entity of symmetry and ordering among males and increased symptoms of cleanliness and washing among females (Bogetto et al., 1999; Stewart et al., 2007).

Though the sample size was rather small for a genetic study, regarding eoOCD it has a considerable size. Since the sample was collected in one facility, it provides a clear stringency regarding a precisely defined phenotype and restrictive exclusion of severe comorbid disorders to assure the predominance of definite OCD in contrast to obsessive-compulsive symptoms in other psychiatric disorders.

In conclusion, our family-based study on genetic variants of NPY and NPY1R could not confirm association with OCD with childhood onset correlating with the outcomes for adult NPY and OCD by Altemus et al. (1999). Unfortunately, there is overall a paucity of studies on NPY and OCD and we can only add another hint for non-existing significant effects. We only know so far that NPY is involved in stress and other anxiety disorders. Therefore, we hope that we could deliver with our study a contribution to the question about an association of NPY with OCD. The major impact of our study is that we could show in our sample—and comprehending the forementioned GWAS-data—that NPY effects on OCD should be very small or not present. The very impact of our study is a sample consisting of early onset patients and therefore of fundamental impact on the database in this specific subgroup. Moreover, the outcome has a meaning for the understanding of the stress response in OCD in general which might be different from other anxiety disorders with an association to NPY. Indeed, in DSM V OCD is no longer listed under anxiety disorders. If our null finding is replicated in larger studies, we might conclude that NPY has no effect on the stress response in OCD and probably might also be a less valuable target for pharmacological research in OCD.

AUTHOR CONTRIBUTIONS

All authors certify that they have participated sufficiently in the work to take public responsibility for the content. TR was responsible for conception and design of the study. TR and MF carried out data acquisition and analysis. TR, MF and AC were mainly involved in drafting the article. TR, MF, AC, EG, AMW, SW, CW, MR, AW and HS contributed to data interpretation and manuscript draft and revision.

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Adverse Childhood Experiences and Telomere Length a Look Into the Heterogeneity of Findings—A Narrative Review

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Background: Adverse childhood experiences (ACEs) have been associated with poor mental and somatic health. Accumulating evidence indicates that accelerated biological aging—indexed by altered telomere-related markers—may contribute to associations between ACEs and negative long-term health outcomes. Telomeres are repeated, non-coding deoxyribonucleic acid (DNA) sequences at the end of chromosomes. Telomeres shorten during repeated cell divisions over time and are being used as a marker of biological aging.

Objectives: The aim of the current paper is to review the literature on the relationship between ACEs and telomere length (TL), with a specific focus on how the heterogeneity of sample and ACEs characteristics lead to varying associations between ACEs and TL.

Methods: Multiple databases were searched for relevant English peer-reviewed articles. Thirty-eight papers were found to be eligible for inclusion in the current review.

Results: Overall, the studies indicated a negative association between ACEs and TL, although many papers presented mixed findings and about a quarter of eligible studies found no association. Studies with smaller sample sizes more often reported significant associations than studies with larger samples. Also, studies reporting on non-clinical and younger samples more often found associations between ACEs and TL compared to studies with clinical and older samples. Reviewing the included studies based on the "Stressor Exposure Characteristics" recently proposed by Epel et al. (2018) revealed a lack of detailed information regarding ACEs characteristics in many studies.

Conclusion: Overall, it is difficult to achieve firm conclusions about associations of ACEs with TL due to the heterogeneity of study and ACE characteristics and the heterogeneity in reported findings. The field would benefit from more detailed descriptions of study samples and measurement of ACEs.

Keywords: early adversity, adverse childhood experiences, stress, childhood trauma, accelerated aging, telomeres, telomere length

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INTRODUCTION

Adverse childhood experiences (ACEs) (e.g., physical abuse, sexual abuse, emotional neglect, loss of a close family member) are a large societal problem, often with long-lasting health consequences. Previous research has shown that ACEs are highly prevalent. In the general population, more than half of people retrospectively report at least one, and more than a quarter two or more, types of ACEs (Felitti et al., 1998; Dube et al., 2001). In addition, ACEs are found to be related to poor health outcomes, including various mental health problems (e.g., depression, anxiety, post-traumatic stress disorder [PTSD], suicidal ideation), substance abuse problems, self-reported illness, obesity, and overall morbidity (Felitti et al., 1998; Widom, 1999; Dube et al., 2001, 2003; Anda et al., 2006, 2010; Widom et al., 2007; Brown et al., 2009; Green et al., 2010; Heim et al., 2010; Kessler et al., 2010; Heim and Binder, 2012; Moffitt and the Klaus-Grawe Think Tank, 2013). ACEs have also been found to be associated with increased risk for many somatic diseases, especially with diseases of aging including cancer, autoimmune, cardiovascular diseases and early mortality (Felitti et al., 1998; Brown et al., 2009; Rich-Edwards et al., 2012; Kelly-Irving et al., 2013; Tomasdottir et al., 2015). Although it is largely accepted that ACEs increase risk for poor health outcomes, mechanisms of the association are still not fully understood (Moffitt and the Klaus-Grawe Think Tank, 2013).

Following a pioneering study by Epel et al. (2004), research on the association of stress and telomere-related processes has rapidly emerged. Accelerated cell aging—indexed by altered telomere maintenance—might be one mechanism that partially explains the association between ACEs and long-term health complaints. Telomeres are repeated non-coding deoxyribonucleic acid (DNA) sequences—TTAGGG nucleotide tandem repeats – at the end of chromosomes, protecting the coded sequences (Blackburn, 1991). Telomeres shorten during cell division, caused by an incomplete replication of the chromosome ends (Blackburn, 2000, 2001). When telomeres are critically short, cells become genomically unstable and can malfunction in cell-specific ways (Blackburn, 2000). Telomeres tend to shorten with age, which makes telomere length (TL) an interesting marker of biological aging (Cawthon et al., 2003; Blackburn, 2005; Aubert and Lansdorp, 2008; Takubo et al., 2010). Interestingly, shorter telomeres are correlated with several psychiatric disorders (Lindqvist et al., 2015; Schutte and Malouff, 2015; Darrow et al., 2016; Ridout et al., 2016; Li et al., 2017a; Epel and Prather, 2018), somatic diseases (Honig et al., 2006; Willeit et al., 2010), and early mortality (Cawthon et al., 2003).

