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NEW MECHANISMS AND THERAPEUTICS IN NEURODEVELOPMENTAL DISORDERS

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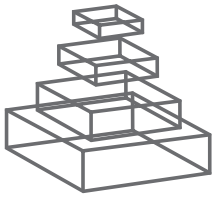

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NEW MECHANISMS AND THERAPEUTICS IN NEURODEVELOPMENTAL DISORDERS

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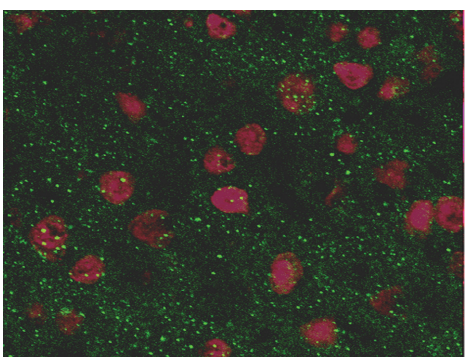

Neurodevelopmental disorders (NDDs) are severe, heritable conditions characterized by impaired brain development and disability in cognition, social interaction and behavior. Genetic predisposition appears to interact with environmental factors to produce the onset of the disease. In the last decade genomic analysis on NDDs advanced the knowledge of the genetic causes of these disorders: most of the genes identified were not predicted from known biology, and almost all of them engage in neurodevelopmental processes. However, the same studies revealed an unexpected outcome regarding the molecular etiology: many of the same susceptibility genes as well as molecular pathways are emerging across hitherto unrelated disorders- such as autism and schizophrenia- challenging how we conceptualize these conditions.

These new discoveries call for the establishment of additional criteria for the classification of the genes and related phenotypes. Beyond the classical distinction according to diagnostic criteria, new parameters should be taken into account: the genetics, the neurobiology of the gene products and the molecular mechanisms that they control. Our broad understanding of NDDs, requires a combination of approaches, including human studies, animal models and theoretical computation. Indeed, progress in technology and the generation of accurate models are advancing the field, and the research is rapidly growing.

This Research Topic presents recent advances in neurodevelopmental disorders from genes to systems, in single conditions and across different phenotypes, and report the newest treatments in use. Recognized experts in the field discuss recent findings in molecular studies, in vivo imaging, neuropsychology, neuropharmacology and computational neuroscience. This multidisciplinary approach enriches our understanding of the mechanisms involved in the onset and progression of NDDs with the perspective of revealing the biological signs of the disorders and identifying new therapeutics.

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New challenges and frontiers in the research for neuropsychiatric disorders

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Research in neuropsychiatric conditions is living a unique time: genomic analysis advanced the knowledge of the genetic causes of these disorders. At the same time technological advances make possible a deep investigation of the structure and function of the brain (Mothersill et al., 2012) and Induced Pluripotent Stem Cells (IPSC) technology gives us access to neuronal cultures from each single patient (Cheung et al., 2012). These progresses hold the promise for a better understanding of neuropsychiatric disorders with a consequent improvement in the tools for diagnosis and treatment.

Nonetheless, the more we deepen our understanding, the more the complexity appears, and this is due to several reasons: first, except for rare monogenic disorders, the majority of neuropsychiatric conditions are polygenic. Second, the molecular etiology is complex: many of the same susceptibility genes and molecular pathways are emerging across hitherto unrelated disorders – such as autism and schizophrenia – challenging how we conceptualize these conditions (Mitchell, 2011). Third, genetic background interacts with environmental factors to produce the onset and progression of the disease (Stolp et al., 2012) with the consequence that genetic analysis alone cannot predict the outcome of specific clinical symptoms, and environmental factors are critical.

Despite recent advances in genetics and imaging, the neurobiology of neuropsychiatric disorders remains largely obscure. Much of the evidence from genomics implicates molecular mechanisms of neurosynaptic development, plasticity, and function (Mitchell, 2011), and research is converging on deficits in the function of neuronal circuitry. As a consequence, we need to establish additional criteria for the classification of the genes and related phenotypes and new parameters should be taken into account such as the genetics, the neurobiology of the gene products, and the molecular and physiological mechanisms that they control. This task requires a combination of approaches, including animal models and human studies.

In autism research, several steps have been undertaken in this direction and in some cases it has been possible to clarify the intracellular pathways and to produce novel therapeutics (Banerjee et al., 2012). This has been possible for monogenic disorders such as Rett syndrome, Angelmann syndrome, and Fragile X, thanks to the genesis of animal models that present similar genetics and symptoms of the patients. However, this is just a minority of disorders as autism has a complex genetic background. Also for psychosis, research is slowly progressing toward the comprehension of the neurobiology of high penetrant variants in schizophrenia (Kamiya et al., 2012).

To foster new ideas in the neurobiology of neuropsychiatric disorders, it is important to explore new hypothesis regarding the mechanisms of action of the candidate genes and to use appropriate substrates to test them. The use of animal models – even considering the genetic variability due to background and species – is critical to access the developmental brain, and to study the onset and progression of the disorders. Even if the clinical investigation in humans cannot be performed on the rodent models, there are parallel signs that can be used as endophenotypes, such as the reduction in the number of dendritic spines, deficits in working memory, and the structural and functional organization of the brain (Mothersill et al., 2012). Other clues can be found in brain areas rarely explored in neuropsychiatric disorders, such as the visual cortex. Recent evidence proved that synaptic reinforcement in evoked visual stimulation is hampered in patients with schizophrenia (Cavuş et al., 2012). This is not surprising, considering that the majority of candidate genes for psychosis relate to the function of synapses, and that the candidate genes are mutated in all brain areas. Therefore, the visual cortex can be a valid model for investigating the role of candidate genes in circuitry function and plasticity both *in vitro* and *in vivo*, as shown in animal models of autism (Banerjee et al., 2012). Together with the exploration of new brain areas, new players should be taken into account, such as inhibitory circuitry (Chattopadhyaya and Di Cristo, 2012) and microRNAs (Mellios and Sur, 2012). Finally, because several genes are mutant in different conditions, we need to understand the overlapping developmental processes common to several disorders (Bozzi et al., 2012).

In conclusion, the deep understanding of neuropsychiatric disorders requires a combination of techniques, including genetic studies, animal models and imaging, and more than that, requires going beyond the hypothesis currently tested, and explore new mechanisms and endophenotypes. Only using this new, multidisciplinary approach it will be possible to clarify the mechanisms involved in the onset and progression of neuropsychiatric disorders with the perspective of revealing the biological signs of the disorders and identifying new therapeutics.

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GABAergic circuit dysfunctions in neurodevelopmental disorders

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GABAergic interneurons control neuronal excitability, integration, and plasticity. Further, they regulate the generation of temporal synchrony and oscillatory behavior among networks of pyramidal neurons. Such oscillations within and across neural systems are believed to serve various complex functions, such as perception, movement initiation, and memory. Alterations in the development of GABAergic circuits have been implicated in various brain diseases with neurodevelopmental origin. Here, we highlight recent studies suggesting a role for alterations of GABA transmission in the pathophysiology of two neurodevelopmental diseases, schizophrenia, and autism. We further discuss how manipulations of GABA signaling may be used for novel therapeutic interventions.

Keywords: GABA, autism, schizophrenia, neurodevelopmental plasticity, basket cells, interneurons, development

Cortical neural networks consist of broadly two classes of neurons: excitatory projection neurons, primarily using glutamate as neurotransmitter, and inhibitory local-circuit interneurons, comprising of about 20% of all cortical neurons, and primarily using gamma-amino butyric acid (GABA) as a neurotransmitter. The last few years have seen a steep increase in our knowledge of the function of GABAergic interneurons. Cortical interneurons have been shown to play a vital role in modulating neuronal excitability (Swadlow, 2003), integration (Pouille and Scanziani, 2001), and in the generation of temporal synchrony and oscillation among networks of glutamatergic neurons (Klausberger and Somogyi, 2008). In addition, GABAergic interneurons contribute to almost all fundamental processes of cortical development; from neuronal proliferation, migration, and differentiation to experience-dependent refinement of local cortical circuits (Owens and Kriegstein, 2002; Hensch, 2005; Sernagor et al., 2010).

These findings have prompted a change in the way we view the role of GABAergic cells in cortical development and processing. Traditionally, GABAergic interneuron function has been described simply as "inhibition" of neuronal activity, similar to a traffic officer who signals simply to let cars stop or go; however it is now clear that the picture is much more complex. We may instead compare GABAergic interneuron function to that of a conductor of a symphony orchestra, whose role is to structure and coordinate the overall musical performance and interpretation of the individual players. Without proper direction, the orchestra cannot produce the right melody.

Considering the multifaceted role played by GABAergic cells in the development, function, and plasticity of cortical networks, it is straightforward to assume that any disturbance in the development of GABAergic interneurons, due to either genetic or epigenetic factors, will strongly affect brain function. Indeed, disruption of GABAergic circuit function has been implicated in various neurodevelopmental and psychiatric disorders including schizophrenia (Gonzalez-Burgos et al., 2011), autism (LeBlanc and Fagiolini, 2011), mental retardation (Cramer et al., 2010), and epilepsy (Rossignol, 2011). Our understanding of the mechanisms underlying development of GABAergic interneurons have started to reveal specific molecular and cellular substrates potentially affected in neurodevelopmental disorders. Further, selective modulation of inhibitory networks is being currently investigated as a tool to re-introduce plasticity and therefore facilitate the restoration of normal function to the diseased brain.

This review will summarize some of the compelling evidence on the role of alterations in GABAergic neuron function in the etiology of two neurodevelopmental disorders, schizophrenia, and autism. An exhaustive review of the genetic causes of schizophrenia and autism is beyond the scope of this paper, and several excellent reviews have already been published on these topics. We will further focus on and discuss recent efforts to modify GABAergic transmission as a targeted therapeutic approach to re-introduce plasticity in the brain.

CORTICAL INTERNEURON DIVERSITY – A CONTINUOUS CHALLENGE

Before describing evidence linking GABAergic dysfunction to neurodevelopmental diseases, we would like to stress that our understanding of GABAergic interneuron function in normal and pathological conditions is challenged by their startling heterogeneity. Indeed different interneuron subtypes display distinctly different morphology, physiological properties, connectivity patterns, and vary in their biochemical composition (Markram et al., 2004). Such complexity makes it difficult to establish a broad consensus regarding a generalized classification scheme of

interneuron subtypes. In parallel however, several studies have singled out multiple criteria to distinguish between major interneuron groups. One criterion for instance is the expression of calcium binding proteins such as parvalbumin (PV), calbindin (CB), or calretinin (CR) and/or neuropeptides such as somatostatin (SST), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), or cholecystokinin (CCK). Such analysis has shown that the cortex has three largely independent populations of interneurons: (1) PV, (2) SST/CB, and (3) CR/VIP expressing interneurons (Somogyi et al., 1998).

Further, different subtypes of interneurons have different spatial and temporal origins. The neocortical interneuron population, as such, is derived from transient ventral telencephalic structures referred to as the ganglionic eminences as well as from the preoptic area (Batista-Brito and Fishell, 2009; Gelman and Marín, 2010). The medial ganglionic eminence (MGE) produces ∼70% of neocortical interneurons, including the PV-expressing and SST-positive interneurons. Meanwhile, the caudal ganglionic eminence (CGE) gives rise to a more heterogeneous group of cortical interneurons that share the expression of 5HT3A ionotropic serotoninergic receptors and are thus highly responsive to the neuromodulatory effects of serotonin (Batista-Brito and Fishell, 2009; Gelman and Marín, 2010; Lee et al., 2010).

Another striking characteristic of GABAergic interneurons is that different subtypes innervate distinct domains of the postsynaptic glutamatergic cells. For example, SST/CB expressing interneurons selectively innervate distal dendrites of target neurons, while PV-expressing, fast-spiking interneurons can be divided in two major classes, basket cells which selectively innervate the soma and proximal dendrites and chandelier cells, which selectively innervate the axon initial segment of pyramidal cells (Huang et al., 2007). The spatial organization of GABAergic synapses along glutamatergic neurons has provided important clues as to the specific functions of various interneuron subtypes (Huang et al., 2007; Rossignol, 2011).

To truly understand how GABAergic circuits develop and function, it is essential to differentiate between distinct GABAergic interneuron subtypes. It is indeed likely that specific interneuron types differentially contribute to network processing that underlies cortical functions ranging from perception to cognition. Consequently, we can hypothesize that deficits in specific interneuron classes may account, at least in part, for different neuropathological conditions.

Recent technical advances have significantly accelerated progress in the interneuron field. In particular, the development of genetic strategies based on interneuron cell type-specific promoters in combination with inducible Cre-loxP knockin driver lines and fluorescent protein reporter lines has allowed efficient high-resolution labeling and manipulation of specific GABAergic interneuron classes in intact or "semi-intact" tissues (such as organotypic brain cultures; Chattopadhyaya et al., 2004, 2007; Taniguchi et al., 2011). Due to the availability of these new tools, it is now possible to study specific GABAergic interneuron types, from cell fate specification, migration, and connectivity, to their function in network dynamics and behavior. Combining insights obtained from basic biological research on GABAergic interneuron development, plasticity, and function with genetic and clinical findings will provide us a detailed picture of the molecular events that result in diseases characterized by GABAergic dysfunction, and will suggest putative useful methods for targeted corrective intervention.

GABAergic ALTERATIONS IN NEURODEVELOPMENTAL DISEASES

SCHIZOPHRENIA

Schizophrenia is a severe brain disorder that afflicts 0.5–1% of the world's population and is usually first diagnosed in late adolescence or early adulthood. The illness manifests with three classes of symptoms: positive or psychotic symptoms (i.e., delusions, hallucinations, and disorganization of thought), negative symptoms (i.e., flat affect, avolition, and alogia), and cognitive impairments such as abnormalities in working memory, inferential thinking, selective attention, as well as defects in executive functions, linguistic fluency, and social-emotional processing.

Although positive symptoms presents the most prominent clinical feature of schizophrenia, disturbances in cognition appear to be the core feature of the illness as they are present before the onset of psychosis and are the best predictor of long-term functional outcome for schizophrenia patients. Therefore, understanding neural mechanisms underlying cognitive and dysfunctions is critical to develop new therapeutic strategies particularly given that currently approved pharmacological treatments for schizophrenia are largely ineffective at improving cognition.

A central cognitive deficit in schizophrenia is the dysfunction of working memory, i.e., the ability to store information and use it while performing complex tasks (Baddeley, 2010). Gamma oscillations (30–80 Hz) are thought to play an important role in normal working memory because gamma band synchrony increases with increasing working memory load (Howard et al., 2003). In patients with schizophrenia, working memory deficits are accompanied by altered patterns of cortical oscillatory activity. In particular, gamma activity does not augment with increasing working memory load in schizophrenia patients (Basar-Eroglu et al., 2007; Barr et al., 2010; Haenschel and Linden, 2011). Working memory related gamma activity, but not theta activity, is reduced in the frontal cortex of first-episode schizophrenia patients independent of medication status, suggesting a deficit related to the disease process as opposed to medication side effects or as a consequence of being chronically ill (Minzenberg et al., 2010). Moreover, subjects with schizophrenia have decreased oscillations in various frequency bands during specific phases of the working memory process including encoding, maintenance, and retrieval (Haenschel et al., 2009).

Among the several mechanisms proposed to play a role in the generation of synchronized oscillations in neocortical circuits, computational modeling studies, and numerous experimental evidence support a leading role of GABA-mediated inhibition. In particular, convergent evidence indicates that synchronous activity in PV-expressing, fast-spiking basket interneurons is key to the emergence of gamma oscillations (Bartos et al., 2007). *In vitro* studies in rodent hippocampus and cortex show that blocking GABA suppresses gamma oscillations, and that driving fast-spiking interneurons by activating metabotropic glutamate receptors generates gamma oscillations (Whittington et al., 1995; Bartos et al., 2002).

Correlative *in vivo* studies similarly show that fast-spiking cells fire action potentials in phase with the gamma oscillations in the neocortex (Hasenstaub et al., 2005; Sirota et al., 2008). Causal evidence for this hypothesis *in vivo* has been recorded in the neocortex using optogenetics, which allow the bidirectional modulation of neuronal activity of selective cell populations that have been made sensitive to light by genetic manipulation. Optogenetic-mediated activation of PV-expressing interneurons selectively amplifies gamma oscillations and modulates sensory responses in rat somatosensory cortex (Cardin et al., 2009). Conversely, suppressing PV-expressing interneuron activity with optogenetic methods significantly reduced or abolished gamma oscillations (Sohal et al., 2009). Finally, mice lacking NMDAR-mediated neurotransmission only in PV-expressing interneurons show altered cortical gamma rhythms with largely normal behavior except for selective cognitive impairments, including deficits in habituation, working memory, and associative learning (Carlén et al., 2011). Therefore, $GABA_AR$-mediated currents from PV-expressing basket cells appear to be the main source of GABA-mediated synchronization underlying cortical gamma oscillations.