A fast-growing body of research describes the association between ACEs and TL over the life course. Various reviews in the broader context of the association between stress and TL have recently been published focusing in detail on early life stress and telomeres (Shalev, 2012; Price et al., 2013; Ridout et al., 2015), perceived stress and TL (Schutte and Malouff, 2014; Mathur et al., 2016), childhood exposure to violence and TL (Moffitt and the Klaus-Grawe Think Tank, 2013), violence and telomeres (Oliveira et al., 2016), caregiving experiences and telomeres (Blaze et al., 2015), and psychosocial factors and TL (Starkweather et al., 2014). Additionally, recent meta-analyses describe the association between early life adversity and TL (Ridout et al., 2017), childhood trauma and accelerated telomere erosion (Li et al., 2017b) and childhood psychosocial stressors and TL (Hanssen et al., 2017). Overall, these analyses reported negative associations between ACEs and TL with aggregated small effect sizes [Ridout et al. (2017) Cohen's d = −0.35; Hanssen et al. (2017) $r = -0.082$; and Li et al. (2017b) $r = -0.05$]. Epel and Prather (2018) summarized the current empirical evidence, concluding that "these meta-analyses demonstrate the robustness of the association [childhood stressors and telomere length] across published studies" (p. 5). However, all three meta-analyses reported a high between-study heterogeneity of effects, which they tried to explain in further moderator analyses. In their moderator analyses Ridout et al. (2017) showed "that differences in developmental timing of adversity exposure and comorbidities likely contributed to the heterogeneity" (p. 12), Li et al. (2017b) concluded that "the heterogeneous feature of childhood trauma may be one of the major potential sources of heterogeneity in outcomes" (p. 68), and Hanssen et al. (2017) found greater effect sizes for categorical compared to continuous measures of stressors, and for shorter durations between stressor and TL measures. Hence, a possible explanation for the observed heterogeneity in findings are attributes related to the characteristics and measurement of stressors. A deeper understanding about the different aspects of ACEs might help to explain the diversity in reported associations.

Epel and Lithgow (2014) stated that research must form a "common knowledge base and taxonomy for describing stressors and stress responses" (p. 11) to bridge the gap between basic and clinical research on aging and stress. Epel et al. (2018) further pointed out that "a large but disjointed literature shows that stress affects slow-acting biological processes in the brain and body, accelerating diseases of aging" (p. 146), but that despite this agreement one major barrier that prevents research progress is the "lack of consistency and thoroughness in stress measurement"(p. 146). This lack of a common knowledge base, consistency and thoroughness in stress measures can also be seen in the field of early life stressors and childhood adversities. Specifically, these conceptual issues lead to a large heterogeneity of reported prevalence and incidence rates of early traumatic stressors and ACEs (Heim and Binder, 2012; Moffitt and the Klaus-Grawe Think Tank, 2013). It can also be seen in the reviews and meta-analyses discussed here that use varying stress -frameworks but overall overlap to a great degree in their included studies.

In search of a common knowledge base and taxonomy, Epel et al. (2018) proposed a working model focusing on stress as "an emergent process that involves interactions between individual and environmental factors, historical and current events, allostatic states, and psychological and physiological reactivity" (p. 146). This model comprises different research perspectives on stress and introduces a more precise language for describing stress measures. Within this framework, stress consists of an exposure within in a specific context that elicits a stress-related response. Stressor exposure characteristics (SECs) are defined along different dimensions: timescale for stress measurements

(acute, event-based, daily, chronic), developmental life stages of stress exposures, stress assessment windows (measurement timeframe; proximity of assessment to the stressor in years), and stressor attributes (duration, severity, controllability, life domain, target of stressor, potential of the stressor to elicit harmful response). However, it is unknown to what extend the proposed SECs can be applied to a diverse body of literature focused on ACEs and TL.

Therefore, the main aim of the current paper is to review the fast-growing body of literature on the associations between ACEs and TL order to find explanations for the heterogeneity in findings. The included sample of studies will be reviewed based on important study design characteristics and the SECs proposed by Epel et al. (2018). This will help us to better understand the complex relationship between ACEs and TL.

METHODS

To be included in the current review, studies had to report on ACEs, assessed by means of a questionnaire or interview, on TL, and on a statistical measure of association between these two. Hereinafter, ACEs are defined as the broad array of harmful, perceived traumatic stressors during a child's development before the age of 18. This includes childhood traumatic experiences, all forms of childhood maltreatment including abuse and neglect, and childhood exposure to violence, and the combination of these factors with further potentially harmful circumstances. Multiple search methods were used to avoid biased retrieval of studies (Rosenthal, 1995). First, a computerized search of relevant databases was conducted: PubMed, PsycInfo, Web of Science, and Google Scholar up to the 26th of April 2018. The following key words were used in varying combinations: "childhood adversit*," "early life stress" or "childhood trauma" and "telomere length." Second, the combination of several instruments reported in the papers to assess ACEs with "telomere length" was examined: Childhood Trauma Questionnaire [CTQ] (Bernstein et al., 1994, 2003); Childhood Trauma Interview [CTI] (Foote and Lovejoy, 1995); Adverse Childhood Experiences [ACE] Questionnaire (Felitti et al., 1998); and the Early Trauma Inventory [ETI] (Bremner et al., 2000). Third, reference lists from relevant reviews on the association between ACEs and TL (Shalev, 2012; Price et al., 2013; Ridout et al., 2015, 2017; Oliveira et al., 2016; Epel and Prather, 2018) were examined for possible additional studies. Finally, reference lists of all included papers were checked for potentially relevant additional articles. One eligible paper by Schaakxs et al. (2015) was excluded, because another paper from the same research group (Schaakxs et al., 2016) used the same sample.

A total of 38 studies were eligible for inclusion in this review. First, we collected information on the following sample characteristics: sample size, sex, age (of the sample), sample origin, study design (cross-sectional [case-control], longitudinal), sample composition, telomere assay approach, and covariates. Additionally, we collected the following ACEs characteristics: questionnaire (specific instrument [e.g., CTQ], modified specific instrument, item, score, total score), and age at adversity exposure. Further, ACEs characteristics were assessed using the proposed SECs defined by Epel et al. (2018). This included: timescale of the used stress measurement (i.e., acute, event-based, daily, chronic); developmental life stages (i.e., childhood only, adolescence only, childhood and adolescence); stress assessment window (i.e., measurement timeframe [e.g., retrospective or prospective]; proximity of assessment to the stressor in years [i.e., duration in years between exposure and assessment]); and stressor attributes (duration, severity, controllability, life domain, target of stressor, potential of the stressor to elicit harmful response). For a detailed definition of the SECs, please refer to Appendix A. "Stress typology for stress measurement" within the model proposed by Epel et al. (2018) (p.163). Moreover, main findings of the ACEs-TL association were summarized and coded (shorter, none, longer, mixed). In a second step, studies were grouped into categories: sample size (<400, >400), age (<25, 25–45, >45), sex (male, female) and population (clinical vs. non-clinical) and reviewed regarding their overall findings.

Information regarding sample characteristics, ACEs characteristics and main findings are presented in **Table 1**. Further information regarding main and sub-findings are presented in **Table 2**. Additional supplementary characteristics including the type of adversity and nature of the ACEs-TL association are provided in the supplementary materials (**Supplementary Table 1**). Information was extracted and coded by the first author (DB) and double checked by one of the co-authors (Dd'H). Differences in extracted information and coding were solved by further discussing these issues.

RESULTS

Study Characteristics

A total of 38 studies were included in this review based on the criteria of eligibility defined in the method section (for an overview see **Table 1**). Sample sizes of included studies ranged from 31 (Tyrka et al., 2010) to 11,670 (Cai et al., 2015). Most studies ($N = 27$) reported on TL in both males and females, seven studies examined only females (Surtees et al., 2011; Malan-Müller et al., 2013; Cai et al., 2015; Mason et al., 2015; Levandowski et al., 2016; Oliveira et al., 2017; Mitchell et al., 2018), and three studies examined only males (Mitchell et al., 2014; Boks et al., 2015; Bersani et al., 2016; Osler et al., 2016). The included studies covered a wide age range of study participants at TL assessment from 5 years (Shalev et al., 2013; Drury et al., 2014) to 93 years of age (Schaakxs et al., 2016). Almost all of the included studies ($N = 32$) are of North-American or European origin, except for six studies that were conducted in Brazil (Levandowski et al., 2016; Oliveira et al., 2017), China (Cai et al., 2015), South-Africa (Malan-Müller et al., 2013), and New Zealand (Jodczyk et al., 2014; Shalev et al., 2014).

Reviewing the design of the studies, all studies, as defined within the inclusion criteria, had to report on TL at a minimum of one time point, and thus were able to associate ACEs and TL cross-sectionally. Of the 38 studies, 14 used a cross-sectional (case-control) approach to investigate differences in TL between groups (e.g., abused vs. non abused)

TABLE 1 | Study and stressor characteristics.

	Study characteristics							ACE characteristics												Findings
												Assessment window		Stressor attributes						
References	Sample size; sex	Age, mean (SD)	Sample origin	Design	Sample composition	Telomere assay	Covariates	Questionnaire	Age	Time scales	Life stage	Time frame	Proximity	Duration	Severity	Controllability	Life domain	Target	Potential	ACEs-TL
Bersani et al., 2016	$N = 76$ 0 f, 76 m	34.64 (9.17)	USA	Cross-sectional (case-control)	Combat-exposed subjects; 41 (healthy), 18 (PTSD), 17 (PTSD + MDD)	qPCR; granulocytes	Age, BMI, antidepressants and ethnicity	ETI-SR	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	17	X	X	X	X	X	X	shorter
Blom et al., 2015	$N = 117$ 64 f, 53 m	15.8 (1.32)	USA	Cross-sectional (case-control)	$n = 22$ (MDD) $n = 25$ (controls) (CTQ only 47 participants)	qPCR; saliva	Total brain volume, MDD and matched healthy controls	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	0	X	✓	X	X	X	X	none
Boks et al., 2015	$N = 96$ 0 f, 96 m	27.0 (9.1)	Netherland	Longitudinal	Clinical sample (PTSD); military combat exposure	qPCR; leukocyte	Baseline methyl. level, time interval, age, gender, alcohol consumption, cigarette smoking, military rank, length, weight, or medication	ETI-SR	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	9	X	X	X	X	X	X	none
Cai et al., 2015	$N = 11,670$ 11,670 f	NA	China	Cross- sectional	CONVERGE study of MDD	qPCR; saliva	NA	Score	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	NA	X	X	X	X	X	X	shorter
Chen et al., 2014	$N = 40$ 25 f, 15 m	36 (10.7)[a]	USA	Cross-sectional (case-control)	20 (MDD) 20 (controls)	qPCR; leukocyte	Age, sex- and ethnicity-matched controls	ACEs	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	18	X	X	X	X	X	X	mixed
Dagan et al., 2017	$N = 78$ 62 f, 16 m	20.5 (1.6)	USA	Cross-sectional	Undergraduate students	qPCR; buccal cells	Age, gender, ethnicity, current income, Health-related factors, smoking history, current physical activity level, and BMI	ACEs	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	2	X	X	X	X	X	X	mixed
Drury et al., 2014	$N = 80$ 39 f, 41 m	10.2 (2.9)	USA	Cross- sectional	High risk families	qPCR; buccal cells	Age, gender, maternal education, parental age at child conception, race	PAPA (mod.)	<15[b]	Event-based, chronic	Children and adolescents (5 to 15)	Retrospective period	0	X	X	X	X	X	X	shorter
Glass et al., 2010	$N = 1,874$ NA	NA	UK	Cross-sectional (case-control)	CM (123/1874) Physical abuse (20/540) Sexual abuse (34/550)	southern blot; leukocyte	Age, sex, BMI, smoking	Items	NA	Event-based, chronic	Whole childhood (NA)	Retrospective period	NA	X	X	X	X	X	X	none
Guarneri-White et al., 2018	$N = 108$ 60 f, 48 m	15.91 (1.65)	USA	Cross-sectional	Adolescence from larger suburban area recruited via school mailing lists and summer camps	qPCR; saliva	BMI, age, and sex	DIAS-VS CSEQ-SR	<19[a]	Event-based, chronic	Children and adolescents (age 11–19)	Retrospective period	0	X	X	X	X	X	X	shorter
Jodczyk et al., 2014	$N = 677$ females and males	Range: 28–30	New-Zealand	Cross-sectional	Population-based; longitudinal Birth cohort; Christchurch Health and Development Study (CHDS)	qPCR; leukocyte	Sex, ethnicity and family SES at birth	Reports; PBI; CTS (mod.); total score	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	0–5	X	X	X	X	X	X	none
Kananen et al., 2010	$N = 974$ 617 f, 357 m	49.8 (12.60)	Finland	Cross-sectional (case-control)	Epidemiological Health 2000 cohort; Anxiety disorder vs. control subjects	qPCR; leukocyte	Comorbidity, psychiatric medication, BMI, blood pressure, serum lipids, glucose, smoking, sleep, exercise	Items; total score	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	34	X	X	X	X	X	X	shorter

(Continued)