In parallel, evidence from post-mortem studies of brains of subjects with schizophrenia supports the notion that specific alterations in GABAergic interneurons are associated with this disease (Lewis et al., 2005). In particular, one of the most reliable findings in schizophrenia research is the decrease in mRNA for GABA-synthesizing enzyme GAD67, which appears to be markedly deficient in PV-positive interneurons in schizophrenic brains (Hashimoto et al., 2003; Lewis et al., 2005). In addition, several GABA-related transcripts including those for GAD67, PV, the GABA transporter GAT1, SST, and the $GABA_AR$ subunits α1 and δ, are decreased in dorsolateral PFC as well as in the anterior cingulate, primary motor, and primary visual cortices (Hashimoto et al., 2008). Overall these data suggest that disturbances in GABAergic neurotransmission in specific cell types could represent a common pathological mechanism for different domains of cortical dysfunction in schizophrenia. It is therefore possible that pharmacological agents with the appropriate specificity for certain GABAergic synapses might be effective for a range of clinical features in the illness. While direct demonstration of a decrease in GABA-mediated synaptic transmission, in particular from basket cells, in the cortex of subjects with schizophrenia is challenging and still missing, the evidence available at the moment strongly points toward a direct link between PV-positive basket cell dysfunction, gamma oscillation alterations, and working memory deficits in schizophrenia patients.

Understanding PV-positive basket cell development in normal conditions should indicate the natural events occurring during this process and point out potential pathogenic mechanisms that can cause GABAergic dysfunction and schizophrenia. GABAergic interneuron development is a prolonged process, which starts in the embryo, progresses through childhood, and is completed by adolescence in all mammalian species studied including rodents and humans. During this prolonged period, the interplay between genetic, epigenetic, and environmental factors can produce subtle developmental alterations in GABAergic circuits that may contribute to the pathophysiology of schizophrenia.

While the molecular mechanisms regulating postnatal development of basket cells are not yet fully understood, recent papers have started to address this issue. In particular, it has been shown that the maturation of perisomatic innervation from GABAergic basket cells is modulated by neuronal activity and sensory experience during a critical postnatal period (Chattopadhyaya et al., 2004; Chattopadhyaya, 2011). A crucial factor regulating the development of innervation patterns by basket cells is GABA release itself, which regulates both the number of postsynaptic targets, as well as the number of synapses made onto each target by a single basket cell, in an age-dependent manner (Chattopadhyaya et al., 2007; Baho and Di Cristo, 2012; Wu et al., 2012). In particular, GAD67 knockdown in single basket cells dramatically decreases the formation of terminal axon branches and perisomatic synapses, as well as the number of postsynaptic neurons innervated by each basket cell (Chattopadhyaya et al., 2007). This finding is particularly interesting since polymorphisms in the 5′ region of the *Gad1* gene (coding for GAD67) have been associated with decreased GAD67 and schizophrenia (Addington et al., 2005; Straub et al., 2007). One possibility, therefore, is that altered inhibitory transmission caused by the genetic impairment of GAD67 gene early in development might alter the developmental trajectory of GABAergic neural circuit maturation, thus causing global network alterations predisposing to schizophrenia.

Another remarkable study showed that the trophic factor Neuregulin-1 and its receptor ErbB4, which are both schizophrenia susceptibility genes, regulate the development of PV-positive basket cell synapses (Fazzari et al., 2010). Further, Neuregulin signaling facilitates activity-dependent GABA release from mature basket cells and selective loss of ErbB4 in these cells causes a disinhibition of prefrontal pyramidal cells and results in schizophrenia-relevant phenotypes in mice (Wen et al., 2010).

In another study, the polysialylated form of the neural cell adhesion molecule NCAM has been shown to regulate the timing of perisomatic GABAergic synapse maturation in mouse cortex (Di Cristo et al., 2007). NCAM is polysialylated by two polysialyltransferases ST8SiaII and ST8SiaIV. SNPs in ST8SIaII have been implicated in schizophrenia (Arai et al., 2006; Tao et al., 2007; Isomura et al., 2011), providing a mechanism by which genetic interference with the complex coordination of NCAM polysialylation and GABAergic synapse maturation may lead to a neurodevelopmental predisposition to psychiatric disease.

In summary, multiple lines of evidence both in humans and animals suggests that the development and function of PV-expressing interneuron are particularly affected in schizophrenia. How can we use this knowledge to design novel targeted therapeutic approaches? Understanding the mechanisms underlying PV-expressing basket cell synapse development and plasticity may give us tools to modulate their function. Further, it has been proposed that combining pharmacological tools to enhance neural synchrony mediated by PV-positive basket cells along with behavioral therapies involving learning paradigms to reduce or reverse the consequences of altered neuronal circuit development could be an effective means of improving cognitive control in individuals with schizophrenia (Gonzalez-Burgos et al., 2011; Lewis, 2011). It is however important to underline that basket neurons are not the only ones affected in schizophrenia and future efforts are

needed to understand how alterations in specific circuits give rise to associated cognitive impairments.

AUTISM

Autism is a complex neurodevelopmental disorder characterized by abnormal socialization, deficits in verbal and non-verbal communication, and a limited interest in the surrounding environment associated with stereotyped and repetitive behaviors. The incidence of this disorder, which varies between 10 and 20 per 10000 children, has risen dramatically over the past two decades mainly because of the use of broader diagnostic criteria and the increased attention of the medical community (Levy et al., 2009). Clinical signs are usually present at the age of 3 years, but prospective studies of infants at risk have demonstrated that deficits in social responsiveness and communication could already be present as early as 6–12 months.

A high concordance rate between monozygotic twins (∼70%) and a significant sibling risk suggests that autism has a strong genetic component (Bailey et al., 1995). Although there is a clear genetic basis to autism, the majority of cases have unknown causes (non-syndromic autism). Similar to other complex brain diseases, it is likely that inherited risk factors, in combination with certain environmental or epigenetic triggers, ultimately causes autism. For many human diseases, the generation and characterization of animal models is an essential bridge between understanding the biological features of the disease and the development of targeted therapeutic approaches. The high genetic inheritance of autism has resulted in the generation of several animal models, however their validation has been controversial. Indeed, in the case of autism, modeling human symptoms requires rigorous behavioral tests to examine socialization, communication, and repetitive behavior, which are rather difficult to do in mice.

The majority of autism research has focused on the higher cognitive symptoms, however, its important to consider that the development and proper execution of higher cognitive processes depends on basic primary processing. The behaviors typically altered in autism arise from multiple sensory areas; for example, communication and socialization involve parallel auditory, visual, and somatosensory information processing. Recent studies suggest that altered low-level perceptual information processing is one of the main problems in autism, and this affects higher integrative areas of the brain (Bertone et al., 2003; Belmonte et al., 2004). Systematic developmental studies in autistic, high-risk infant siblings and control subjects are necessary to better understand the role of sensory processing and perception in the pathogenesis of autism. Nevertheless, these studies suggest that testing sensory processing in mouse models of autism is a promising approach that could allow for a better understanding of the biological features altered in the autistic brain.

Studies in autistic patients strongly suggest a correlation with dysfunction in the GABAergic system. For example, analysis of post-mortem tissues from autistic patients has shown that the GABA-synthesizing enzymes GAD65 and GAD67 are significantly reduced (∼50%) in the parietal cortex and cerebellum (Fatemi et al., 2002; Yip et al., 2007). Multiple studies have also found both $GABA_A$ and $GABA_B$ receptor alterations in autistic brains (Collins et al., 2006; Fatemi et al., 2009, 2010; Oblak et al., 2010). Reduced benzodiazepine binding to hippocampal $GABA_A$ receptors further suggests altered modulation of $GABA_A$ receptors in the presence of GABA (Guptill et al., 2007). The cortex is organized in vertical mini columns of functionally related glutamatergic and GABAergic neurons that process thalamic inputs. Local GABAergic circuits contribute to control the functional integrity of minicolumns via lateral inhibition. Interestingly, the number of mini columns is increased while their width is decreased in autistic brains indicating abnormal cortical organization regulated by inhibitory circuitry (Casanova et al., 2003). Further, a meta-analysis study of PV expression across multiple autism mouse models found a consistent reduction of PV-positive cells in the neocortex of autism mouse models as compared to controls (Gogolla et al., 2009). Maybe one of the most convincing indications of altered GABAergic function in autism is the high co-morbidity of autism with epilepsy. Altered GABA function may reduce the threshold for developing seizures. Indeed, ∼30% of autistic patients also have epilepsy, and inter-ictal epileptic activity recorded on scalp EEG occurs in up to 85% of autistic children (Gillberg and Billstedt, 2000; Yasuhara, 2010).

Non-idiopathic autism has been difficult to study because of the lack of suitable animal models. By comparing the molecular mechanisms underlying different single-gene disorders, it may be possible to discover commonalities and general principles that might hold true even for those cases in which no specific genetic cause has been identified. Dysfunction of GABAergic signaling has been shown to occur in the majority of animal models of autism obtained by experimentally manipulating candidate genes for autism susceptibility. Here, we summarize the GABAergic dysfunctions found in animal models carrying either rare mutations identified in autism patients or mutations known to cause diseases that are comorbid with autism. In fact, autism is comorbid with a number of other diseases, including Rett and Angelman Syndrome, which have known genetic causes and have been modeled in transgenic mice.

Mutations in neuroligin-neurexin adhesion complex

A small percentage of autistic patients carry single mutations in genes encoding for synaptic cell adhesion molecules of the neurexin (NRXN)-neuroligin (NLG) families. These include mutations in genes encoding for NRXN1 (Szatmari et al., 2007; Kim et al., 2008), NLG3, and NLG4 (Jamain et al., 2003; Laumonnier et al., 2004). Neuroligins are postsynaptic transmembrane molecules localized at both excitatory and inhibitory synapses, where they bind with presynaptic neurexins. Neuroligin-neurexin complex has been shown to regulate the formation of both excitatory and inhibitory synapse (for a review see Craig and Kang, 2007; Huang and Scheiffele, 2008; Südhof, 2008). In particular, a point mutation (R451C) that replaces an arginine with a cysteine in the extracellular portion of neuroligin-3 was identified in two brothers, one with severe autism and the other with Asperger syndrome (Jamain et al., 2003). In addition, a mutation in neuroligin-4 has been discovered in another set of autistic brothers (Jamain et al., 2003). Interestingly, mice carrying the mutation R451C show an upregulation of inhibitory markers, including the vesicular GABA transporter (VGAT) and the postsynaptic scaffolding protein gephyrin. Further, inhibitory transmission in

the somatosensory cortex is functionally augmented as shown by increased frequency of miniature IPSCs, increased amplitude of evoked IPSCs, and increased IPSC amplitude in response to GABA application. Mutant mice show behaviors relevant to autism, including altered socialization and enhanced spatial learning (Tabuchi et al., 2007; but see also Chadman et al., 2008). However, none of these molecular, physiological, and behavioral phenotypes were found in neuroligin-3 knockout mice, suggesting that this particular mutation results in a gain-of-function though the mechanism is still not well understood. In addition, mice lacking neuroligin-4 show lack of reciprocal social interaction and reduced ultrasonic vocalization, providing further evidence that mutant neuroligin mouse models may be useful to study neuronal circuit alterations in autism (Jamain et al., 2008).

Rett syndrome

Mutations in the X-linked MeCP2 gene, which encodes the transcriptional regulator methyl-CpG-binding protein 2 (MeCP2), causes the majority of Rett syndrome the majority of Rett syndrome cases. Rett syndrome is characterized by an apparently normal early development followed by developmental regression, including loss of language skills, motor abnormalities, cognitive deficits, stereotyped behavior, respiratory dysrhythmias, and seizures sometimes leading to premature death.

To uncover the molecular changes that underlie Rett syndrome, mouse models with different MeCP2 mutations have been generated. The analysis of synaptic circuit structure and function in these mouse lines showed several defects in the formation/maturation of neuronal GABAergic connectivity, which seem to be specific to selected brain regions, including the ventro-lateral medulla, the ventral basal complex, the reticular thalamic nucleus, and the cerebellum (reviewed in Boggio et al., 2010). Interestingly, wildtype GABAergic neurons express 50% more MeCP2 than wildtype non-GABAergic cells. Based on this observation, a conditional mutant mouse was generated where MeCP2 was exclusively disrupted in GABAergic cells using a VGAT-Cre mouse line. These mice develop nearly all of the symptoms that arise in germline MeCP2 knockout mice, including limb clasping, self-injury from excessive grooming, motor deficiencies, increased prepulse inhibition, altered socialization, and decreased lifespan. Further, GABAergic neurons, in these mice, exhibit reduced inhibitory quantal size, reduced GAD65 and GAD67 levels, and reduced GABA immunoreactivity. In addition, specific knockout of MeCP2 in forebrain GABAergic neurons recapitulates many of the symptoms observed in germline MeCP2 knockout mice (Chao et al., 2010), therefore suggesting that disruption of MeCP2 exclusively in inhibitory neurons is sufficient to cause Rett syndrome in mice.

Angelman syndrome

Linkage and association studies have revealed that the chromosomal region 15q11-q13 is strongly implicated in autism spectrum disorders (Shao et al., 2003). Maternal duplications of this region remains one of the most common cytogenetic abnormalities found in cases of idiopathic autism, which account for 1–2% of the total cases. Deletion of this region results in either Angelman or Prader-Willi syndrome, depending on which parent the deletion comes from. Within this chromosomal region, there is a gene cluster of $GABA_A$ receptors: Gabrb3, Gabra5, and Gabrg3, encoding for β3, α5, and γ3 subunits, respectively. Individuals with 15q11–13 deletions usually have more severe epilepsy than those with more specific mutations for example in the gene E3 ubiquitin ligase, which are sufficient to cause Angelman syndrome, but spares the $GABA_AR$ gene clusters (Minassian et al., 1998). Among different $GABA_A$ receptor genes, the targeted deletion of Gabrb3 gene, encoding for the β3 subunit, leads to abnormalities in social behavior, cognitive deficits, motor stereotypes, and seizures, reminiscent of the autistic phenotype (DeLorey et al., 1998).

In summary, numerous studies both in human and animal research suggests that GABAergic interneuron development and function are affected in autism. It remains to be seen however, whether these alterations are brain region and/or circuit specific and most importantly, if they are a general feature of all autism cases. So far autism is defined as a "spectrum disorder" due to the extraordinary heterogeneity of intellectual ability, associated symptoms, and possible etiology. Future studies, therefore, are critical to establish specific network alterations that are reliably and consistently associated with the autism phenotype. Moreover, the generation of animal models carrying human mutations will be invaluable in dissecting the network, cellular, and molecular changes underlying autism.

MODULATION OF GABAergic TRANSMISSION AS A TOOL TO RESTORE PLASTICITY

Multiple lines of evidence critically implicate GABAergic dysfunction in neurodevelopmental diseases, including schizophrenia and autism. It is important to note that alterations in GABAergic functions are most likely not limited to these disorders. For example, a mouse model of neurofibromatosis Type 1, a neurodevelopmental disorder associated with cognitive impairments, including difficulties with visuospatial skills and executive function, as well as an increased incidence of autism, shows increased GABA release, which underlies LTP and learning deficits (Cui et al., 2008). These findings open the question as to whether rescuing specific GABAergic defects might ameliorate at least in part the cognitive deficits. Since the GABAergic network is truly heterogenous with distinct GABAergic cell types, and multiple GABAergic receptor isoforms, further studies are essential to develop a targeted approach toward a specific class of GABAergic cells or/and its associated specific cognitive function(s).

Altered GABA function, caused either by genetic/epigenetic or environmental factors, or most likely by a combination of the above, affects the developmental trajectory of interneuron connectivity (Chattopadhyaya et al., 2007; Baho and Di Cristo, 2012; Wu et al., 2012). In turn, the formation of interneuron connectivity during early development is critical to the emergence of optimal network architecture that subserve different cognitive functions in the adult brain. Therefore it is likely that factors that cause the initial perturbation in GABAergic circuits would consequently affect the processing of incoming sensory stimuli and drive the developmental process along an abnormal trajectory. This leads to important, recurrent questions for the appropriate therapeutic strategies: What is the developmental window of opportunity for pharmacological treatment of neurodevelopmental diseases? Can existing symptoms be corrected or improved, or is it necessary to initiate

treatment early in pre- or postnatal development prior to the onset of symptoms? In other words, is it possible to change abnormal neuronal circuits after their formation and consolidation? Recent studies aiming at reversing cognitive and neural circuit deficits using genetic, pharmacological, or environmental strategies in different mouse models of neurodevelopmental diseases have shown encouraging results. For example, in a mouse model of Rett syndrome, MeCP2 function was restored during or following onset of symptoms using a tamoxifen-inducible Cre-LoxP strategy, resulting in the robust reversal of several associated phenotypes (Guy et al., 2007). Further, early enriched environment starting before the second postnatal week dramatically improved several phenotypes of MeCP2 mutant mice (Lonetti et al., 2010). In addition, pharmacological treatments have been shown to improve memory impairments in mouse models of Tuberous Sclerosis (Ehninger et al., 2008; Auerbach et al., 2011) or Fragile X Syndrome (Krueger and Bear, 2011). All together, these studies constitute an important proof of principle that the course of neurodevelopmental diseases could be arrested and even reversed with manipulations begun in late childhood and early adolescence, at least in animal models.