TABLE 1 | Continued

	Study characteristics							ACE characteristics												Findings
												Assessment window		Stressor attributes						
References	Sample size; sex	Age, mean (SD)	Sample origin	Design	Sample composition	Telomere assay	Covariates	Questionnaire	Age	Time scales	Life stage	Time frame	Proximity	Duration	Severity	Controllability	Life domain	Target	Potential	ACEs-TL
Kiecolt-Glaser et al., 2011	*N* = 132 95 f, 37 m	69.7 (10.14)	USA	cross-sectional	Caregivers vs. control subjects; 42/132 (abuse); 74/132 (adverse event)	southern blot; PBMCs	Age, sex, BMI, exercise, sleep, alcohol use, caregiving status	CTQ; total score	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	52	X	X	X	X	X	X	shorter
Kuffer et al., 2016	*N* = 120 57 f, 63 m	74.1 (6.1)[a]	Switzerland	Cross-sectional (case-control)	62 (child laborers, with or without PTSD), 58 (healthy controls)	qPCR; buccal cell	Age, sex, years of education, self-evaluated financial situation, depression, and mental and physical functioning	CTQ-SF	<18	Event-based, chronic	Whole childhood (<18)	Retrospective pe riod	56	X	✓	X	X	X	X	longer
Levandowski et al., 2016	*N* = 176 176 f, 0 m	Subst.: 28.6 (7.3)[a] Contr.: 68.3 (7.4)	Brazil	Cross-sectional (case-control)	Crack cocaine addiction: CRACK-ELS (*n* = 93) CRACK (*n* = 34) ELD (*n* = 49)	qPCR; blood	Age, educational level, BMI	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	11	X	X	X	X	X	X	shorter
Liu et al., 2017	*N* = 894 590 f, 304 m	Range: 28–60 Median = 46	Sweden	Cross-sectional (case-control)	Longitudinal population-based cohort study (PART); 337 (recent depress-ion diagnosis), 574 (non-depressed controls)	qPCR; saliva	Age, alcohol, number of somatic diseases, sex, education, BMI, smokers, physical exercise regularly	Items; total score	<18	Event-based, chronic	Whole childhood	Retrospective period	28	X	X	X	X	X	X	mixed
Malan-Müller et al., 2013	*N* = 128 128 f, 0 m	Range: 18–50 Mean = 29.8	South-Africa	Cross-sectional (case-control)	HIV-positive (83/128) Childhood Trauma (66/128)	qPCR; PBMCs	Age, education, BMI, trauma-subtype, traumatic life experiences, PTBS symptomatology, alcohol abuse	CTQ-SF	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	12	X	X	X	X	X	X	none
Mason et al., 2015	*N* = 1,135 1,135 f, 0 m	45.5 (4.1)	USA	Cross-sectional	Population-based Nurses' Health Study II (NHSII)	qPCR; leukocyte	Age, own and parental education, parental occupation, parental morbidity before age 60	CTS; SES	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	28	X	✓	X	X	X	X	mixed
McFarland et al., 2017	*N* = 1,108 594 f, 514 m	45.6 (11.6)	USA	Cross-sectional	Nashville Stress and Health Study (NSHS)	qPCR; leukocyte	Age, gender, depressive symptoms	LHC	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	28	X	X	X	X	X	X	shorter
Mitchell et al., 2018	*N* = 81 81 f, 0 m	25.48 (4.27)	USA	Cross-sectional	Pregnant women from the Ohio State University Wexner Medical Center (OSUWMC)	qPCR; PBMCs	Age, race, current household, income, education level, marital status, BMI, exercise, smoking and depressive symptoms	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	7	X	X	X	X	X	X	none
Mitchell et al., 2014	*N* = 40 0 f, 40 m	9	USA; African American Boys	Cross-sectional (case-control)	Fragile Families and Child Wellbeing Study (FFCWS)	qPCR; saliva	Mother's age at birth, BMI	CTS (mod.)	<10	Event-based, chronic	Childhood (<10)	Retrospective period	0	X	X	X	X	X	X	shorter
O'Donovan et al., 2011	*N* = 88 45 f, 43 m	30.55 (7.44)	USA	Cross-sectional (case-control)	43 adults with chronic PTSD (*n* = 18 with multiple childhood trauma) and 47 controls	qPCR; leukocyte	Age, sex, BMI, smoking, education	LSC (mod.)	<15	Event-based, chronic	Whole childhood (<15)	Retrospective period	16	X	X	X	X	X	X	shorter

(Continued)

TABLE 1 | Continued

	Study characteristics							ACE characteristics												Findings
												Assessment window		Stressor attributes						
References	Sample size; sex	Age, mean (SD)	Sample origin	Design	Sample composition	Telomere assay	Covariates	Questionnaire	Age	Time scales	Life stage	Time frame	Proximity	Duration	Severity	Controllability	Life domain	Target	Potential	ACEs-TL
Oliveira et al., 2017	$N = 83$ 83 f, 0 m	Range: 65–74	Brazil	Cross-sectional	42 women with less than secondary education and 41 with secondary or more education	qPCR; leukocyte	Age, parental abuse of alcohol	Items	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	54	X	X	X	X	X	X	longer
Osler et al., 2016	$N = 324$ 0 f, 324 m	56	Denmark	Cross-sectional	Copenhagen metropolitan birth-cohort	qPCR; leukocyte	Chronic diseases, and lifestyle, BMI, body weight and height	Items; total score	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	38	X	X	X	X	X	X	shorter
Puterman et al., 2016	$N = 4598$ 2,724 f, 1874 m	Median: 70 Range: 50–90	USA	Cross-sectional	US Health and Retirement Study: longitudinal, nationally representative sample of >26,000 US residents	qPCR; saliva (cell type not specified)	Age, ethnicity, sex, education, current partnership status, BMI, smoking, medical conditions, high blood pressure, diabetes, cancer, lung disease, heart disease, stroke, psychiatric problems, and arthritis	Items; total score	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	52	X	X	X	X	X	X	shorter
Revesz et al., 2016	$N = 2,936$ 1,950 f, 986 m	Baseline: 41.8 (13.1) Follow-up: 48.5 (13.0)	Netherlands	Longitudinal (6-year follow-up)	Population-based	qPCR; leukocyte	Sex, age, smoking, triglycerides, BP	CTI	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	26	X	X	X	X	X	X	mixed
Riley et al., 2018	$N = 66$ 27 f, 39 m	40.9 (9.8)		Cross-sectional (case-control)	48 adults with DSM-5 schizophrenia and 18 healthy controls	qPCR; lymphocytes	Age	ETI	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	23	X	✓	X	X	X	X	mixed
Robles et al., 2016	$N = 39$ 28 = f, 19 m	11.3 (1.5) Range = 8–13	USA	Cross-sectional	Population-based	qPCR; leukocyte	Age, gender, BMI, parent educational status and leukocyte composition	Score; total score	[8; 13]	Daily, chronic	Childhood (age 8–13)	Daily ratings for 56 days	0	X	X	X	X	X	X	shorter
Savolainen et al., 2014	$N = 1,486$ 817 f, 674 m	61.5 (2.9)	Finland	Cross-sectional	Population-based; Helsinki Birth Cohort Study	qPCR; leukocyte	Age, sex, stock of DNA, mental disorder, depression, education, alcohol, father's education and mother's age at delivery, coronary heart disease and stroke, BMI, diabetes II	National Archive Finland; TEC	Life time (median 10)	Event-based, chronic	Whole childhood + early childh., childhood and adolescence	Retrospective period, archive information	0 + 42	X	X	X	X	X	X	mixed
Schaakxs et al., 2016	$N = 496$ 323 f, 173 m	70.6 (7.4)	Netherlands	Cross-sectional	Netherlands Study of Depression in Older Persons (NESDO); 44.2% childhood abuse at least once, 23.4% one or more events	qPCR; leukocyte	Sex, age, chronic disease	Score	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	55	X	X	X	X	X	X	mixed
Shalev et al., 2013	$N = 236$ 116 f, 120 m	Baseline: 5 Follow-up: 10	UK	Longitudinal	Population-based; Environmental-Risk (E-Risk) Study, subset of epidemiological sample; 108/236 (Exp./N)	qPCR; buccal cells	Age, sex, BMI, SES	CTS; clinical interview; items; total score	[5; 10]	Event-based, chronic	Childhood (age 5–10)	Prospective, longitudinal + retrospective period	0	X	X	X	X	X	X	shorter

(Continued)

TABLE 1 | Continued

	Study characteristics							ACE characteristics												Findings
												Assessment window		Stressor attributes						
References	Sample size; sex	Age, mean (SD)	Sample origin	Design	Sample composition	Telomere assay	Covariates	Questionnaire	Age	Time scales	Life stage	Time frame	Proximity	Duration	Severity	Controllability	Life domain	Target	Potential	ACEs-TL
Shalev et al., 2014	$N = 1037$ 498 f, 539 m	Baseline: 26 Follow-up: 38	New-Zealand	Longitudinal	Population-based; Dunedin Multidisciplinary Health and Development Study, birth cohort; $n = 234$: int. dis. $n = 524$: no int. dis.	qPCR; leukocyte	CM, tobacco smoking, substance dependence, psychiatric medication use	Total Score	[3; 11]	Event-based, chronic	Childhood (age 3–11)	Prospective, longitudinal + retrospective periods	0–15	X	X	X	X	X	X	none
Surtees et al., 2011	$N = 4,441$ 4,441 f, 0 m	Median: 62 Range: 41–80	UK	Cross-sectional	European Prospective Investigation into Cancer (EPIC)-Norfolk Population Study	qPCR; lymphocyte	Age, physical health score, self- reported health, social class, obesity, smoking, preexisting disease	HLEQ	<17	Event-based, chronic	Whole childhood (<17)	Retrospective period	45	X	X	X	X	X	X	shorter
Tyrka et al., 2010	$N = 31$ 22 f, 9 m	26.9 (10.1)	USA	Cross-sectional (case-control)	No history of CM ($n = 21$) or a history of moderate or severe CM ($n = 10$)	qPCR; leukocyte	Age, sex, oral contraceptives, smoking, BMI, race, education, SES, perceived stress	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	9	X	X	X	X	X	X	shorter
Tyrka et al., 2016	$N = 289$ 177 f, 113 m,	31.0 (10.7)	USA	Cross-sectional (case-control)	No disorder/no adversity ($n = 113$) No disorder/ adversity ($n = 66$) Disorder/no adversity ($n = 39$) Disorder/adversity ($n = 72$)	qPCR; leukocyte	Race, smoking, oral contraceptive, psychiatric disorder	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	13	X	X	X	X	X	X	shorter
van Ockenburg et al., 2015	$N = 1,094$ 588 f, 506 m	53.1 (11.4)	Netherlands	Longitudinal (prospective)	Population-based	qPCR; leukocyte	Sex, age, comorbidity, BMI, smoking, exercise, education	LTE (mod.)	<12	Event-based, chronic	Childhood (<12)	Retrospective period	41	X	X	X	X	X	X	none
Verhoeven et al., 2015	$N = 2,936$ 1,950 f, 986 m	41.8 (13.1)	Netherlands	Cross-sectional	Longitudinal cohort study examining the course and consequences of depressive and anxiety disorders; 57% current depression	qPCR; leukocyte	Sex, age, comorbidity, depression, BMI, smoking, alcohol use, exercise, education	CTI; CTQ; score	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	26	X	X	X	X	X	X	none
Vincent et al., 2017	$N = 180$ 103 f, 77 m	50 (15.65)	UK	CROSS- sectional	80 depressed subjects and 100 control subjects	qPCR; blood (cell type not specified)	Age, sex	CTQ	<18	Event-based, chronic	Whole childhood (<18)	Retrospective period	32	X	X	X	X	X	X	mixed
Zalli et al., 2014	$N = 333$ 167 f, 166 m	63.2 (5.5)[a]	UK	Cross-sectional	Heart Scan Study, a subsample of the Whitehall II epidemiological cohort	qPCR; PBMC	Age, SES and BMI	Item	<16	Event-based, chronic	Whole childhood (<16)	Retrospective period	47	X	X	X	X	X	X	shorter

ACEs, adverse childhood experiences; BMI, body mass index; BP, blood pressure; BTL, buccal cell telomere length; CA, childhood adversity; CM, childhood maltreatment; CPA, child physical abuse; CSEQ, children's social experiences questionnaire; CTI, childhood trauma interview; CTQ, childhood trauma questionnaire; CTS, conflict tactics scale; DIAS-VS, direct and indirect aggression scale-victim version; DNA, deoxyribonucleic acid; DNAm age, DNA methylation age; ELD, elderly people; ELS, early life stress; ETI, early trauma inventory; Exp., exposed; f, female; HIV, human immunodeficiency virus; HC, healthy controls; HLEQ, health and life experiences questionnaire; int.dis., internalizing disorders; LHC, life history calendar; LSC, life stressor checklist; LTE, list of threatening events; LTL, leukocyte telomere length; m, male; MDD, major depressive disorder; mod., modified; N, number of participants; NA, not available; NLE, adulthood negative life events; PBI, parental bonding instrument; PAPA, preschool age psychiatric assessment; PBMC, peripheral blood mononuclear cells; PTSD, posttraumatic stress disorder; qPCR, quantitative polymerase chain reaction; SES, socioeconomic status; SF, short form; SR, self-report; SLE, stressful life events in early life; TA, Telomerase Activity; TEC, traumatic experiences checklist; TL, Telomere length; UK, United Kingdom; USA, United States of America.

[a] *weighted mean ages.*

[b] *Range up till the age of participants (oldest participant 15 years).*

(Glass et al., 2010; Kananen et al., 2010; Tyrka et al., 2010, 2016; O'Donovan et al., 2011; Malan-Müller et al., 2013; Chen et al., 2014; Mitchell et al., 2014; Blom et al., 2015; Bersani et al., 2016; Kuffer et al., 2016; Levandowski et al., 2016; Liu et al., 2017; Riley et al., 2018). Five studies measured TL at more than one time point and were therefore able to examine TL longitudinally (Shalev et al., 2013, 2014; Boks et al., 2015; van Ockenburg et al., 2015; Revesz et al., 2016). The type of samples and the sample composition of the included papers varied widely. Some studies examined general population samples, such as birth cohorts (Jodczyk et al., 2014; van Ockenburg et al., 2015; Osler et al., 2016), whereas others had a focus on specific clinical populations, such as on depressed patients (Chen et al., 2014; Blom et al., 2015; Cai et al., 2015; Liu et al., 2017; Vincent et al., 2017), patients with anxiety disorders (Kananen et al., 2010), patients with post-traumatic stress disorder (PTSD) (O'Donovan et al., 2011; Boks et al., 2015; Kuffer et al., 2016), or patients with substance use disorders (Levandowski et al., 2016).