Although the human brain retains plasticity through life, continuously reorganizing its connections in the face of new experiences, the foremost period of heightened plasticity is during childhood when there exist "critical periods" during which experience can produce permanent, large-scale changes in neuronal circuits. Understanding the mechanisms that underlie activation and regulation of such critical periods in the central nervous system may help us develop rational pharmacological approaches to correct alterations in the brain of children with neurodevelopmental disorders involving altered synapse formation and/or plasticity.

The most widely studied model of experience-dependent plasticity is ocular dominance (OD) plasticity in the visual cortex. Electrophysiological recordings from neurons in the primary visual cortex show activation to different degrees by visual stimuli presented to one eye or the other, a property termed OD. Closing one eye during a specific postnatal time period causes synaptic reorganization of neural circuits in visual cortex, resulting in lifelong, irreversible reduction of the ability of the deprived eye to drive neuronal responses in the cortex, and an increase in the number of neurons responsive to stimuli presented to the open eye. This time period specific change in eye preference following manipulation of visual inputs is called OD plasticity (Hubel and Wiesel, 1970). In adults, however, such plasticity in OD, while not eliminated, is strongly restricted.

Convergent studies show that development of inhibitory circuitry in the cortex is critical for controlling the onset and time course of critical periods. For example, the onset of visual cortical plasticity is delayed by genetic disruption of GABA synthesis (Hensch et al., 1998). In parallel, application of benzodiazepines or other treatments that accelerate GABA circuit function triggers premature plasticity (Huang et al., 1999; Fagiolini and Hensch, 2000; Di Cristo et al., 2007). Further, recent results implicate intracortical inhibition as a fundamental limiting factor for adult cortical plasticity. In adult animals, where inhibitory circuits are fully developed, a brief reduction of GABAergic inhibition is sufficient to reopen a window of plasticity in the visual cortex well after the normal closure of the critical period (Harauzov et al., 2010). In addition different pharmacological and environmental strategies shown to enhance plasticity in the adult visual cortex, act at least in part, through a reduction of GABAergic inhibition (Sale et al., 2007; Maya-Vetencourt et al., 2008).

In an elegant study, Southwell et al. (2010) showed that transplanted MGE GABAergic precursors are sufficient to trigger a plasticity response in the host cortex of young mice. Interestingly, grafted interneurons promote plasticity only when they reach a cellular age comparable to that of the endogenous counterpart during the critical period, suggesting that plasticity is successfully initiated by a cell-autonomous program present in interneuron progenitors and is minimally influenced by the age of the host tissue. It is still unknown, however, whether transplantation of interneurons could induce visual plasticity in adults; nevertheless these findings suggest that cell transplantation might be effective in triggering plasticity processes.

In addition to GABAergic inhibition, several other factors have been implicated in limiting adult plasticity (Pizzorusso et al., 2002; McGee et al., 2005; Syken et al., 2006; Bavelier et al., 2010; Morishita et al., 2010). Therefore, targeting multiple pathways maybe key to successfully increase plasticity in the adult brain at a level sufficient enough to trigger long-lasting neural circuitry reorganization. Further along the road, coupling cognitive rehabilitation with pharmacological treatments that facilitate brain plasticity, could correct neuronal network connectivity and function and, ultimately, improve cognitive deficits.

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Epilepsy as a neurodevelopmental disorder

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Epilepsy is characterized by spontaneous recurrent seizures and comprises a diverse group of syndromes with different etiologies. Epileptogenesis refers to the process whereby the brain becomes epileptic and can be related to several factors, such as acquired structural brain lesions, inborn brain malformations, alterations in neuronal signaling, and defects in maturation and plasticity of neuronal networks. In this review, we will focus on alterations of brain development that lead to an hyperexcitability phenotype in adulthood, providing examples from both animal and human studies. Malformations of cortical development (including focal cortical dysplasia, lissencephaly, heterotopia, and polymicrogyria) are frequently epileptogenic and result from defects in cell proliferation in the germinal zone and/or impaired neuronal migration and differentiation. Delayed or reduced arrival of inhibitory interneurons into the cortical plate is another possible cause of epileptogenesis. GABAergic neurons are generated during early development in the ganglionic eminences, and failure to pursue migration toward the cortex alters the excitatory/inhibitory balance resulting in aberrant network hyperexcitability. More subtle defects in the developmental assembly of excitatory and inhibitory synapses are also involved in epilepsy. For example, mutations in the presynaptic proteins synapsins and SNAP-25 cause derangements of synaptic transmission and plasticity which underlie appearance of an epileptic phenotype. Finally, there is evidence that defects in synapse elimination and remodeling during early "critical periods" can trigger hyperexcitability later in life. Further clarification of the developmental pathways to epilepsy has important implications for disease prevention and therapy.

Keywords: cortex, hippocampus, GABA, glutamate, critical period, sodium channels

INTRODUCTION

Epilepsy is one of the most common neurological disorders, characterized by the repeated occurrence of spontaneous bursts of neuronal overactivity, known as seizures. Seizures typically arise in restricted regions of the brain and may remain confined to these areas or spread to the whole cerebral hemispheres. The hippocampal formation and cerebral cortex are considered the most epileptogenic regions of the brain (Pitkanen and Sutula, 2002; Avanzini and Franceschetti, 2003). The behavioral manifestations of seizures, as well as the severity of the epileptic condition, strictly relate to the brain regions that are affected by overactivity. Epilepsy comprises a large group of syndromes with different etiologies. A large series of recent studies demonstrated that several developmental factors (including congenital brain malformations, altered neuronal signaling during embryonic life, and defects in postnatal maturation of neuronal networks) contribute to epileptogenesis, leading to the concept of epilepsy as a neurodevelopmental disorder.

In the first part of the review, we will focus on those alterations in embryonic development of the cerebral cortex that lead to an hyperexcitability phenotype in postnatal life. Taking examples from both animal and human studies, we will describe the role of a number of key developmental genes controlling the migration of projection (excitatory) and local circuit (inhibitory) neurons, and describe how their altered function may result in epileptogenesis. In the second part of the review, we will describe the defects in the developmental assembly of excitatory and inhibitory synapses that are also involved in epileptogenesis. Specifically, we will focus on defects in synapse elimination and remodeling during early "critical periods" that may trigger hyperexcitability later in life. The deep understanding of the complex developmental processes involved in epileptogenesis may have important implications for disease prevention and therapy.

DEVELOPMENTAL DEFECTS OF THE CEREBRAL CORTEX LEAD TO EPILEPSY

The development of the mammalian cerebral cortex can be subdivided into three partially overlapping phases (Rubenstein, 2000, 2011; Lui et al., 2011). During the first phase, stem cells located in the ventricular and subventricular zones of the telencephalon proliferate and differentiate into neuronal precursors or glial cells. During the second phase, neurons migrate from their place of origin and reach their final destination in the cerebral cortex (Marin and Rubenstein, 2003; Kriegstein et al., 2006; Fishell, 2007; Fishell and Hanashima, 2008). The mature cerebral cortex is organized in six layers (Molyneaux et al., 2007) and contains two major types of projection neurons. The vast majority (∼80%) are glutamatergic (excitatory) neurons extending their long axon into the ipsilateral or contralateral cortex (cortico-cortical neurons, located in layers 2/3) or toward subcortical regions (cortico-fugal neurons, located

in layers 5/6). The remainder (∼20%) are GABAergic local circuit neurons (inhibitory interneurons) that establish synaptic contacts with excitatory neurons located in their proximity.

Migration of glutamatergic and GABAergic cortical neurons occurs in two different ways. Glutamatergic projection neurons are generated from neuronal precursors located in the neocortical neuroepithelium via asymmetric cell divisions of cortical primary progenitors (radial glia) located in the ventricular and subventricular zones (Malatesta et al., 2003; Kriegstein et al., 2006; Hansen et al., 2010). Asymmetric divisions generate immature projection neurons, that migrate toward the cortical plate along radial glial processes (Rakic, 2007) and hence reach their final destination in specific cortical layers through the interaction with local molecular cues, such as Reelin (Frotscher, 2010). Cortical neurons are generated in an inside-out pattern, layer 6 neurons being the first to be born. The majority of GABAergic interneurons are instead generated outside of the cerebral cortex, in the ganglionic eminences of the basal forebrain (Wonders and Anderson, 2006). The immature interneurons initially migrate tangentially along the subventricular zone of the basal forebrain, and then change direction by following a radial or an oblique path to enter the cortical plate, from where they reach their final destination into the layers of the cerebral cortex (Kriegstein and Noctor, 2004).

After completion of neuronal migration, the third, last phase of cortical development involves a complex series of apoptotic and synaptogenic events aimed at finely regulating the number of mature neurons and their connections, ultimately leading to the shaping of cortical circuits.

The embryonic development of the cerebral cortex is a complex process, tightly controlled by a series of gene expression cascades (Guillemot et al., 2006). Alterations of these gene regulatory pathways during development may lead to cortical malformations, resulting in malfunction during postnatal life. Accordingly, several cortical abnormalities have been identified that are caused by mutations in key genes involved in the different phases of cortical development. Cortical malformations may result from abnormal neuronal proliferation, migration defects of both excitatory and inhibitory neurons, or altered synaptogenesis/circuit formation, and are usually epileptogenic (Barkovich et al., 2005; Guerrini and Parrini, 2010; Manzini and Walsh, 2011).

Defects of neuronal and glial proliferation during embryonic development result in epileptogenic cortical lesions. Tuberous sclerosis complex (TSC) is a genetic disorder characterized by the widespread development of benign tumors (hamartomas) in multiple organ systems including the brain. Cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas represent the typical lesions observed in TSC. These malformations result in early-onset seizures, that are often accompanied by intellectual disability and autism. TSC results from mutations of TSC1 (hamartin) or TSC2 (tuberin) genes that lead to hamartomatous growths of neuronal and glial cells (Holmes and Stafstrom, 2007).

Neuronal migration disorders are a heterogeneous group of neurological conditions characterized by abnormal neuronal positioning in the cerebral cortex. Smooth cortex/lissencephaly (absent or reduced convolutions resulting in cortical thickening and smooth cerebral surface) and heterotopias (typically, ectopic nodules of gray matter located in a periventricular or subcortical position) have been associated to mutations in a number of genes regulating cortical neuron migration and are characterized by severe neurological impairment and epilepsy (Guerrini and Parrini, 2010). Migration defects of both excitatory and inhibitory neurons contribute to these conditions and lead to an altered excitation/inhibition balance and aberrant network hyperexcitability.

Following the initial generation and migration of excitatory and inhibitory neurons, immature neural networks are transformed into organized circuits through a process of refinement that is largely controlled by electrical activity (Katz and Shatz, 1996). During sensitive phases of the early postnatal life, called "critical periods," initially exuberant connections are eliminated and the remaining synapses undergo a functional maturation (Katz and Shatz, 1996; Berardi et al., 2000). Perturbations in this developmental refinement of neuronal circuitry during critical periods may trigger hyperexcitability and epilepsy later in life. In the following sections, we will detail some of the most significant molecular mechanisms involved in neuronal proliferation, neuronal migration, and synaptic refinement (schematically illustrated in **Figure 1** and summarized in **Table 1**), whose perturbation during embryonic or postnatal development results in epileptogenesis.

NEURONAL PROLIFERATION DEFECTS

Tuberous sclerosis complex is a neurocutaneous syndrome characterized by benign tumors, early-onset epilepsy, intellectual disability, and autism (Holmes and Stafstrom, 2007). TSC results from loss-of-function mutations of TSC1 or TSC2 genes, which are crucially involved in the control of neuronal and glial cell proliferation during embryonic development. TSC1 encodes a protein (hamartin) containing two coiled-coil domains, while TSC2 encodes a GTPase activating protein (tuberin) that inhibits small G-proteins belonging to the Ras-related super-family. Hamartin and tuberin are both expressed in neurons and astrocytes of specific central nervous system (CNS) regions such as forebrain, cerebellum, and brainstem, where they form a protein–protein complex that constitutively inhibits mammalian target of rapamycin (mTOR), a serine–threonine kinase positively regulating protein synthesis, cell proliferation, and survival. The function of the TSC1/2 complex is controlled by multiple intracellular signaling pathways converging on AKT, a pro-survival and pro-oncogenic kinase that directly phosphorylates TSC2 inhibiting its function. Loss of TSC1/2 function leads to activation of the mTOR cascade and results in increased cell proliferation; conversely, inhibition of mTOR function (e.g., by rapamycin) results in growth suppression and reduced cell size (Jozwiak, 2006; Holmes and Stafstrom, 2007). The occurrence of tubers in animal models of TSC has been a point of controversy in the field. In contrast with the main feature of the human disease, *Tsc1*$^{+/-}$ or *Tsc2*$^{+/-}$ mice do not develop tubers (Kobayashi et al., 2001; Uhlmann et al., 2002; Goorden et al., 2007; Ehninger et al., 2008; Bonnet et al., 2009). However, conditional inactivation of *Tsc1* or *Tsc2* in astrocytes leads to tuber-like lesions and severe seizures in mice (Zeng et al., 2008, 2011; Way et al., 2009). More recently, *in utero* electroporation was used to induce homozygous (*Tsc1*$^{-/-}$) cell clones on a TSC1 heterozygous (*Tsc1*$^{+/-}$) background. This strategy resulted in cortical tuber-like

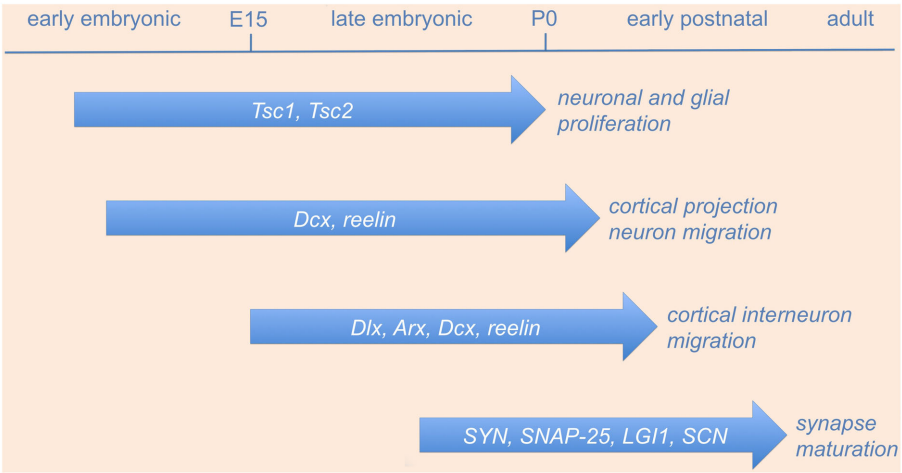

FIGURE 1 | Genes involved in neurodevelopmental causes of epilepsy. The picture shows a schematic time-line of the most significant gene functions involved in neuronal proliferation, neuronal migration, and synaptic refinement, whose perturbation during embryonic or postnatal development results in epileptogenesis (see text and **Table 1** for details). Abbreviations are as in the text.

lesions and a lower seizure threshold, suggesting that cells inside the tubers might have an additional somatic mutation that might contribute to the pathological phenotype (Feliciano et al., 2011). These studies clearly indicate that altering TSC1/2 signaling in specific CNS cell types is at the origin of TSC, but also point out the difficulty of modeling TSC in mice.

The histopathological features of cortical tubers in TSC reflect the antiproliferative role normally exerted by the TSC1/2 signaling complex. Cortical tubers in TSC patients are characterized by giant, dysplastic, and heterotopic neurons with aberrant dendrites and axons, as well as by proliferating astrocytes (Holmes and Stafstrom, 2007). Cortical hyperexcitability arises in the proximity of tubers, but its causes remain largely unknown (Major et al., 2009). Several hypotheses have been proposed to explain epileptogenesis in TSC. Increased expression of NMDA glutamate receptors and decreased expression of the GABA synthetic enzyme glutamic acid decarboxylase (GAD65), GABA vesicular transporter (vGAT), and GABA receptor subunits have been described in cortical tubers from human TSC patients (White et al., 2001), suggesting that excitation/inhibition imbalance in cortical circuits may contribute to epileptogenesis. Another intriguing hypothesis is that cortical tubers may alter thalamocortical connectivity during early brain development, thus resulting in hyperexcitable cortical circuits (Holmes and Stafstrom, 2007).