Because there are different ways to measure telomere length (Montpetit et al., 2014), information on the telomere assay method was collected. In our sample of eligible papers, almost all studies ($N = 36$) investigated TL using a quantitative polymerase chain reaction (qPCR). Only two papers used a southern blot analysis as TL assay method (Glass et al., 2010; Kiecolt-Glaser et al., 2011). TL was examined in different cell types: six papers reported that DNA was extracted from saliva samples (Kiecolt-Glaser et al., 2011; Mitchell et al., 2014; Blom et al., 2015; Cai et al., 2015; Puterman et al., 2016; Liu et al., 2017; Guarneri-White et al., 2018), and four studies used epithelial buccal cells (Shalev et al., 2013; Drury et al., 2014; Kuffer et al., 2016; Dagan et al., 2017). The other studies ($N = 28$) extracted DNA from peripheral blood samples. Most of these studies assayed leukocyte DNA for TL ($N = 22$), four studies extracted DNA from peripheral blood monocular cells (PBMCs) (Kiecolt-Glaser et al., 2011; Malan-Müller et al., 2013; Zalli et al., 2014; Mitchell et al., 2018), and two studies extracted DNA from lymphocytes (Surtees et al., 2011; Riley et al., 2018). Although a wide variety of covariates were included across the studies, almost all studies controlled for age, sex, body mass index (BMI) and smoking.

ACEs Characteristics

Assessments of ACEs varied substantially across studies (see **Table 1**). Studies examined various age ranges: 18 studies included ACEs before the age of 18 (Tyrka et al., 2010, 2016; Kiecolt-Glaser et al., 2011; Malan-Müller et al., 2013; Chen et al., 2014; Boks et al., 2015; Mason et al., 2015; Bersani et al., 2016; Kuffer et al., 2016; Levandowski et al., 2016; Osler et al., 2016; Puterman et al., 2016; Dagan et al., 2017; Liu et al., 2017; McFarland et al., 2017; Vincent et al., 2017; Mitchell et al., 2018; Riley et al., 2018), one study reported on ACEs before the age of 17 (Surtees et al., 2011), eight studies investigated ACEs before the age of 16 (Kananen et al., 2010; Jodczyk et al., 2014; Zalli et al., 2014; Cai et al., 2015; Verhoeven et al., 2015; Revesz et al., 2016; Schaakxs et al., 2016; Oliveira et al., 2017), one study before the age of 15 (O'Donovan et al., 2011) and two studies before the age of 12 (Shalev et al., 2014; van Ockenburg et al., 2015). Additionally, six studies assessed ACEs up till the time of assessment (Shalev et al., 2013; Drury et al., 2014; Mitchell et al., 2014; Blom et al., 2015; Robles et al., 2016; Guarneri-White et al., 2018). Furthermore, the eligible papers used different ACEs assessments. About half of the studies ($N = 20$) used standardized validated questionnaires or interviews to assess adversities. The most commonly used questionnaire was the retrospective, self-report CTQ (Bernstein et al., 1994) that was used in 10 studies (Tyrka et al., 2010, 2016; Kiecolt-Glaser et al., 2011; Malan-Müller et al., 2013; Blom et al., 2015; Verhoeven et al., 2015; Kuffer et al., 2016; Levandowski et al., 2016; Vincent et al., 2017; Mitchell et al., 2018). The other studies ($N = 18$) used modified versions of other questionnaires or interviews or used novel items to create adversity scores (see **Table 1**, column assessment; **Supplementary Material**, column type of adversity).

TABLE 2 | Overview results.

	Shorter	None	Longer	Mixed
Total association				
ACEs and TL ($N = 38$)	18	9	2	9
ACEs and ΔTL ($N = 5$)	2	3	–	–
Sub-findings				
Sample size ($N = 38$)				
<400 ($N = 23$)	13	4	2	4
>400 ($N = 15$)	5	5	–	5
Sex ($N = 37$)				
Only male ($N = 3$)	2	1	–	–
Only female ($N = 7$)	3	2	1	1
Both ($N = 27$)	13	5	1	8
Age ($N = 36$)				
<25 ($N = 7$)	5	1	–	1
25–45 ($N = 13$)	5	6	–	2
>45 ($N = 16$)	7	1	2	6
Sample Composition ($N = 38$)				
Clinical ($N = 16$)	6	5	1	4
Non-clinical ($N = 22$)	12	4	1	5

ΔTL, telomere attrition or within subject TL change.

With the SECs in mind, it was shown that almost all studies ($N = 37$) either had an event-based, or event-based/chronic stress measurement timescale. The only exception was Robles et al. (2016), who based their adversity score on current ratings of daily emotions to family conflict. Regarding the developmental life stage, all papers reported on ACEs before the age of 18. Most studies did not differentiate between childhood and adolescence. However, certain studies only included ACEs in childhood or did differentiate between childhood and adolescence (Shalev et al., 2013; Drury et al., 2014; Mitchell et al., 2014; Blom et al., 2015; Robles et al., 2016; Guarneri-White et al., 2018). Some studies used smaller age ranges (Shalev et al., 2014) or built subcategories of their larger ranges (Savolainen et al., 2014; van Ockenburg et al., 2015). Looking at the stress assessment window—in particular the measurement timeframe of ACEs assessments—most studies ($N = 34$) assessed ACEs retrospectively. Some studies used combined retrospective and prospective assessments (Shalev et al., 2013, 2014), a combination of retrospective

self-reports and archive information (Savolainen et al., 2014), or an adversity score based on daily ratings (Robles et al., 2016). In terms of the time between the ACEs exposure and the age at ACEs assessment, the duration varied between 0 and 56 years. Aggregating all duration measures across studies, the mean time between the end of the ACEs measure and age at ACEs assessment was approximately 23 years.

Regarding the six reviewed stressor attributes, almost no information is included and specified in the included sample of studies. First, only one study reported on the duration of ACEs (the duration of being separated from their parents) (Savolainen et al., 2014). Second, four studies reported on the severity of ACEs on a continuous scale (Blom et al., 2015; Mason et al., 2015; Kuffer et al., 2016; Riley et al., 2018). Most studies ($N = 34$), however, did not report on the severity of the stressor on a continuous measure. Instead, they reported exposure categories, defined by using self-developed items or certain cut-off scores on continuous measures. Third, none of the studies explicitly measured controllability on a continuous scale. Fourth, looking at specific life-domains, no study reported on ACEs from a specific life-domain. However, many ACEs in childhood are of interpersonal and interpersonal-intimate nature, resulting from multiple life domains, mainly family, peers and school. Fifth, no study explicitly reported on the attribute "target of the stressor," though, most studies assessed ACEs that targeted participants themselves, or close others. Last, focusing on the attribute "potential of the stressor to elicit potential harmful responses," none of the study described in detail the qualities inherent to the adversities that were measured.

Overall, the eligible studies reported on stressors from a broad range of potentially harmful experiences. However, a lot of information is unknown, missing or not specified. Therefore, more research using a common language and taxonomy to describe certain characteristics of stressors—in particular with regard to ACEs—is needed.

Main Findings: ACEs and TL

In total, 18 paper reported a negative association between ACEs and TL or higher odds for shortened TL among individuals reporting exposure to ACEs compared to those who were less or non-exposed (Kananen et al., 2010; Tyrka et al., 2010, 2016; Kiecolt-Glaser et al., 2011; O'Donovan et al., 2011; Surtees et al., 2011; Shalev et al., 2013; Drury et al., 2014; Mitchell et al., 2014; Zalli et al., 2014; Cai et al., 2015; Bersani et al., 2016; Levandowski et al., 2016; Osler et al., 2016; Puterman et al., 2016; Robles et al., 2016; McFarland et al., 2017; Guarneri-White et al., 2018). Additionally, nine papers showed no association between ACEs and TL (Glass et al., 2010; Malan-Müller et al., 2013; Jodczyk et al., 2014; Shalev et al., 2014; Blom et al., 2015; Boks et al., 2015; van Ockenburg et al., 2015; Verhoeven et al., 2015; Mitchell et al., 2018). Furthermore, two studies even reported a trend toward longer telomeres among individuals reporting more ACEs (Kuffer et al., 2016; Oliveira et al., 2017). Finally, nine papers reported mixed findings, with studies reporting some associations within their data, but no conclusive association within their total sample (Chen et al., 2014; Savolainen et al., 2014; Mason et al., 2015; Revesz et al., 2016; Schaakxs et al., 2016; Dagan et al., 2017; Liu et al., 2017; Vincent et al., 2017; Riley et al., 2018).

Beyond that, five studies have examined TL at more than one time point (Shalev et al., 2013, 2014; Boks et al., 2015; van Ockenburg et al., 2015; Revesz et al., 2016). Hence, these studies were able to assess telomere attrition, which is the change in telomere length within a subject. Two of these studies showed ACEs to be associated with TL change (Shalev et al., 2013; Revesz et al., 2016), whereas three papers reported no association between ACEs and TL change (Shalev et al., 2014; Boks et al., 2015; van Ockenburg et al., 2015).

Possible Moderators

To attempt to explain the variety in findings, comparisons were made based on sample size, age, sample composition, and sex of study samples. First, focusing on the study characteristics, the results of studies with more than 400 participants ($N = 15$) seemed to be less conclusive than studies with <400 participants ($N = 23$). Of these studies with larger samples, five papers reported a cross-sectional association between early adversity and TL (Kananen et al., 2010; Surtees et al., 2011; Cai et al., 2015; Puterman et al., 2016; McFarland et al., 2017), five studies reported mixed results (Savolainen et al., 2014; Mason et al., 2015; Revesz et al., 2016; Schaakxs et al., 2016; Liu et al., 2017), and five reported no associations (Glass et al., 2010; Jodczyk et al., 2014; Shalev et al., 2014; van Ockenburg et al., 2015; Verhoeven et al., 2015). Second, subdividing the age of study samples indicated that studies investigating TL during childhood, adolescence or emerging adulthood ($N = 7$) more often find associations of ACEs and shorter TL (Shalev et al., 2013; Drury et al., 2014; Mitchell et al., 2014; Blom et al., 2015; Robles et al., 2016; Dagan et al., 2017; Guarneri-White et al., 2018). Findings in older samples are more inconclusive. Third, considering the sample composition, comparing clinical (with mental disorders) ($N = 16$) and non-clinical samples (without mental disorders) ($N = 22$) indicated that studies in non-clinical samples more often find negative associations between ACEs and TL than do studies in clinical populations. Fourth, with regard to the sex of participants, there were no observable differences in reported results.

DISCUSSION

The aim of the current review was to review the literature on the associations between ACEs and TL in an attempt to highlight how heterogeneity in sample and stressor characteristics contributes to findings. Overall, the sample of studies we reviewed indicates a negative association between ACEs and TL, although many papers presented mixed findings and a quarter of eligible studies found no relationship between ACEs and TL. These findings are consistent with recently published meta-analyses investigating the association between early adversity, childhood trauma and childhood psychosocial stressors and TL. All three studies showed significant small negative associations with TL (Hanssen et al., 2017; Li et al., 2017b; Ridout et al., 2017). These meta-analyses further reported high between-study heterogeneity of effects. Considering possible moderators within our sample of

studies indicates that results of larger samples seem to be less conclusive than results of smaller samples. In addition, studies investigating participants younger than 25 more often find ACEs to be negatively associated with TL compared to older samples. Furthermore, results from studies of non-clinical samples more often report negative associations between ACEs and TL than do studies of clinical samples. Using the SECs proposed by Epel et al. (2018) to examine characteristics of the included ACEs shows a lack of detailed information on SECs in many studies. At least four findings (sample size, age, psychopathology, and ACEs characteristics) need to be discussed in more detail to find explanations for the heterogeneity and inconclusiveness of reported findings.

First, with regard to sample size, we observed that findings of larger samples are less conclusive compared to findings of smaller samples. This might be explained by the fact that larger samples can control for more additional variables and potential confounds. These additional factors might moderate, mediate, conceal or suppress the direct, independent impact of ACEs, as many of these variables in larger models are inter-correlated (e.g., adversities, mental health problems, negative life-styles, and smoking status).

Second, we observed that studies with younger participants more often find negative associations than studies with older participants. This is in line with Ridout et al. (2017) who reported in their moderator analyses that the smaller the duration between ACEs exposure and age at TL assessment, the larger the magnitude of effect sizes. They explained this finding by pointing to the fact that studies of children assume no smoking amongst participants, and that adversities early in childhood tend to be associated with larger effects (Ridout et al., 2017). Similar results were found by Hanssen et al. (2017). Another potential explanation, according to the healthy survivor effect, might be that participants within older samples drop out due to morbidity or early mortality, which is in turn associated with shorter telomeres (Mather et al., 2011; Kuffer et al., 2016; Schaakxs et al., 2016; Oliveira et al., 2017). Moreover, Schaakxs et al. (2016) argued that "a possible explanation for these null findings in older adults may be that older adults have been exposed to numerous competing causes for shortened TL, such as somatic diseases or an unhealthy lifestyle over the life span. These other TL-damaging factors may suppress the independent impact of psychosocial stressors." (p. 441).