Seizure suppression in TSC remains a difficult task to be achieved. Infantile spasms in TSC often respond to vigabatrin (a GABA-transaminase inhibitor), but not to other antiepileptic drugs (AEDs), and the surgical removal of tubers remains in many cases the only therapeutic option (Holmes and Stafstrom, 2007). For these reasons, much emphasis has been put to the potential beneficial effects of the mTOR inhibitor rapamycin as a novel anticonvulsant and antiepileptogenic drug. Indeed, rapamycin is able to reduce seizures and prevent epileptogenesis in various animal models. For example, rapamycin treatment blocked epilepsy progression in conditional mutant mice lacking TSC1 or the TSC-positive regulator PTEN (Zeng et al., 2008; Sunnen et al., 2011), and reversed learning deficits in a $Tsc2^{+/-}$ mice (Ehninger et al., 2008). Importantly, rapamycin administered to chronically epileptic rats following kainic acid (KA; Zeng et al., 2009) or pilocarpine (Huang et al., 2010) treatment has been shown to suppress acquired epilepsy, even though these results have not been replicated in mice (Buckmaster and Lew, 2011). However, some authors pointed out that rapamycin treatment in animal models is still far to be optimal, since seizures may reappear after treatment cessation, and continuous rapamycin exposure might severely affect animal growth and health (Sunnen et al., 2011). In this respect, valid alternative strategies might be represented by a high-dose pulse treatment (Raffo et al., 2011) or even prenatal exposure (to be applied in cases of familial TSC predisposition; Anderl et al., 2011), that have been successfully tested in rodents. According to these results obtained in animal models, preliminary findings in human patients are encouraging: rapamycin has been shown to induce the regression of astrocytomas in a small group ($n = 5$) of TSC cases (Franz et al., 2006), and to reduce seizure frequency in a single young TSC patient (Muncy et al., 2009). However, it is important to point out that while the effects of rapamycin on tumor growth in TSC patients are now well documented and reproduced in many cases, its efficacy on seizure control and other neurological deficits needs to be further investigated.

MIGRATION DEFECTS OF CORTICAL PROJECTION NEURONS

The classification and neuropathological features of genetic neuronal migration disorders have been described in other reviews (Guerrini and Marini, 2006; Guerrini and Parrini, 2010). Different forms of lissencephaly ("smooth brain") and heterotopias have been associated to mutations in genes involved in cortical neuron migration (LIS1, DCX, ARX, TUBA1A, RELN, FLNA, and ARFGEF2). Here we will focus on two of these genes, doublecortin (DCX) and reelin (RELN), whose function in neuronal migration and epilepsy has been investigated in more detail.

Table 1 | Genes involved in neurodevelopmental epileptic syndromes and their corresponding mouse models.

Syndrome (*phenotype*)	Affected genes	Animal models (*phenotype*)	Reference
TUBEROUS SCLEROSIS COMPLEX			
Benign tumors (hamartomas) in multiple organs (brain, skin, heart, kidneys, lung), renal and skin lesions, epilepsy, behavioral and learning disabilities, autism.	TSC1 (hamartin)	*Tsc1*$^{+/-}$ mice. *Renal and hepatic tumors.* Increased number of astrocytes but *no cerebral lesions. No spontaneous seizures, but impaired social behavior and impaired learning in hippocampal-dependent tasks. Tsc1*$^{-/-}$ *mice are lethal.*	Kobayashi et al. (2001), Uhlmann et al. (2002), Goorden et al. (2007)
		Conditional mutant mice lacking *Tsc1* in glia. *Glial proliferation, enlarged brain size, progressive epilepsy, and premature death.*	Zeng et al. (2008), Feliciano et al. (2011)
	TSC2 (tuberin)	*Tsc2*$^{+/-}$ mice. *Renal and hepatic defects. Cognitive deficits in the absence of neuropathology or seizures. Tsc2*$^{-/-}$ *mice are lethal.*	Uhlmann et al. (2002), Ehninger et al. (2008), Bonnet et al. (2009)
		Conditional mutant mice lacking *Tsc2* in glia. *Cortical and hippocampal lamination defects, hippocampal heterotopias, enlarged dysplastic neurons and glia, abnormal myelination, astrocytosis, megalencephaly, epilepsy, and premature death.*	Way et al. (2009), Zeng et al. (2011)
LISSENCEPHALY			
Different forms of the disease, due to different genetic mutations. Absent or decreased cerebral convolutions, resulting in cortical thickening and smooth cerebral surface. Developmental delay, myoclonic jerks, seizures.	LIS1	Lis1 knockout mice. *Lethal Lis1*$^{-/-}$ *mice. Neuronal migration defects in Lis1*$^{+/-}$ *mice (aberrant morphology of cortical neurons and radial glia, slower neuronal migration, cortical plate splitting, and abnormal thalamocortical innervation). Behavioral defects not reported.*	Cahana et al. (2001)
	TUBA1A	*N*-ethyl-*N*-nitrosourea-induced mouse with S140G mutation in the TUBA1A gene. *Abnormal neuronal migration in layers II/III and IV of the visual, auditory, and somatosensory cortices. Fractured pyramidal cell layer in the hippocampus. Impaired spatial working memory, reduced anxiety, abnormal nesting.*	Keays et al. (2007)
SUBCORTICAL BAND HETEROTOPIA (SBH)			
Bands of gray matter interposed between the cortex and lateral ventricles ("double cortex"). Developmental delay, myoclonic jerks, seizures. DCX mutations are also associated to lissencephaly.	DCX (doublecortin)	Hemizygous *Dcx*$^{-/y}$ male mice. *Hippocampal lamination defects, reduced number of hippocampal interneurons, no gross anatomical defects of the cerebral cortex. Learning deficits in hippocampus-dependent tasks. Spontaneous seizures with hippocampal onset, hyperexcitability.*	Corbo et al. (2002), Nosten-Bertrand et al. (2008)
		Dcx$^{-/y}$ *Dclk1*$^{-/-}$ mice. *Perinatal lethality, disorganized neocortical, and hippocampal layering.*	Deuel et al. (2006), Koizumi et al. (2006), Kerjan and Gleeson (2007)
		Dcx in utero electroporation in rats. *SBH-like migration defects of cortical neurons, seizures. Rescued by postnatal re-expression of Dcx.*	Bai et al. (2003), Ackman et al. (2009), Lapray et al. (2010), Manent et al. (2009), Kerjan and Gleeson (2009)
X-LINKED LISSENCEPHALY WITH ABNORMAL GENITALIA			
Intellectual disability, autism, and epilepsy without cortical malformations.	ARX	*Arx*$^{-/-}$ mice. *Perinatal lethality. Absent interneuron migration from the lateral and medial ganglionic eminences. Reduced number of CR, NPY, CB but not PV interneurons. Conditional deletion of Arx in Dlx5/6 expressing interneurons results in early-onset seizures in hemizygous (Arx*$^{-/y}$*) male mice.*	Kitamura et al. (2002), Colombo et al. (2007), Friocourt et al. (2008), Marsh et al. (2009), Friocourt and Parnavelas (2010)
		Arx knock-in mice for human ARX mutations. *P355R mutants die at P0, with anatomical defects similar to Arx*$^{-/-}$ *mice. 330ins(GCG)7 and P355L mutants die after birth, with cortical malformations and altered development of GABAergic and cholinergic neurons. More severe seizures and learning deficits in 330ins(GCG)7 than P355L mutants.*	Kitamura et al. (2009)

LISSENCEPHALY WITH CEREBELLAR HYPOPLASIA			
Moderate lissencephaly, severe cerebellar hypoplasia, dysmorphic facial features, developmental delay, and epilepsy.	RELN (reelin)	*Reeler* mice. *Impaired migration of cortical projection neurons, resulting in disorganized and dyslaminated cerebral cortex. Increased susceptibility to hippocampal and neocortical epileptiform activity.*	D'Arcangelo and Curran (1998), D'Arcangelo (2006), Patrylo et al. (2006)
PERIVENTRICULAR HETEROTOPIA (PH)			
Periventricular nodules, seizures.	FLNA (filamin A)	Flna-null mice. *Die in utero due to cardiovascular defects (abnormal vessels, persistent truncus arteriosus, incomplete cardiac septation). No CNS analysis available*	Feng et al. (2006)
PH WITH MICROCEPHALY			
Periventricular nodules, microcephaly, seizures, developmental delay.	ARFGEF2	Not available.	Guerrini and Parrini (2010)
AUTISM SPECTRUM DISORDER (ASD)			
SNPs in DLX1 and DLX2 are associated to ASD, a neurodevelopmental disorder often associated to epilepsy.	DLX1, DLX2	*Dlx1*$^{-/-}$ and *Dlx2*$^{-/-}$ mice. *Selective apoptotic cell death of CR, SOM, and NPY cortical interneurons in Dlx1/2*$^{-/-}$ *mice. Noise- or handling-induced behavioral seizures and generalized electrographic activity in Dlx1*$^{-/-}$ *mice.*	Cobos et al. (2005b, 2007), Liu et al. (2009)
EPILEPTIC SYNDROMES			
Autosomal dominant lateral temporal lobe epilepsy (ADLTE)	LGI1	Overexpression of an Lgi1 gene with a truncating mutation. *Arrested postnatal maturation of presynaptic and postsynaptic function in the hippocampus, lowered seizure threshold.*	Zhou et al. (2009)
		LGI1$^{-/-}$ mice: *Severe seizures during the second postnatal week and premature death. Seizure-induced cell loss and reorganization in the hippocampus. Altered excitatory synaptic transmission in the hippocampus*; LGI1$^{+/-}$ mice: *Lowered seizure threshold.*	Chabrol et al. (2010), Fukata et al. (2010), Yu et al. (2010)
GEFS+	SCN1A	Mice homozygous for the R1648H mutation causing GEFS+. *Spontaneous generalized seizures and premature death.*	Martin et al. (2010)
		Heterozygous mutants for the R1648H mutation. *Infrequent spontaneous generalized seizures, reduced threshold for induced seizures. Impairments in inhibitory neuron function.*	
Dravet's syndrome	SCN1A	Scn1a$^{+/-}$ and Scn1a$^{-/-}$ mice. *Spontaneous seizures, reduced threshold for induced seizures, high mortality. Reduction of sodium currents in hippocampal interneurons.*	Yu et al. (2006), Oakley et al. (2009)
		Knock-in mice carrying a truncation mutation in the Scn1a gene. *Spontaneous seizures, alterations in parvalbumin interneuron firing.*	Ogiwara et al. (2007)
Benign familial infantile seizures	SCN2A	Transgenic mice (Q54) carrying a missense mutation of SCN2A. *Spontaneous seizures and hippocampal sclerosis reminiscent of human MTLE.*	Kearney et al. (2001), Manno et al. (2011)
Epilepsy (X-linked, with variable learning disabilities and behavior disorders; associated to SYNI mutations)	SYNI	Syn I and Syn II null mice. *Spontaneous and sensory stimuli-evoked epileptic seizures of unspecified type. SynI null mice: the readily releasable pool is reduced at inhibitory synapses and increased at excitatory synapses.*	Rosahl et al. (1995), Garcia et al. (2004), Baldelli et al. (2007), Chiappalone et al. (2009)
Absence epilepsy	SNAP-25	Coloboma mice. *Cortical spike-wave discharges and increased thalamic T-type currents.*	Zhang et al. (2004)

The cited literature essentially refers to animal model studies; for the clinical features of the listed syndromes, the reader is referred to the "Online Mendelian Inheritance in Man" database (OMIM; www.omim.org). Abbreviations, if not otherwise specified, are as in the text.

DOUBLECORTIN

Dominant, X-linked DCX mutations cause lissencephaly or subcortical band heterotopia (SBH; "double cortex"), syndromes characterized by mental retardation and epilepsy in humans. Specifically, DCX mutations cause lissencephaly in hemizygous males, whereas heterozygous females show a mosaic phenotype with a normal cortex and a second band of heterotopic neurons beneath the cortex ("double cortex"; des Portes et al., 1998; Matsumoto et al., 2001). DCX encodes a 40-kDa microtubule-associated protein (doublecortin) expressed in migrating neuroblasts and containing two conserved tandem repeats required for tubulin binding and microtubule stabilization (des Portes et al., 1998; Gleeson et al., 1998; Gleeson and Walsh, 2000). How doublecortin influences microtubule dynamics during brain embryonic development remains largely unclear.

Mice lacking *Dcx* (hemizygous $Dcx^{-/Y}$ male and heterozygous $Dcx^{+/-}$ female mice) have been generated in the attempt to reproduce the human phenotype of DCX mutations (Corbo et al., 2002). $Dcx^{-/Y}$ male mice present hippocampal lamination defects and a reduced number of hippocampal interneurons, but have no gross anatomical defects at the level of the cerebral cortex (Corbo et al., 2002; Nosten-Bertrand et al., 2008). Similar defects are observed also in $Dcx^{+/-}$ female mice. Importantly, $Dcx^{-/Y}$ male mice have hippocampal-dependent learning deficits (Corbo et al., 2002), are hyperexcitable and show spontaneous seizures with hippocampal onset (Nosten-Bertrand et al., 2008).

Severe migration defects of cortical projection neurons and cortical malformation have been instead detected in mice lacking both doublecortin and doublecortin-like kinase 1 (*Dclk1*; Deuel et al., 2006; Koizumi et al., 2006; Kerjan and Gleeson, 2007). More importantly, *Dcx* RNA interference (RNAi) performed by *in utero* electroporation results in migration defects of pyramidal cortical neurons and spontaneous seizures in adult rats (Bai et al., 2003; Ackman et al., 2009; Lapray et al., 2010), similar to those observed in human SBH. Manent et al. (2009) tested whether the postnatal re-expression of *Dcx* could rescue these anatomical and neurological deficits. To this aim, the *Dcx* knockdown RNAi vector was *in utero* electroporated into cortical neurons together with a tamoxifen-inducible *Dcx* expression vector system. In this way, it was possible to knockdown *Dcx* in cortical neurons at embryonic day (E)14 and then re-activate it between postnatal day (P)0 and P10 by treating newborn pups with tamoxifen. Postnatal re-expression of *Dcx* induced in this way was able to reduce neuronal migration defects and seizures in this model of SBH (Kerjan and Gleeson, 2009; Manent et al., 2009).

Dcx and doublecortin-like kinase have also been implicated in the migration of cortical interneurons (Friocourt et al., 2007; see section below). Taken together, these data indicate that doublecortin is required for hippocampal lamination as well as migration of neocortical pyramidal neurons and interneurons; as a consequence of this important role, an altered function of doublecortin during embryonic forebrain development results in epilepsy in the postnatal life.

REELIN

Mutation of the RELN gene, coding for the extracellular glycoprotein Reelin, causes a neuronal migration disorder called lissencephaly with cerebellar hypoplasia (Zaki et al., 2007; Guerrini and Parrini, 2010). Reelin binds several receptors, including a complex composed by the apolipoprotein E receptor 2 (ApoER2) and the very low-density lipoprotein receptor (VLDLR) (Honda et al., 2011). Following receptor binding, Reelin activates the intracellular protein Disabled homolog 1 (Dab1), whose phosphorylation enhances signal transduction pathways leading to axonal growth, neuronal migration, and synaptic plasticity (Frotscher, 2010; Honda et al., 2011). Reelin-activated pathways include the PI3K/Akt/Gsk-3β and Notch cascades (Honda et al., 2011). During embryonic development, Reelin is mainly expressed by Cajal–Retzius cells in the marginal zone of the neocortex and outer molecular layer of the hippocampus, whereas in the adult brain it is expressed in GABAergic interneurons (Alcantara et al., 1998). Mice lacking Reelin (*reeler* mice) show a dramatic impairment of migration of cortical projection neurons, that results in a highly disorganized and dyslaminated cerebral cortex (D'Arcangelo and Curran, 1998; D'Arcangelo, 2006). The final outcome is an increased susceptibility to hippocampal and neocortical epileptiform activity (Patrylo et al., 2006). During embryogenesis, the firstly generated cortical neurons migrate from the germinal zone and form the preplate. At the arrival of subsequently generated neurons, the preplate splits into two layers: the Cajal–Retzius layer (expressing Reelin) and the subplate. Successive waves of neurons migrate past the subplate, but not past the Cajal–Retzius layer, that will form the most superficial layer of the mature cerebral cortex. In the absence of Reelin, neuronal migration does not occur properly and neocortical lamination is perturbed. The preplate does not split, and newly generated neurons pile up behind the previously generated ones, resulting in an inverted layering of the neocortex. Several hypotheses have been proposed to explain the role of Reelin in neuronal cortical migration: (i) Reelin might function as a chemoattractant for migrating neurons; (ii) it might control the preplate splitting that initiates the inside-out lamination; (iii) it might determine the detachment of neurons from radial glial processes and their subsequent differentiation (D'Arcangelo and Curran, 1998; Cooper, 2008; Gaiano, 2008). More recent studies suggest that Reelin and Notch signaling cooperate to regulate neuronal migration in the neocortex (Hashimoto-Torii et al., 2008).

Reelin is also implicated in the migration of excitatory neurons of the hippocampus; a marked dispersion of projection neurons is observed in the stratum pyramidale and dentate granule cell layer in *reeler* mice (D'Arcangelo and Curran, 1998). Granule cell dispersion (GCD) is often observed in human mesial temporal lobe epilepsy (MTLE), and recent studies investigated the role of Reelin in this form of epileptic condition. In the adult hippocampus, Reelin-positive interneurons (Cajal–Retzius cells) are normally found in the CA1 and CA3 regions, in the hilus, and along the hippocampal fissure, both in mouse (Heinrich et al., 2006) and human samples (Haas et al., 2002). Indeed, GCD correlates with hippocampal Reelin loss in MTLE patients, as shown by studies performed on hippocampal tissues resected from adult subjects who underwent temporal lobectomy for intractable MTLE (Haas et al., 2002). GCD and loss of Reelin was also observed in rodent models of MTLE (Heinrich et al., 2006; Gong et al., 2007; Antonucci et al., 2008, 2009; Duveau et al., 2010). The causative role of Reelin deficiency in GCD is confirmed by the observation

that GCD is induced by the infusion of Reeling-blocking antibody, and prevented by the administration of exogenous Reelin (Haas and Frotscher, 2010). It remains still to be determined how Reelin loss and subsequent GCD contribute to epileptogenesis, and whether they are a cause or an effect of seizures (Haas and Frotscher, 2010).

MIGRATION DEFECTS OF CORTICAL INTERNEURONS

Cortical GABAergic interneurons are generated in ganglionic eminences of the basal forebrain, from where they migrate to reach their final location (Wonders and Anderson, 2006). Several genes have been implicated in interneuron differentiation and migration; the role of specific interneuron types in epilepsy is a developing field, which may help us to better understand the complex neurodevelopmental processes underlying seizure onset and control. Here we will focus on *Dlx*, *Arx*, *Dcx*, and Reelin genes, whose role in interneuron migration and possible implications in epileptogenesis has been extensively studied in mouse models.

Dlx

Migration and differentiation of cortical GABAergic interneurons has been shown to depend on the action of Dlx homeobox transcription factors, namely *Dlx1/2* and *Dlx5/6*. *Dlx* genes are expressed in GABAergic neuron progenitors of the developing forebrain and in subsets of mature GABAergic interneurons of the adult cerebral cortex (Cobos et al., 2005b, 2007; Wang et al., 2010). Loss-of-function mutations of pairs of mouse *Dlx* genes (*Dlx1/2* or *Dlx 5/6*) severely affect the migration and differentiation of distinct subpopulations of cortical interneurons. Specifically, *Dlx1/2* have a crucial role in the migration and survival of calretinin (CR), somatostatin (SOM), and neuropeptide Y (NPY) positive interneurons (Anderson et al., 1997; Cobos et al., 2005b, 2007) whereas Dlx5/6 preferentially control parvalbumin-positive interneurons (Wang et al., 2010).

Migration defects and subsequent reduced number of cortical interneurons result in epilepsy in Dlx mutant mice. Mice lacking *Dlx1* ($Dlx1^{-/-}$ mice) show selective apoptotic cell death of CR, SOM, and NPY cortical interneurons, resulting in decreased synaptic inhibition at 1 month of age. Accordingly, adult $Dlx1^{-/-}$ mice show noise- or handling-induced behavioral seizures and generalized electrographic activity (Cobos et al., 2005b). Mice homozygous for both *Dlx5* and *Dlx6* ($Dlx5/6^{-/-}$ mice) show a normal layering of the cerebral cortex and a normal positioning of MGE-derived cortical interneurons, but die perinatally. A reduced number of PV cortical interneurons is observed in these mutants, and the surviving PV interneurons show an increased *dendritic* branching (Wang et al., 2010). Conversely, heterozygous $Dlx5/6^{+/-}$ mice do not present interneuron loss nor other gross anatomical abnormalities, but develop epilepsy; this has been ascribed to functional deficit and aberrant connectivity of PV neurons (Wang et al., 2010).

Taken together, these studies clearly demonstrate that *Dlx* genes regulate the development and function of forebrain interneurons, which are crucial to maintain the inhibitory tone of the cerebral cortex and other brain structures. The reduced function of *Dlx* transcription factors (or of their transcriptional targets, such as GAD and vGAT; Stuhmer et al., 2002; Rubenstein, 2011) might increase the excitation/inhibition ratio thus predisposing the cerebral cortex to hyperexcitability and epilepsy.

Arx

The aristaless-related homeobox gene (*Arx*) belongs to the family of paired-class homeobox transcription factors, and its expression is controlled by the Dlx genes in cells derived from basal ganglia progenitor domains (Cobos et al., 2005a). *Arx* plays a pivotal role in the development of the CNS. ARX large deletions, protein truncating mutations and missense mutations in the homeobox region lead to X-linked lissencephaly with abnormal genitalia, while other ARX mutations cause intellectual disability, autism, and epilepsy without cortical malformations (Kitamura et al., 2002). *Arx* expression has been detected in the developing lateral and medial ganglionic eminence and later in cortical progenitors and migrating interneurons (Colombo et al., 2004; Friocourt et al., 2006). Studies in animal models showed that *Arx* is critical for radial migration of cortical progenitors and is crucially involved in the development and migration of GABAergic interneurons (Colombo et al., 2007; Friocourt et al., 2008; Marsh et al., 2009). In *Arx*-null embryos, interneuron migration from the lateral and medial ganglionic eminences is nearly absent, whereas migration through the cortical layers is only partially impaired. As a consequence, CR, NPY, and calbindin (CB) interneurons are severely reduced (Kitamura et al., 2002; Colombo et al., 2007). No loss of PV interneurons was instead observed in hemizygous $Arx^{-/-}$ mice suggesting the *Arx* loss results in specific interneuron subtype deficits (Friocourt and Parnavelas, 2010). These studies also revealed that abnormal expression of transcription factors potentially important for neuronal differentiation and migration occurs in $Arx^{-/-}$ mice.

Dcx

Cortical interneuron migration is also controlled by both *Dcx* and *Dclk*. Dcx knockdown by RNAi slows the migration of interneurons from the ganglionic eminence to the cerebral cortex (Friocourt et al., 2007), and cortices of *Dcx*, *Dclk*, and *Dcx/Dclk* mutant mice contain less CB-positive interneurons as compared to control mice (Friocourt et al., 2007; Nosten-Bertrand et al., 2008). An increased number of CR-positive interneurons is detected in the dentate gyrus of *Dcx* mutant mice (Nosten-Bertrand et al., 2008), whereas no defects of PV interneurons are detected in *Dcx*, *Dclk*, and *Dcx/Dclk* mutant mice (Kerjan and Gleeson, 2009).

REELIN

A large subset of cortical and hippocampal GABAergic interneurons express Reelin. These interneurons derive from the caudal ganglionic eminence (CGE), and occupy superficial layer of the cerebral cortex (Miyoshi et al., 2010) and strata lacunosum moleculare and deep radiatum of the mature hippocampus (Tricoire et al., 2011). The role of Reelin in interneuron migration and positioning in forebrain areas has been debated. Some authors showed that in *reeler* mice, GABAergic interneurons migrate normally into the developing forebrain, but fail to acquire proper layer position in the mature cerebral cortex (Hammond et al., 2006; Yabut et al., 2007). Using transplants to analyze the mechanisms underlying the positioning of cortical interneurons, other authors instead

found that layer acquisition by these cells does not directly depend on Reelin signaling (Pla et al., 2006). Interestingly, a recent study showed that neuronal activity is essential for the migration and morphology of CGE-derived Reelin- and CR-positive interneurons (De Marco García et al., 2011). To suppress excitability in CGE-derived interneurons, the authors electroporated *in utero* the inward rectifying potassium channel Kir2.1 under the control of a *Dlx5/6* enhancer element at E15.5, and then analyzed the positioning and morphology of Reelin and CR interneurons. Results showed that neuronal activity is required before P3 for the correct migration of CGE-derived interneurons, and that after P3, glutamate-mediated activity controls the development of their connections (De Marco García et al., 2011). These findings indicate that the genetic program underlying the development of Reelin-expressing interneurons is strongly modulated by activity, and may have implications for the role of Reelin in neurodevelopmental epileptic syndromes.

GABA AND GLUTAMATE CONTROL NEURONAL MIGRATION IN THE FOREBRAIN

Studies performed on corticohippocampal organotypic co-cultures from EGFP-expressing mouse embryos demonstrated that GABA and glutamate may modulate neuronal migration in the developing forebrain. Specifically, GABA and glutamate modulate the migration of hippocampal pyramidal neurons respectively acting on $GABA_A$ and NMDA receptors (Manent et al., 2005), while glutamate controls the migration of GABAergic interneurons via AMPA receptor activation (Manent et al., 2006). These results led the authors to postulate that the migrations of glutamatergic and GABAergic interneurons are inter-dependent: glutamate released from pioneer glutamatergic neurons controls the migration of GABAergic interneurons, which in turn would facilitate glutamate neuron migration via GABA release (Manent and Represa, 2007). In keeping with these observation, the same authors showed that prenatal exposure to some AEDs acting on GABA signaling (such as vigabatrin, valproate, and lamotrigine) results in hippocampal and cortical dysplasias in the developing embryos (Manent et al., 2007, 2008), thus raising serious concerns about the possible consequences of AEDs use during pregnancy.

DEVELOPMENTAL REMODELING OF NEURONAL CIRCUITS

In the previous sections we have considered how defects in the early stages of brain development (i.e., neuronal proliferation and migration) can induce an epileptic phenotype. Following the initial assembly of excitatory and inhibitory neurons, immature neural networks are transformed into organized circuits that subserve adult brain function (Katz and Shatz, 1996). This process of network refinement is largely controlled by electrical activity. In particular, work in the sensory cortices has clearly established the existence of so-called "critical periods," during which patterns of activity generated by sensory experience play a critical role for maturation of cortical function (Berardi et al., 2000). During these sensitive phases of development, initially exuberant connections are eliminated and neuronal dendrites are correspondingly pruned and narrowed. The remaining synapses functionally mature (Katz and Shatz, 1996). In particular, in the visual system, synapse elimination and remodeling depends upon the amount and patterning of neural activity within the visual pathway (Fagiolini et al., 1994; Caleo et al., 2007).

Perturbations in this developmental refinement of neuronal circuitry during critical periods may trigger hyperexcitability and epilepsy later in life. One clear example comes from a study on blockade of hippocampal activity during early development (Galvan et al., 2000). In the rodent hippocampus, CA3 pyramidal neurons display an exuberant growth of axon collaterals during postnatal weeks 2 and 3; this developmental time period corresponds to a critical phase, when CA3 networks have a marked propensity to generate epileptic seizures (Swann and Brady, 1984). Exuberant CA3 axons are then remodeled with maturation, so that about half of the branches are eliminated (Gomez-Di Cesare et al., 1997). To prevent this axonal remodeling, Swann and colleagues infused tetrodotoxin (a blocker of voltage-gated sodium channels) into the rat hippocampus for about 10 days starting from P12 (Galvan et al., 2000). This transient blockade of neuronal activity resulted in the establishment of a chronic epileptic focus in adulthood, with prolonged electrographic seizures originating from the infused hippocampus (Galvan et al., 2000). These results are consistent with the idea that blockade of neuronal activity during early critical phases can enhance seizure susceptibility later in life by preventing developmental remodeling of neural circuits. Indeed, the immature brain is more prone to seizures than the adult brain (Ben-Ari and Holmes, 2006).

Recently, a persistent immaturity of glutamatergic circuitries in the hippocampus was found to underlie seizure susceptibility in autosomal dominant lateral temporal lobe epilepsy (ADLTE; Zhou et al., 2009). This study investigated the function of LGI1 (leucine-rich, glioma-inactivated 1), a gene that is implicated in about half the cases of ADLTE (Kalachikov et al., 2002; Striano et al., 2011). LGI1 encodes a protein that localizes to glutamatergic synapses, binds its receptors ADAM22 and ADAM23 (disintegrin and metalloproteinase domain 22 and 23), and copurifies with presynaptic and postsynaptic regulatory molecules (Fukata et al., 2006; Schulte et al., 2006). LGI1 expression increases in the rodent hippocampus exactly during the third postnatal week, when glutamatergic synapses are pruned and mature (Fukata et al., 2006; Zhou et al., 2009). In particular, excitatory neurons downregulate their presynaptic vesicular release probability and reduce their postsynaptic NMDA-receptor subunit NR2B; during this same period, dendritic arbors and spines are pruned and remodeled. Anderson and colleagues generated mice expressing a truncated form of the LGI1 gene, found in ADLTE patients, and showed that hippocampal glutamatergic synapses did not properly mature in these mice (Zhou et al., 2009). Specifically, (i) the normal, age-dependent decrease of probability of glutamate release was blocked in mutant mice; (ii) developmental changes in glutamate receptor composition were impaired by mutated LGI1; and (iii) dendrites of adult mutant mice remained immature, with a high density of branches and spine protrusions (Zhou et al., 2009). Importantly, mutant LGI1 mice displayed a lower threshold for the development of pharmacologically induced seizures. There was no significant effect of mutated LGI1 on GABAergic neurotransmission (Zhou et al., 2009; Yu et al., 2010). All together, these data suggest that mutated LGI1 blocks the normal functional maturation of both presynaptic and postsynaptic compartments and halts structural pruning,

thus maintaining a high density of excitatory synaptic inputs converging onto hippocampal neurons and leading to a hyperexcitability phenotype (Caleo, 2009; Zhou et al., 2009). Other reports are consistent with the idea that LGI1 regulates the development of glutamatergic synapses (Fukata et al., 2010; Yu et al., 2010). In keeping with this findings, recent data show that heterozygous mice lacking LGI1 (*LGI1*$^{+/-}$ mice) display a lower threshold to auditory stimuli induced seizures, and homozygous *LGI1*$^{-/-}$ mice develop spontaneous recurrent seizures followed hippocampal neuronal loss, mossy fiber sprouting, astrocyte reactivity, and GCD (Chabrol et al., 2010).

EPILEPTOGENIC ION CHANNEL MUTATIONS: FOCUS ON SCN2A

In the last years, a number of epileptogenic mutations have been identified in different genes (Noebels, 2003). The majority of these genes code for voltage- or ligand-gated ion channels. Mutations in different subunits of sodium, potassium, and calcium channels underlie different forms of genetic epilepsies ("channelopathies"; Kullmann, 2010; Mantegazza et al., 2010). Mutations of the nicotinic acetylcholine receptor subunits are associated with autosomal dominant nocturnal frontal lobe epilepsy (De Fusco et al., 2000). Mutations in the voltage-gated potassium channel subunit genes KCNQ2 and KCNQ3 are associated with benign familial neonatal seizures (Leppert and Singh, 1999). Mutations in the $GABA_A$ receptor, which is the primary mediator of synaptic inhibition, have also been found to contribute to several idiopathic epilepsies and febrile seizures (Galanopoulou, 2010). The functional consequences of these epileptogenic mutations have been amply studied, and the data have provided significant knowledge on the pathogenic mechanisms that lead to epilepsy. For more details on these findings, the reader is referred to a series of excellent reviews (Noebels, 2003; Kullmann, 2010; Mantegazza et al., 2010).

Mutations in voltage-gated sodium channel genes (Nav) are the most common genetic cause of familial epilepsy. Specifically, mutations in the Nav1.1 alpha subunit gene (SCN1A) are responsible for "generalized epilepsy with febrile seizures plus" (GEFS+; Scheffer and Berkovic, 1997) and Dravet's syndrome (Mantegazza et al., 2010; Meisler et al., 2010). Missense mutations in Nav1.2 alpha subunit (SCN2A) are found in patients with benign familial neonatal-infantile convulsions (Avanzini et al., 2007). Functional studies demonstrate that Nav1.2 mutations result in modifications of the gating properties of the channel, with a net amplification of the sodium current and resulting greater depolarization, consistent with the hyperexcitability phenotype (Scalmani et al., 2006).

It is important to point out that it is often difficult to predict the epileptic phenotype based solely from the change in the behavior of the mutated channel. First, it is crucial to determine cell-specific patterns of expression of the mutated subunit, as expression in excitatory vs. inhibitory neurons can lead to completely opposite effects on excitability status of the neuronal network (Yu et al., 2006). Second, the cellular background (i.e., the modifier effect of other channels expressed in the same neuron) has to be considered (Glasscock et al., 2007). "Humanized" mouse models (in which human mutated channel sequences are inserted in genetically engineered mice) offer an unique opportunity to study the modulatory role of the genetic background and of the interactions between different genes. One example is represented by transgenic mice (Q54) carrying a human, epileptogenic missense mutation of the sodium channel SCN2A (Nav1.2; Kearney et al., 2001). Mice carrying the Q54 transgene on a SJL strain background exhibit severe spontaneous seizures originating in the hippocampus with onset at about 4 weeks, and progressive hippocampal sclerosis with extensive cell loss and gliosis in areas CA1, CA3, and hilus (Kearney et al., 2001; Manno et al., 2011). However, on a pure C57BL/6J background, onset of seizures is delayed and the epileptic phenotype is mild. Two modifier loci responsible for the difference in severity between strains C57BL/6J and SJL have been mapped, and the evidence points to the voltage-gated potassium channel gene Kcnv2 as one modifier (Bergren et al., 2005, 2009). These data indicate that severity of the epileptic condition may be significantly impacted by gene interactions.