Third, the sample composition of included studies varied strongly. Some of the studies focused on specific clinical populations and the impact of psychiatric disorders on TL. These studies included ACEs in their models as control variables. In contrast, other studies focused on the impact of ACEs on TL controlling for psychiatric conditions. We observed that studies with non-clinical populations more often report negative associations between ACEs and TL. This is in line with Ridout et al. (2017), who found effect sizes of smaller magnitude regarding the association of ACEs and TL in their moderator analyses, when looking at studies that included subjects with mental disorders. Epel and Prather (2018) recently proposed a triad model of stress exposures, psychopathology and telomere biology combining the meta-analytic evidence between the associations of stress and telomeres, stress and psychopathology, and psychopathology and telomeres. Having this triad in mind, when approaching TL from a psychopathological perspective, studies have to acknowledge that "expression of psychopathology may be strongly influenced by exposure to maltreatment" (Teicher and Samson, 2013, p. 1,114). This distinctive phenotypical expression of a psychiatric disorder (with vs. without maltreatment) might reveal distinct subtypes of disorders that are important to account for when determining the biological bases of these mental disorders (Teicher and Samson, 2013; Teicher et al., 2016). Moreover, possible direct associations of ACEs on TL might be mediated by the later development of mental disorders. Assuming that early adversities often precede psychopathology, psychiatric disorders might mediate the association of ACEs and TL. Hence, research on TL should acknowledge both perspectives: distinct subtypes of psychiatric disorders (with vs. without maltreatment) within clinical samples and the potential mediating effect of psychopathology in non-clinical samples.

Fourth, the current study further examined ACEs using the SECs recently proposed by Epel et al. (2018). Results showed an overall lack of details and lots of missing information. This makes it indeed very difficult to understand the adverse nature of these experiences with important characteristics and attributes not being measured or articulated. Differentiating between event-based and chronic exposures, the target of the exposure, and the duration, for instance, is very important in the context of trauma research as many childhood adversities are interpersonal and traumatic in nature (e.g., abuse and neglect, interpersonal loss, interpersonal conflict, interpersonal violence) and are targeted at either participants themselves or at close others (e.g., siblings or family members) (Widom et al., 2008; Moffitt and the Klaus-Grawe Think Tank, 2013). Chronic-occurring interpersonal events are often followed by a broad range of trauma-associated psychopathologies that are not captured within the classical framework of PTSD (Cook et al., 2005). These harmful responses can lead to diverse behavioral and emotional alterations, often referred to as complex trauma symptoms, as for example affective dysregulation, attentional and behavioral problems, self and relational deregulation (Briere et al., 2008; Greeson et al., 2011; Schmid et al., 2013). For this reason many experts emphasized the need for a more developmentally sensitive diagnostic system that takes account of the heterogeneity of psychopathology following early trauma (Cloitre et al., 2009; van der Kolk et al., 2009; D'Andrea et al., 2012; Schmid et al., 2013). This led to the inclusion of complex trauma symptoms within the PTSD section in the Diagnostic and Statistical Manual of Mental Disorders—Fifth Edition (DSM-5) and the inclusion of a complex PTSD disorder in the International Classification of Diseases 11th Revision (ICD-11). These complex trauma symptoms contain symptoms of affect dysregulation, negative self-concepts and interpersonal problems that are related to the traumatic exposure (Cloitre et al., 2013). Overall, the adversities included are all of a stressful, adverse, and traumatic nature. Most of these stressors have the potential to elicit harmful emotional responses (e.g., social threat, loss of control, shame) and behavioral alterations

(e.g., role-change, impulsivity), but detailed and differentiating information is missing.

Limitations

The current review needs to be seen in light of some limitations. First, this review is not a systematic review as defined by PRISMA or Cochrane guidelines. The narrative approach, however, allowed us to discuss the complexity of exposure characteristics in an overall heterogenous sample of studies and adds to recently published systematic meta-analyses. Second, most studies assessed ACEs retrospectively with self-reported questionnaires, sometimes with several decades between adversity and assessment of adversity, which leads to recall biases. Hardt and Rutter (2004) extensively discussed biases of retrospective self-reports and concluded that they easily lead to an underreporting of events and that the validity of details assessed retrospectively might be low, but false-positive reports are rare. In contrast, a recently published meta-analysis reported only weak associations between prospective and retrospective measures of adversity concluding that these measures identify different groups of individuals (Baldwin et al., 2019). This should be taken into account in future studies. Third, this review focused on the ACEs part of the ACEs-TL association. Besides that, methodological issues with regard to the TL measurement approach are also of high interest and might explain some of the heterogeneity in findings. These issues are extensively reviewed and discussed elsewhere and beyond the scope of this review (Montpetit et al., 2014; Lai et al., 2018). Fourth, publication bias is likely to occur because we only included papers that were published in peer-reviewed journals. Last and most important, as described in the method section, studies were included that measured ACEs before the age of 18 by means of a questionnaire or an interview. Studies reporting on early adversities solely based on high-risk status, on low socio-economic status (SES), on neglectful, non-supportive parenting styles, on maternal depression, and on maternal stressors during pregnancy, were not included due to their lack of direct measurement of adverse experiences. Being at risk for ACEs is highly correlated with incidence of ACEs but not all at-risk individuals are exposed. This approach was used because the focus of this review was on the harmful long-term consequences of experiencing ACEs. Still, as a substantial overlap between different operationalization's of stressors exist, it is therefore very difficult to draw clear boundaries.

Implications

Future research might benefit from a differentiated look into ACEs, articulating multiple domains of stressors such as in the SECs (Epel et al., 2018). This will help to improve our understanding of the adverse nature of these exposures and uncover different exposure-related emotional and behavioral responses that mediate the association between ACEs and long-term health outcomes. This might help to further our understanding of the complex associations of stress and TL, beyond what can be explained by simply summing potentially harmful incidents in childhood. In addition, resilience factors that protect children and adolescents from sustained physiological consequences need further investigation.

CONCLUSION

Overall, the included sample of studies indicates a negative association between ACEs and TL, but the diversity in sample and stressor characteristics makes it difficult to achieve a final and confident conclusion. From a developmental perspective, a more comprehensive evaluation of adversities using a common language and dimensional approaches to SECs might help to improve understanding of the complex associations between (early) stressors and health outcomes. Individuals are exposed to numerous competing and interacting exposures that might shorten TL over the life course. A focus on developmental trajectories combining early adversities, psychopathology and protective factors might help to develop enhanced approaches to reduce the stress-related health burden of our societies.

AUTHOR CONTRIBUTIONS

DB, CB, MS, and KS contributed to the conception of the paper. DB extracted all study and stressor characteristics and wrote the first draft of the manuscript. AOD, CB and DdH wrote sections of the manuscript and edited the paper. AOD, AE, JF, AdG, MS, KS, and CB critically revised the paper. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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