Finally, in the study of channelopathies it is important to underlie the possible effects of the complex interaction between mutated genes and the environment. As we have described in the previous section, early environmental conditions shape neuronal connectivity, and the resulting changes in network organization can potently affect clinical expression of a mutated channel. In keeping with this notion, Q54 transgenic mice (carrying an epileptogenic mutated SCN2A; see above) exposed to environmental enrichment from birth show a dramatic reduction of spontaneous seizures and hippocampal cell loss (Manno et al., 2011). The data indicate that an enriched housing from birth may have profound antiepileptic and neuroprotective effects. Environmental enrichment may exert these actions by up-regulation of neurotrophic factors, plastic rearrangements in excitatory/inhibitory circuits, and stimulation of neurogenesis (Dhanushkodi and Shetty, 2008; Sale et al., 2009). Thus, changes in network organization due to environmental influences can halt epileptogenic changes and dampen hyperexcitability of neural networks.

EPILEPTOGENIC ALTERATIONS OF PRESYNAPTIC PROTEIN FUNCTION

In addition to channelopathies, defects in the control of neurotransmitter release account for a wide variety of epileptic syndromes. Presynaptic proteins that have been found to be involved in epilepsy include the synaptic vesicle (SV) protein SV2A (Crowder et al., 1999; Janz et al., 1999), the SV-anchoring phosphoproteins synapsins (Rosahl et al., 1995; Baldelli et al., 2007) and the plasma membrane fusion protein SNAP-25 (Zhang et al., 2004). Not all disruptions of the neurotransmitter machinery are equally epileptogenic, as shown by the lack of seizures in mice lacking other presynaptic proteins, such as synaptotagmin (Fernandez-Chacon et al., 2001) or synaptobrevin/VAMP (Schoch et al., 2001). Data obtained in mice mutant for synapsins and SNAP-25 are particularly interesting, since they suggest that hyperexcitability might result from developmental alterations causing an unbalance in the activity of excitatory and inhibitory neurons.

SYNAPSINS

Synapsins (Syns) comprise a family of neuron-specific proteins encoded by three distinct genes (SYNI, SYNII, and SYNIII) located in chromosome X, 3, and 22, respectively. The Syns are phosphoproteins implicated in the regulation of neurotransmitter release

and synapse formation. In particular, Syns are responsible for the formation and maintenance of SV pools within the nerve terminal (Benfenati et al., 1992) and their expression has been causally related to the formation and maturation of synaptic connectivity during neural development and synaptogenesis (Valtorta et al., 1995; Bonanomi et al., 2005). Despite the absence of gross defects in brain morphology, Syn I and Syn II (but not Syn III) knockout (KO) mice exhibit early-onset spontaneous and sensory stimuli-evoked epileptic seizures (Rosahl et al., 1995). Syn I mutations can also contribute to human epilepsy (Garcia et al., 2004). In Syn KO mice, spontaneous recurrent seizures develop after 2 months of age and the animals become more susceptible with age (Rosahl et al., 1995). This latent period of "epileptogenesis" suggests that alterations in the developmental assembly of neural networks may underlie hyperexcitability in these mice.

Several theories have been put forward to explain the nexus between the molecular function of Syns at the neuronal level and the onset of the epileptic phenotype. Since Syns are involved in crucial steps of presynaptic physiology and play a role in the formation of the synaptic connectivity during development, it is likely that mutations affecting their expression and/or phosphorylation-dependent functions will result in significant changes in synaptic transmission, plasticity and development which could be potentially related to the appearance of an epileptic phenotype. Thus, the epileptic phenotype of Syn KO animals might be due to a defect in the overall brain connectivity (Chin et al., 1995), or to an impairment in synaptogenesis within confined neuronal populations (Gitler et al., 2004; Bonanomi et al., 2005). There is also support for the idea that lack of Syns produces an imbalance in the activity of excitatory and inhibitory neurons. Indeed, Syn I KO reduces the readily releasable pool at inhibitory synapses, and increases it at excitatory synapses (Baldelli et al., 2007; Chiappalone et al., 2009).

SNAP-25

A further example of presynaptic protein involved in epilepsy is SNAP-25. SNAP-25, together with syntaxin and synaptobrevin/VAMP, is a member of the SNARE (soluble NSF attachment protein receptor) complex which contributes to the formation of the fusion complex required for SV exocytosis. Beside participating in SV fusion, SNAP-25 has been proposed to regulate exocytosis at additional steps. Through its interaction with other proteins, such as synaptotagmin or complexins (Jahn et al., 2003) or through putative calcium binding sites (Sorensen et al., 2002), SNAP-25 appears to participate in the calcium-dependent regulation of exocytosis. SNAP-25 has also been shown to interact with and modulate the activity of various voltage-activated calcium channels (Atlas, 2001; Pozzi et al., 2008; Condliffe et al., 2010). The possible involvement of SNAP-25 in neuronal hyperexcitability and epilepsy comes from the neurological mouse mutant Coloboma (Cm/+), bearing an *autosomal dominant deletion* on chromosome 2, including the SNAP-25 gene (Hess et al., 1992, 1994). Cm/+ mice show 50% reduction in SNAP-25 mRNA and protein, and impaired evoked neurotransmitter release (Raber et al., 1997; Wilson, 2000). Interestingly, Cm/+ mice show robust cortical cortical spike-wave discharges and increased thalamic T-type currents (Zhang et al., 2004), two typical features of "absence" epilepsy (Tsakiridou et al., 1995; Coenen and Van Luijtelaar, 2003).

Similarly to what already described for Syns, the role of SNAP-25 in the etiopathology of epilepsy may involve alterations of synaptic formation/refinement during development. The epileptic phenotype of SNAP-25 mutant mice may be produced by a defect in the overall brain connectivity consequent to the reduced expression of the protein, which is known to play a crucial role in neurite extension (Osen-Sand et al., 1996). Alternatively, it may be the consequence of unbalances in neurotransmitter release due to deficits in the SNARE complex. Interestingly, SNAP-25 is expressed at much higher levels at excitatory vs. inhibitory synapses (Verderio et al., 2004; Bragina et al., 2007). Thus, hyperexcitability could result from perturbations of the processes that balance the developmental assembly of inhibitory and excitatory circuits. Furthermore, recent studies have shown a role of SNAP-25 in the regulation of calcium homeostasis in neurons. In particular, it has been shown that phosphorylated SNAP-25 negatively modulates calcium dynamics by inhibiting voltage-gated calcium channels (Verderio et al., 2004; Pozzi et al., 2008). Thus, reduced levels of SNAP-25 may lead to increased calcium currents (Condliffe et al., 2010) and consequently to the onset of epileptic discharges.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we have highlighted some of the neurodevelopmental pathways that lead to a chronic epileptic condition in adulthood. Abnormal development of the cerebral cortex (due to perturbations of neuronal proliferation and/or migration) is a frequent cause of epilepsy. In addition, more subtle alterations in the assembly and fine-tuning of neuronal networks may also lead to an hyperexcitability phenotype later in life. A better understanding of the biology of these epileptogenic mechanisms has important implications for the development of novel therapeutic approaches.

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X-chromosome inactivation in Rett syndrome human induced pluripotent stem cells

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Rett syndrome (RTT) is a neurodevelopmental disorder that affects girls due primarily to heterozygous mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2). Random X-chromosome inactivation (XCI) results in cellular mosaicism in which some cells express wild-type (WT) MECP2 while other cells express mutant MECP2. The generation of patient-specific human induced pluripotent stem cells (hiPSCs) facilitates the production of RTT-hiPSC-derived neurons *in vitro* to investigate disease mechanisms and identify novel drug treatments. The generation of RTT-hiPSCs has been reported by many laboratories, however, the XCI status of RTT-hiPSCs has been inconsistent. Some report RTT-hiPSCs retain the inactive X-chromosome (post-XCI) of the founder somatic cell allowing isogenic RTT-hiPSCs that express only the WT or mutant MECP2 allele to be isolated from the same patient. Post-XCI RTT-hiPSCs-derived neurons retain this allele-specific expression pattern of WT or mutant MECP2. Conversely, others report RTT-hiPSCs in which the inactive X-chromosome of the founder somatic cell reactivates (pre-XCI) upon reprogramming into RTT-hiPSCs. Pre-XCI RTT-hiPSC-derived neurons exhibit random XCI resulting in cellular mosaicism with respect to WT and mutant MECP2 expression. Here we review and attempt to interpret the inconsistencies in XCI status of RTT-hiPSCs generated to date by comparison to other pluripotent systems *in vitro* and *in vivo* and the methods used to analyze XCI. Finally, we discuss the relative strengths and weaknesses of post- and pre-XCI hiPSCs in the context of RTT, and other X-linked and autosomal disorders for translational medicine.

Keywords: Rett syndrome, human induced pluripotent stem cells, X-chromosome inactivation

INTRODUCTION

Rett syndrome [RTT (MIM 312750)] is a neurodevelopmental disorder that primarily affects young girls at an incidence of 1 in 10,000 live female births (Chahrour and Zoghbi, 2007). RTT girls develop normally until 6–18 months of age when they enter developmental arrest. Clinical features of RTT include microcephaly, stereotypic hand-washing movements, autistic features, loss of language, and mental retardation (Hagberg et al., 1983). Genetically, over 95% of RTT patients carry a heterozygous mutation in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2; Amir et al., 1999). MECP2 functions as a transcriptional regulator by binding to the genome in a DNA methylation-dependent manner via its methyl-CpG binding domain and recruiting chromatin remodeling proteins via its transcriptional repression domain (Nan et al., 1993, 1997, 1998; Chahrour et al., 2008; Ben-Shachar et al., 2009; Skene et al., 2010). Other genes less commonly implicated in RTT include *CDKL5* and *FOXG1*, which are located on chromosome X and 14, respectively (Scala et al., 2005; Ariani et al., 2008).

A complexity of the RTT story is that the *MECP2* gene is located on the X-chromosome and is influenced by X-chromosome inactivation (XCI). XCI is the mammalian strategy to equalize X-linked gene dosage between XX females and XY males and involves transcriptionally silencing the majority of genes on one X-chromosome in females (Escamilla-Del-Arenal et al., 2011; Yang et al., 2011). This process initiates early in development; in the embryo proper this occurs at about the time of implantation. At its onset, XCI is random and either the maternally or paternally inherited X-chromosome is silenced in each cell. Subsequently, that X-chromosome remains the inactive X-chromosome (Xi) throughout all future cell divisions (Escamilla-Del-Arenal et al., 2011).

Regulation of XCI in both human and mouse requires the presence in *cis* of *XIST*, a 17 kb non-coding RNA (ncRNA; Brockdorff et al., 1991; Brown et al., 1991, 1992; Penny et al., 1996). *XIST* is only expressed from the Xi and its RNA product closely associates with or "coats" the chromosome (Brown et al., 1991, 1992; Clemson et al., 1996). Therefore, a key developmental event is to upregulate *Xist* from the future Xi. Both *cis* and *trans*-acting factors have been identified in mouse through the use of transgenes and targeted deletions (Donohoe et al., 2009; Barakat et al., 2011). In *cis* *Xist* is positively and negatively regulated by adjacent sequences and transcripts that include at least four ncRNAs (Debrand et al., 1999; Lee et al., 1999; Ogawa and Lee, 2003; Augui

et al., 2007; Tian et al., 2010; Barakat et al., 2011). Perhaps the best characterized negative regulator is *Tsix*, an ncRNA antisense to *Xist* (Lee et al., 1999). While *XIST* is conserved between human and mouse, at least some events at the onset of XCI must differ between the species, as most ncRNAs including *TSIX* are poorly conserved (Chureau et al., 2002; Migeon et al., 2002).

Upon Xist upregulation, the Xi is heavily epigenetically remodeled, in many ways similar to other silenced genes throughout the genome. Epigenetic marks associated with the Xi include CpG island promoter DNA methylation (Hellman and Chess, 2007; Sharp et al., 2011), incorporation of histone variant MacroH2A (Costanzi and Pehrson, 1998), and modification of core histones (Keohane et al., 1996; de Napoles et al., 2004; Kohlmaier et al., 2004). An early event that follows *XIST* accumulation is the recruitment of the polycomb complex PRC2 that induces histone H3 trimethylation at lysine 27 (H3K27me3; Plath et al., 2003; Silva et al., 2003; Marks et al., 2009). Other epigenetic features, such as DNA methylation, accumulate later, and are important in the maintenance of XCI. Altogether these many alterations function with *XIST* to create a silenced nuclear compartment (Chow et al., 2010) that is spatially sequestered to the periphery of the nucleus and is cytologically recognizable as the darkly staining Barr body (Barr and Bertram, 1949).

Because of the random nature of XCI, RTT females are mosaic with both wild-type (WT) cells that inactivate the X-chromosome harboring the mutant allele and mutant cells that have inactivated the WT allele. Although XCI is random in most cases resulting in a 50:50 XCI ratio, it can deviate from this ratio. This results in a wide range of clinical presentations for RTT patients, even among related individuals carrying identical *MECP2* mutant alleles, depending on the extent of favorable skewing (Archer et al., 2007).

One model to study RTT is to employ the technology of induced pluripotency. Human induced pluripotent stem cells (hiPSCs) are pluripotent stem cells generated from somatic cells by the introduction of a combination of pluripotency associated genes such as *OCT4*, *SOX2*, along with either *KLF4* and *c-MYC* or *NANOG* and *LIN28* via retroviral or lentiviral vectors (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008). Most importantly, hiPSCs are similar to human embryonic stem cells (hESCs) functionally as they are pluripotent and can potentially differentiate into any desired cell type when provided with the appropriate cues, but do not have the ethical issues surrounding hESCs (Murry and Keller, 2008). For these reasons, hiPSCs have huge potential in translational medicine such as disease modeling, drug screening, and cellular therapy. Indeed, patient-specific hiPSCs have been generated for a multitude of diseases, including many with a neurological basis, in which disease phenotypes have been recapitulated *in vitro* and proof-of-principle drug screening has been performed (Saha and Jaenisch, 2009; Ross and Ellis, 2010; Han et al., 2011; Marchetto et al., 2011; Zhu et al., 2011).

The generation of patient-specific hiPSCs from RTT girls has been an area of intense research as several groups have reported the generation of such cells (Hotta et al., 2009; Marchetto et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011). RTT-hiPSCs generated by different groups have similar properties as they carry pathogenic mutations in *MECP2* or *CDKL5* and are pluripotent *in vitro* and *in vivo*. Most importantly, RTT-hiPSCs can be differentiated into affected neurons and exhibit RTT-associated phenotypes *in vitro* and can be rescued by transgene expression or drug treatments. However, with the generation of RTT-hiPSCs from multiple groups, the XCI status of RTT-hiPSCs, and more generally, female hiPSCs, has been variable. Some groups (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011) reported the generation of RTT-hiPSCs that retain the Xi (post-XCI) from the founder somatic cell it was derived from, while others (Kim et al., 2009; Marchetto et al., 2010) reported the generation of some RTT-hiPSCs that reactivate the Xi of the founder somatic cell and hence carry two active X-chromosomes (pre-XCI).

Here we review the differences in XCI status reported in the RTT-hiPSC literature and attempt to discuss the differences and interpret the inconsistencies between post- and pre-XCI RTT-hiPSCs by considering the XCI status described in other pluripotent systems from mouse and human, both *in vitro* and *in vivo*. Given the variations in XCI status reported in the human pluripotent stem cell (hPSC) literature, we discuss different methods to accurately evaluate the XCI status. We discuss the advantages and disadvantages of post- and pre-XCI RTT-hiPSCs for translational medicine. Finally, we discuss the significant impact XCI has on hiPSCs generated from female individuals, especially those affected by X-linked disorders other than RTT, but also apparently unaffected females who may or may not be carriers of X-linked mutations, and perhaps also autosomal disorders.

XCI IN THE MOUSE PLURIPOTENT SYSTEM

To place the relationship between XCI and RTT-hiPSCs into context, it is important to consider the XCI status of other pluripotent stem cell systems such as hiPSCs, hESCs, mouse induced pluripotent stem cells (miPSCs), and mouse embryonic stem cells (mESCs), and how they compare to their *in vivo* counterparts (Fan and Tran, 2011; van den Berg et al., 2011; Minkovsky et al., 2012). At embryonic day (e)3.5 of mouse embryogenesis, preimplantation epiblast cells carry two active X-chromosomes (Xa; Rastan, 1982; Takagi et al., 1982). Subsequently, random XCI ensues at gastrulation at around e5.5 (Rastan, 1982; Takagi et al., 1982). *Xist* RNA is expressed at low levels on both Xa until random XCI ensues in which the Xa represses *Xist* RNA while the Xi upregulates *Xist* RNA (Mak et al., 2004; Okamoto et al., 2004). Consistently, mESCs isolated from the preimplantation epiblast cells of the blastocysts at e3.5 carry two Xa, expressing *Xist* RNA in a biallelic manner (Evans and Kaufman, 1981; Martin, 1981; Lee et al., 1999). Similarly, the generation of miPSCs is accompanied by reactivation of the Xi in the founder somatic cell and hence carry two Xa followed by random XCI upon differentiation (Maherali et al., 2007; Stadtfeld et al., 2008a). This indicates that the generation of miPSCs involves the complete erasure of XCI. Subsequently, pluripotent stem cells known as mouse epiblast stem cells (mEpiSCs) have been isolated from the postimplantation epiblast at around e5.5 (Brons et al., 2007; Tesar et al., 2007). mEpiSCs carry an Xi similar to their *in vivo* counterpart in which random XCI has already ensued (Guo et al., 2009).

XCI IN hESCs

HESCs are isolated from the preimplantation epiblast cells of the blastocyst (Thomson et al., 1998; Reubinoff et al., 2000). Studies of XCI in human embryos are much more limited than in mouse, but intriguingly suggest differences in XCI timing and *XIST* RNA expression (van den Berg et al., 2009; Okamoto et al., 2011). Unlike mouse, *XIST* RNA is upregulated in preimplantation blastocysts (van den Berg et al., 2009; Okamoto et al., 2011). The role of *XIST* RNA association in XCI at this early timepoint is not yet clear as two recent studies have shown different results; female preimplantation blastocysts had monoallelic *XIST* RNA upregulation and XCI hallmarks in one study (van den Berg et al., 2009), whereas another reported *XIST* RNA accumulation from all X-chromosomes, males and females, without gene silencing (Okamoto et al., 2011). Whether such heterogeneity exists between different human embryos or variability is introduced upon culturing, it appears that human *XIST* RNA coating and XCI are not strictly coupled.

Similarly, the XCI status of hESCs has been highly variable (Dhara and Benvenisty, 2004; Enver et al., 2005; Hoffman et al., 2005; Adewumi et al., 2007; Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Liu and Sun, 2009; Dvash et al., 2010; Diaz Perez et al., 2012). The XCI status of hESCs can be categorized into three classes as proposed by Lee and colleagues (Silva et al., 2008). Class I hESCs are in a pre-XCI state, express X-linked genes in a biallelic fashion, lack XCI marks such as *XIST* RNA and H3K27me3, and initiate XCI upon differentiation accompanied by upregulation of the same marks (Dhara and Benvenisty, 2004; Hall et al., 2008; Silva et al., 2008; Dvash et al., 2010; Diaz Perez et al., 2012). The most defining feature of Class I hESCs is initiation of random XCI upon differentiation, resulting in random monoallelic expression of X-linked genes (Dhara and Benvenisty, 2004). To date, few Class I hESCs have been isolated using conventional hESC conditions in atmospheric oxygen concentrations (20% O_2) and basic FGF (bFGF)/knockout serum (KOSR; Dhara and Benvenisty, 2004; Hall et al., 2008; Silva et al., 2008; Dvash et al., 2010; Diaz Perez et al., 2012). Most hESCs have initiated XCI and thus are in a post-XCI state that can be subdivided according to the presence (Class II) or absence (Class III) of XCI marks such as *XIST* RNA and H3K27me3 (Hoffman et al., 2005; Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Liu and Sun, 2009; Dvash et al., 2010; Diaz Perez et al., 2012). Class II hESCs carry an Xi with a non-random skewing pattern resulting in non-random monoallelic expression of X-linked genes (Hoffman et al., 1987; Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Liu and Sun, 2009). This is likely due to clonal expansion of cells with one of the parental X-chromosomes inactivated (Liu and Sun, 2009). Class III hESCs can also retain an Xi despite the absence of such XCI marks (Shen et al., 2008; Silva et al., 2008; Diaz Perez et al., 2012). This indicates that XCI marks are not an accurate evaluation of XCI status in hESCs. Although most genes stay silenced in Class III hESCs, previously silenced X-linked genes may reactivate on a small scale accompanied by DNA hypomethylation of their promoters (Shen et al., 2008; Dvash et al., 2010). A recent analysis suggests that this reactivation may occur on a much larger scale with entire regions of the X-chromosome arms being reactivated (Bruck and Benvenisty, 2011). Overall, such sporadic X-linked gene reactivation appears higher than in somatic cells but is likely akin to levels in mouse cells that have lost *Xist* RNA (Wutz and Jaenisch, 2000; Csankovszki et al., 2001; Zhang et al., 2007). A defining feature of Class III hESCs is that they have already initiated XCI despite having lost the XCI marks. Therefore, they cannot initiate XCI again and do not upregulate XCI marks upon differentiation (Shen et al., 2008; Silva et al., 2008; Dvash et al., 2010; Diaz Perez et al., 2012).

HESCs with these three classes of XCI are hypothesized to be in a continuum and interrelated (Silva et al., 2008; Diaz Perez et al., 2012). It is thought that Class I hESCs represent the most pristine pluripotent stem cells present in the human blastocyst which contains two Xa (Okamoto et al., 2011). However, the culturing of Class I hESCs can result in a spontaneous transition into Class II hESCs in which XCI initiates and upregulates XCI marks (Silva et al., 2008). Class II hESCs, upon culture and/or cellular stresses such as freeze/thaw cycles, can also lose XCI marks such as *XIST* RNA and repressive chromatin marks, and thus transition into Class III hESCs (Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Dvash et al., 2010; Diaz Perez et al., 2012). Together, these data suggest that the XCI status of hESCs is subject to extensive epigenetic fluidity with respect to XCI.

Given the lack of correlation between XCI marks and XCI status in hESCs, Benvenisty and colleagues used an X-chromosome wide microarray analysis to investigate the expression level of a large number of X-linked genes as a functional read out of XCI in hPSCs, including hESCs and hiPSCs (Bruck and Benvenisty, 2011). Their microarray analysis revealed that hPSCs can be classified into *No XCI*, *Full XCI*, or *Partial XCI* categories. These three categories are defined by the proportion of X-linked genes that express twofold (i.e., two Xa) in comparison to male hPSCs. No XCI indicates most X-linked genes (>85%) express at twofold levels compared to male hPSCs. Full XCI indicates few X-linked genes (<15%) express at twofold levels compared to male hPSCs. Partial XCI indicates reactivation of a proportion (15–85%) of X-linked genes. Interestingly, it was observed that the location of partial inactivation tends to surround the *XIST* locus. *XIST* RNA expression, by quantitative reverse transcription polymerase chain reaction (qRT-PCR), was only partially correlated with these three states and would not have been able to distinguish between Full XCI and Partial XCI states. The term No XCI used in this study is fitting as it is not clear whether these hPSCs were generated directly with a pre-XCI phenotype, or from partial-XCI hPSCs that have reactivated X-linked genes on a chromosomal level resulting in an apparent pre-XCI phenotype. Thus, X-chromosome wide expression analysis via microarray could be used in determining the XCI status of the entire X-chromosome, but this method alone cannot identify pre-XCI hPSCs.

The two classifications above proposed by Lee and colleagues and Benvenisty and colleagues are both invaluable (Silva et al., 2008; Bruck and Benvenisty, 2011). XCI based solely on XCI marks such as *XIST* RNA and H3K27me3 is insufficient and suggests that candidate X-linked gene expression analysis in an allele-specific manner is crucial to functionally read out the XCI status of hESCs. In contrast, Benvenisty and colleagues categorized the hPSCs in their study based solely on expression of the entire X-chromosome. For the purpose of this review and to be

consistent with the terminology used in most of the hESC conversion studies and the hiPSC literature discussed below, we prefer to categorize hPSCs as either *pre-XCI* or *post-XCI*. Pre-XCI hPSCs are identical to Class I hPSCs and carry two Xa without any signs of XCI initiation. Upon differentiation, pre-XCI hPSCs will initiate XCI and upregulate XCI marks in a random pattern resulting in random monoallelic expression of X-linked genes. Post-XCI hPSCs encompass both Class II and III hPSCs in that they have already initiated the process of XCI and carry an Xi regardless of the presence or absence of XCI marks. Differentiation of post-XCI hPSCs will yield a non-random XCI skewing pattern resulting in a non-random monoallelic expression of X-linked genes.

The largely post-XCI state of hESCs may be explained by the fact that they are thought to represent a cell type that is developmentally later than mESCs (Rossant, 2008; Silva and Smith, 2008; Nichols and Smith, 2009). It is thought that although hESCs are isolated from the preimplantation epiblast of the blastocyst, where there could be two Xa (Okamoto et al., 2011), they may in fact represent cells of the postimplantation epiblast where XCI has likely ensued as in the murine postimplantation epiblast (Rastan, 1982; Takagi et al., 1982). This is supported by the fact that hESCs are more similar to the murine *in vitro* counterpart of the postimplantation epiblast, mEpiSCs, than the murine *in vitro* counterpart of the preimplantation epiblast, mESCs. The pluripotent state of mESCs is maintained by cytokines including leukemia inhibitory factor (LIF) and bone morphogenic protein (BMP) 4 which activates the Jak/Stat pathway and induces inhibitor-of-differentiation proteins, respectively (Smith et al., 1988; Williams et al., 1988; Niwa et al., 1998; Matsuda et al., 1999; Ying et al., 2003). On the other hand, hESCs, similar to mEpiSCs, are maintained by fibroblast growth factor (FGF) which induces mitogen-activated protein kinases Erk1/2 and activin/nodal signaling (Thomson et al., 1998; Vallier et al., 2004; James et al., 2005; Brons et al., 2007; Kunath et al., 2007; Stavridis et al., 2007; Tesar et al., 2007). Furthermore, gene expression profiling reveals similarities between hESCs and mEpiSCs, but distinct from mESCs (Tesar et al., 2007). Therefore it is not surprising that hESCs are mostly in a post-XCI state similar to mEpiSCs (Guo et al., 2009).

CONVERSION OF MOUSE AND HUMAN PLURIPOTENT STEM CELLS TO THE NAÏVE STATE

To explain why hESCs isolated from the preimplantation epiblast would more closely resemble cells isolated from the postimplantation epiblast, it is important to consider that upon isolation, they are not frozen in developmental time in culture (Nichols and Smith, 2009). Indeed, it may be unnatural for hESCs (and mESCs) to expand in a pre-XCI state as this does not occur in development (Hall et al., 2008). Therefore, hESCs may preferentially undergo XCI during derivation and expansion. On the other hand, female mESCs appear to utilize a different strategy to accommodate for two Xa by frequently losing one X-chromosome (Zvetkova et al., 2005). Therefore, although hESCs may have a pre-XCI status during their isolation, they may continue to progress into the postimplantation epiblast stage (representative of mEpiSCs) and become post-XCI. To distinguish these two pluripotent stages, mESCs are classified to be in a *naïve state*, whereas mEpiSCs (and likely hESCs) are classified to be in a *primed state* (Silva and Smith, 2008; Nichols and Smith, 2009). Further evidence that these two stages are biologically relevant comes from experiments that show mESCs and mEpiSCs are distinct states that can be converted between one another. When cultured in conditions consisting of small molecules (known as the *2i* cocktail) that inhibit FGF stimulation of mitogen-activated protein kinases Erk1/2 and constitutive activity of glycogen synthase kinase-3, mESCs are maintained in the most pristine naïve state of pluripotency (Ying et al., 2008). On the other hand, mESCs can be differentiated into mEpiSCs when placed in the culture conditions of the latter while introduction of a *Klf4* transgene and growth in 2i conditions can convert mEpiSCs back to a naïve state reminiscent of mESCs (Guo et al., 2009). These studies suggest that the external milieu to which pluripotent stem cells are exposed has a significant impact on their pluripotent state and their XCI status.

Since the identification of optimal conditions for naïve pluripotency for mESCs and the hypothesis that hESCs may be more similar to mEpiSCs, attempts have been made to define conditions to isolate hESCs in a more naïve state of pluripotency in order to derive hESCs equivalent to mESCs. One approach that was examined was the isolation of hESCs under physiological oxygen concentrations (5% O_2; Lengner et al., 2010). It was proposed that the atmospheric oxygen concentration (20% O_2), in which conventional propagation of hESCs is performed is hyperoxic in comparison to the blastocyst *in vivo*, and may represent a suboptimal culture condition for hESCs. Indeed, when hESCs were established from human embryos in 5% O_2, pre-XCI hESCs were derived that were capable of random XCI upon differentiation. On the other hand, hESCs derived under 20% O_2 were all post-XCI with a non-random monoallelic expression pattern. Furthermore, when 5% O_2 pre-XCI hESCs were subsequently exposed to 20% O_2 or cellular stress (such as freeze/thaw), XCI ensued. This suggests that conventional hESC culture conditions (i.e., 20% O_2) and other cellular stresses are detrimental for capturing and maintaining hESCs in a pre-XCI state. Conversely, post-XCI hESCs were not able to go through X-chromosome reactivation (XCR) when placed back in hypoxic conditions or after treatment with antioxidants. This suggests that XCI is irreversible (Lengner et al., 2010) or that other strategies are required for XCR (Hanna et al., 2010).

To that end, primed hESCs, with an Xi, have been converted to naïve hESCs, with two Xa, by continuous transgene expression of *OCT4* and *KLF4*, or *KLF4* and *KLF2*, combined with defined conditions including 2i and LIF (Hanna et al., 2010). The converted naïve hESCs revert back to a primed state when placed back in conventional hESC conditions (bFGF and KOSR) demonstrating that the naïve and primed states are interchangeable in hESCs when given the correct environmental cues along with exogenous transgenes. However, naïve hESCs were only maintained under continuous transgene expression demonstrating the need for further optimal culture conditions without genetic manipulation to capture this naïve pluripotency state. To this end, the authors identified Forskolin, a protein kinase A pathway agonist which induces the expression of KLF4 and KLF2. Forskolin can substitute for the continuous ectopic transgene expression although its effect was transient as naïve hESCs were only maintained for a limited number of

passages. Similar methods in generating naïve hiPSCs can also be achieved by the exogenous transgene expression of *OCT4*, SOX2, *KLF4*, *c-MYC*, and *NANOG* in the presence of LIF although the XCI status of these cells was not examined (Buecker et al., 2010). Together, these data suggest that XCR is possible via the introduction of exogenous transgenes. However, if naïve hESCs were to be used clinically, non-integrating approaches will be preferred.

To that end, two small molecules, Sodium Butyrate (SB) and 3-deazaneplanocin A (DZNep), have been recently identified to promote the conversion and isolation of Class I hESCs (Diaz Perez et al., 2012). Note that here we employ the Class I, II, III, system to be consistent with the description by the authors (Diaz Perez et al., 2012). SB is a histone deacetylase inhibitor which can convert hESCs to a more naïve state similar to mESCs (Ware et al., 2009). DZNep depletes enhancer of zeste homolog2 (EZH2) resulting in a global decrease of H3K27me3 (Tan et al., 2007; Fiskus et al., 2009; Musch et al., 2010). When Class II hESCs were treated with SB or SB + DZNep, Class I hESCs emerged as indicated by the absence of H3K27me3 and lack of H3K4me3 exclusion on the X-chromosome, indicative of XCR (Diaz Perez et al., 2012). However, this effect was not 100% efficient, as only a proportion of cells within a culture of Class II hESCs can be converted. In contrast, Class III hESCs were not able to proceed through XCR by the same treatment. Conversely, treatment with SB or DZNep or both prevented Class I hESCs from transitioning into Class II, at least during the time frame (10 passages) analyzed by the authors. Altogether, these data suggest that although pre-XCI hESCs can be isolated, suboptimal culture conditions prevent facile maintenance of naïve hESCs in a pre-XCI state (Hanna et al., 2010; Lengner et al., 2010; Diaz Perez et al., 2012). Hence, hESCs continue to progress along the developmental timeline to stabilize in a primed state as post-XCI hESCs (Nichols and Smith, 2009).

XCI IN hiPSCs

The XCI status of female hiPSCs has been extensively studied by Plath and colleagues. They found that most female hiPSCs in their study were post-XCI as indicated by *XIST* RNA and enrichment of the polycomb repressive complex EZH2 which mediates enrichment of H3K27me3, and depletion of active histone marks H3K18ac and H3K4me3 from the Xi (Tchieu et al., 2010). Furthermore, post-XCI hiPSCs expressed the X-linked genes *XIST*, *ATRX*, and *PDHA1* in a non-random monoallelic fashion revealed by allele-specific SNP analysis. This suggests that during reprogramming, hiPSCs inherit the Xi from the founder somatic cell. Post-XCI hiPSCs were prone to losing XCI marks such as *XIST* RNA, EZH2, macroH2A1, and H4K20me1 upon extended passaging. However, these hiPSCs retained a transcriptionally silent Xi. This reiterates that evaluation of XCI marks such as *XIST* RNA and chromatin marks and their mediators is not sufficient to determine XCI status in hPSCs (Shen et al., 2008; Silva et al., 2008; Lengner et al., 2010; Diaz Perez et al., 2012). Finally, these data suggest that the non-random XCI nature of female hiPSCs can be exploited to generate isogenic control (expressing WT protein) and mutant (expressing mutant protein) hiPSCs from the same individual carrying heterozygous mutations in X-linked genes. Indeed, the authors were able to isolate isogenic control and mutant hiPSCs from Duchenne muscular dystrophy (DMD) carriers with a heterozygous mutation in the X-linked *DYSTROPHIN* gene (Tchieu et al., 2010).

Several groups have generated hiPSCs from RTT patients (Marchetto et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011). The heterozygous mutation of the X-linked gene *MECP2* in RTT-hiPSCs has prompted extensive analysis of their XCI status as it directly affects their functional read out (**Figure 1**). Post-XCI RTT-hiPSCs with non-random XCI will yield isogenic WT or mutant MECP2 expressing RTT-hiPSCs. Differentiation of post-XCI RTT-hiPSCs will yield cultures that maintain this non-random monoallelic expression pattern allowing the direct comparison of WT and mutant neurons (**Figure 1A**). Conversely, pre-XCI RTT-hiPSCs will carry two Xa and upon differentiation would yield a mosaic culture of WT or mutant MECP2 expressing neurons similar to RTT patients (**Figure 1B**).

POST-XCI RTT-hiPSCs

Four studies report the generation of RTT-hiPSCs in a post-XCI state (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011). In our study, X-chromosome activity and mosaicism were evaluated by *XIST* RNA-FISH and H3K27me3 immunofluorescence (IF; Cheung et al., 2011). We found RTT-hiPSCs that were positive or negative for these XCI marks within the same culture. The RTT-hiPSCs that are negative for XCI marks could be interpreted as: (1) loss of an X-chromosome, (2) two Xa, or (3) an Xi that has subsequently lost XCI marks but remains inactive. We rejected the first hypothesis as RTT-hiPSCs carried two X-chromosomes as assayed by X-centromere DNA-FISH and karyotyping. The second possibility, two Xa, was ruled out as RTT-hiPSC-derived neurons had a non-random XCI skewing pattern, as measured by the androgen receptor methylation (AR) assay (Allen et al., 1992), which is inconsistent with the presence of two Xa which would have otherwise resulted in random XCI upon differentiation. Therefore we favored the explanation that RTT-hiPSCs negative for XCI marks are post-XCI with an Xi that has subsequently lost those marks. Post-XCI RTT-hiPSCs had non-random XCI consistent with the fact that reprogramming retains the Xi in the founder somatic cell (Cheung et al., 2011). Finally, expression analysis, by direct sequencing of cDNA or qRT-PCR using WT *MECP2*-specific primers, confirmed that post-XCI RTT-hiPSCs and their neuronal derivatives expressed WT or mutant MECP2 in non-random monoallelic manner. This confirms the findings by Plath and colleagues that the non-random monoallelic post-XCI status of female hiPSCs can be exploited to generate isogenic control (expressing WT MECP2) and mutant (expressing mutant MECP2) hiPSCs from the same individual (Tchieu et al., 2010). Similarly, Renieri and colleagues and Chang and colleagues isolated isogenic control and mutant RTT-hiPSCs from some female patients carrying heterozygous mutations in the X-linked *CDKL5* gene and *MECP2*, respectively (Amenduni et al., 2011; Ananiev et al., 2011). RTT-hiPSCs were post-XCI as they displayed non-random XCI skewing by AR assay and expressed *CDKL5* or *MECP2* in a non-random monoallelic manner allowing the isolation of isogenic control and mutant RTT-hiPSCs. Altogether, these studies demonstrate that post-XCI RTT-hiPSCs can

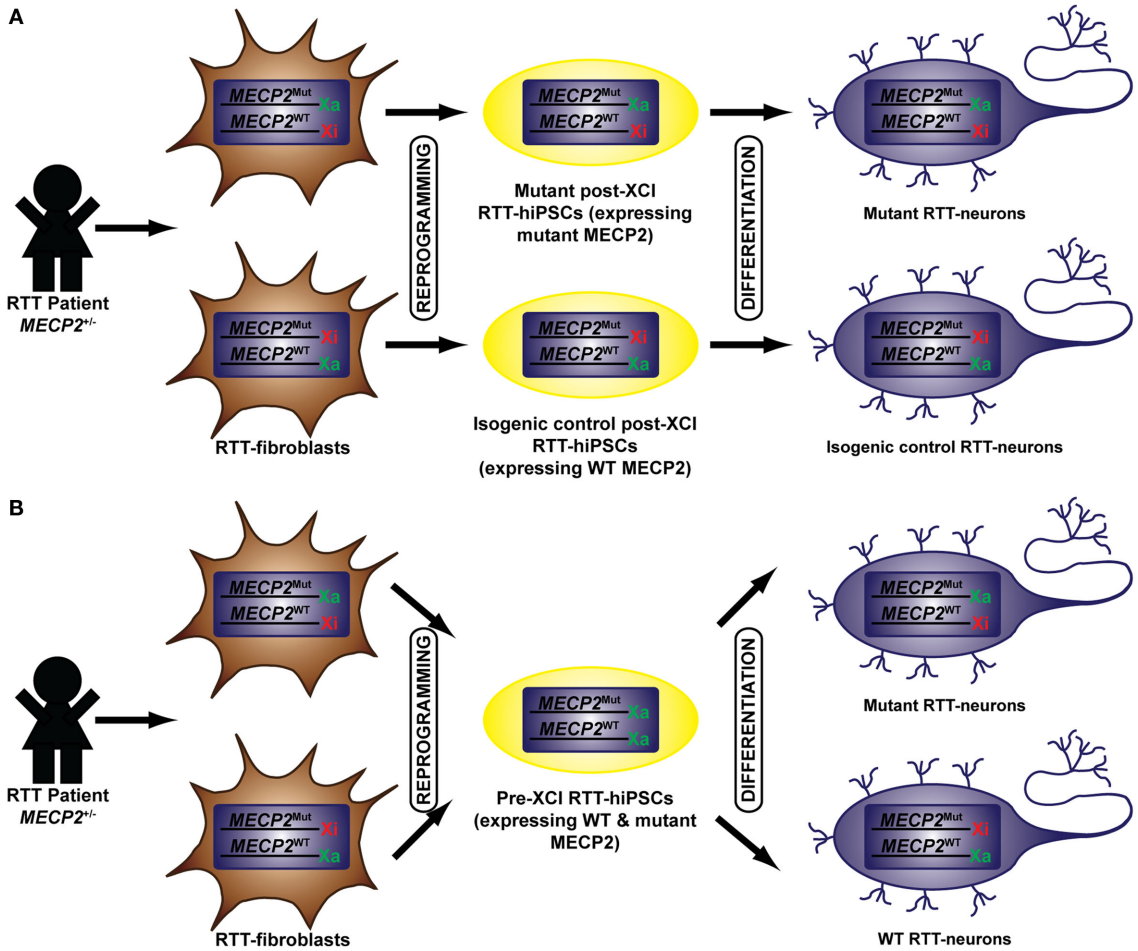

FIGURE 1 | Generation of pre- and post-XCI RTT-hiPSCs. RTT-hiPSCs have been generated from RTT-fibroblasts isolated from RTT patients who carry a heterozygous mutation in *MECP2*. The reprogramming of RTT-fibroblasts have yielded RTT-hiPSCs that are in a post-XCI **(A)** or pre-XCI **(B)** state. Post-XCI RTT-hiPSCs retains an Xi from the founder somatic cell and express MECP2 in a non-random monoallelic manner. This results in the generation of mutant and isogenic control RTT-hiPSCs depending on whether the Xi harbors the WT or mutant *MECP2*. The differentiation of post-XCI RTT-hiPSCs retains this XCI pattern allowing homogeneous cultures of neurons that express WT or mutant MECP2. Pre-XCI RTT-hiPSCs carry two Xa and express the WT and mutant MECP2 in a biallelic fashion. The differentiation of RTT-hiPSCs into neurons initiate random XCI resulting in a mosaic culture of neurons that either express WT or mutant MECP2. Mut, mutant.

be isolated, and become a particularly strong model system that allows direct comparison of mutant and isogenic control RTT-hiPSCs from the same individual (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011).

Colman and colleagues examined the dynamics of XCI during RTT-hiPSC establishment in order to implement strategies that were necessary to isolate pairs of mutant and isogenic control RTT-hiPSCs (Pomp et al., 2011). Similar to the RTT-hiPSCs described above (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011), RTT-hiPSCs displayed a non-random post-XCI pattern (Pomp et al., 2011). However, they were unable to isolate pairs of mutant and isogenic control post-XCI RTT-hiPSCs from the same individual. For example, RTT-hiPSCs generated from p.T158M *MECP2* fibroblasts were all expressing WT *MECP2*, while RTT-hiPSCs generated from 1155del32 *MECP2* fibroblasts were all expressing mutant *MECP2*. They referred to the expressing and silent X-chromosome as dominant (X_d) and unfavored (X_u), respectively. Fibroblasts carrying an active X_d had a higher reprogramming efficiency (50 ~ 100 fold) than those carrying an active X_u. Interestingly, although early passage fibroblasts were mosaic for the two MECP2 alleles, serial passaging skewed the XCI toward the X_d in a predictable manner mirrored during reprogramming. This loss of mosaicism was due to a proliferative advantage of those fibroblasts carrying an active X_d, and an increase in DNA damage in fibroblasts carrying an active X_u. The authors hypothesized that this was a consequence of critically shortened telomeres resulting in telomere-associated DNA damage and senescence in fibroblasts expressing X_u.

To address the role of telomeres in reprogramming, Colman and colleagues introduced an exogenous telomerase reverse transcriptase (TERT) transgene into fibroblasts to prevent skewing (Pomp et al., 2011). Such TERT expression allowed the generation

of isogenic control and mutant RTT-hiPSCs from the same individual. The reprogramming bias toward fibroblasts with an active X_d can also be overcome by increasing the number of hiPSC lines characterized, or by subcloning the fibroblasts expressing X_u prior to reprogramming. However, this remedy will not work on fibroblasts with a totally skewed population in which all the fibroblasts with an active X_u may have already been lost. To restore this lost population, they promoted naïve conversion of post-XCI RTT-hiPSCs to pre-XCI RTT-hiPSCs by culturing in 2i/LIF conditions as discussed above (Hanna et al., 2010). The resulting pre-XCI RTT-hiPSCs expressed both the WT and mutant *MECP2* allele in a biallelic fashion and lost H3K27me3 suggesting XCR (Pomp et al., 2011). Upon differentiation, pre-XCI RTT-hiPSCs underwent random XCI and generated a mosaic culture of fibroblasts that expressed either the WT or mutant MECP2, thereby restoring the lost population expressing X_u. Although not demonstrated, the reprogramming of such fibroblasts expressing X_u should also yield isogenic control and mutant post-XCI RTT-hiPSCs. Together, these data suggest that there is an inherent difference in the two parental X-chromosomes, independent of the *MECP2* mutation, which impacts the reprogramming efficiency of any given cell. This will ultimately impact one's ability to generate isogenic control and mutant post-XCI RTT-hiPSCs from fibroblasts that have been extensively passaged. For example, we were only able to isolate isogenic control and mutant RTT-hiPSCs from primary fibroblasts carrying the Δ3–4 *MECP2* mutation (Cheung et al., 2011). Similarly, Chang and colleagues and Renieri and colleagues isolated isogenic control RTT-hiPSCs from primary Q347X-fibroblasts (Amenduni et al., 2011), and primary R294X-fibroblasts (Ananiev et al., 2011), respectively. This is consistent with the findings by Colman and colleagues who suggest that the mosaic fibroblast culture is lost overtime. Therefore, the generation of isogenic control and mutant post-XCI RTT-hiPSCs should be more efficient from primary fibroblasts at earlier passages while the mosaic culture is still present (Pomp et al., 2011).

PRE-XCI RTT-hiPSCs

Two groups have isolated pre- and post-XCI RTT-hiPSCs (Marchetto et al., 2010; Kim et al., 2011). To evaluate the XCI status of RTT-hiPSCs, Muotri and colleagues performed *XIST* RNA-FISH and H3K27me3 IF (Marchetto et al., 2010). They reported both pre- and post-XCI RTT-hiPSCs based on the absence or presence of these XCI marks, respectively, in which the former was the focus for the rest of their study. Their pre-XCI RTT-hiPSCs initiated XCI upon differentiation into neurons as they induced the expression of *XIST* RNA and H3K27me3. To determine whether there was random XCI, the authors focused on RTT-hiPSCs from a patient carrying an 1155del32 mutation which results in a truncated MECP2 protein. By using a C-terminus MECP2 antibody, they could distinguish between the WT and mutant protein via IF. The pre-XCI RTT-hiPSCs were homogeneously MECP2-positive, suggesting biallelic expression of MECP2 as expected for two Xa. When pre-XCI RTT-hiPSCs were differentiated into neurons, there was a mosaic expression of MECP2-positive and -negative neurons. Furthermore, western blot analysis of pre-XCI RTT-hiPSC-derived neurons showed a reduction in MECP2 protein levels. Based on these findings, the authors conclude that pre-XCI RTT-hiPSCs initiate random XCI upon differentiation. However, the AR assay revealed that the pre-XCI RTT-hiPSC-derived neurons showed extreme skewing (96:4 to 98:2) inconsistent with random XCI. Thus, the neurons preferentially inactivated the *AR* gene on one parental X-chromosome but not randomly as would be expected. These results have been interpreted as suggesting that the neurons preferentially inactivated the parental X-chromosome carrying WT *MECP2* (Walsh and Hochedlinger, 2010). However, it is also possible that during neuronal differentiation, dividing neuronal progenitors carrying a particular Xi may have a proliferative advantage causing the observed skewing in the resulting neurons (Pomp et al., 2011).

Park and colleagues were also able to isolate both pre- and post-XCI RTT-hiPSCs (Kim et al., 2011). Pre-XCI and post-XCI RTT-hiPSCs expressed MECP2 in a biallelic and monoallelic manner, respectively. Furthermore, the authors measured the total transcript levels of MECP2 via qRT-PCR and found that pre-XCI RTT-hiPSCs expressed MECP2 twofold in comparison to male hESCs while post-XCI RTT-hiPSCs expressed MECP2 at similar levels compared to male hESCs. With regards to XCI marks, pre-XCI and post-XCI RTT-hiPSCs were absent and present for H3K27me3, respectively. Based on these findings, the authors also concluded that they had isolated pre-XCI RTT-hiPSCs which carry two Xa and express MECP2 in a biallelic manner.

INCONSISTENCIES BETWEEN THE XCI STATUS OF RTT-hiPSCs

The generation of RTT-hiPSCs with different XCI status is worth reviewing (Marchetto et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011). These differences are unlikely to be due to the starting fibroblasts as identical fibroblast cell lines were often used, as were similar culture conditions using bFGF/KOSR on mouse embryonic fibroblasts (MEFs; **Table 1**). One exception was that Muotri and colleagues used feeder-free conditions with mTeSR1 on matrigel (Marchetto et al., 2010). Whether this subtle difference can affect the XCI status of RTT-hiPSCs remains to be determined, although it is worth noting that the mTeSR1/matrigel conditions act as a substitute for the bFGF/KOSR conditions (Ludwig et al., 2006). Finally, reprogramming methods were similar between the different groups, as all used retroviral transduction of *OCT4*, *SOX2*, *KLF4*, and *c-MYC*, consistent with the lack of correlation between reprogramming methods and XCI status of female hiPSCs (Tchieu et al., 2010; Ananiev et al., 2011). Therefore, the starting cell type, culture conditions, and reprogramming methods were similar and are not strong candidates to explain the differences observed in the XCI status of RTT-hiPSCs.

Although we have opted to use the post- and pre-XCI nomenclature for this review, it is worth revisiting the classification system proposed by Lee and colleagues (Silva et al., 2008). Most RTT-hiPSCs reported are either Class II or III as they are post-XCI, exhibit non-random XCI skewing resulting in non-random monoallelic expression of MECP2 and maintain this expression pattern upon differentiation into neurons (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011). XCI marker analyses have identified examples of RTT-hiPSCs that can be classified as Class II or III based on *XIST* RNA-FISH and/or

Table 1 | Summary of RTT-fibroblasts reprogrammed, method of reprogramming, and the outcome of XCI status in RTT-hiPSCs.

Group	Fibroblasts reprogrammed	Reprogramming method	Culture condition	XCI status of RTT-hiPSCs
Muotri (Marchetto et al., 2010)	GM11270 (R306C), GM11272 (1155del32), GM16548 (Q244X), GM17880 (T158M)	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*)	Feeder free – mTesr1	Pre- and post-XCI
Ellis (Cheung et al., 2011)	Δ3–4 (Exon 3–4 delete), GM11270 (R306C), GM17880 (T158M)	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*)	bFGF/KOSR MEFs	Post-XCI
Renieri (Amenduni et al., 2011)	*CDKL5* (Q347X), *CDKL5* (T288I) – male	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*)	bFGF/KOSR MEFs	Post-XCI
Park (Kim et al., 2011)	GM07928 (E235fs), GM11270 (R306C), GM16548 (Q244X), GM17567 (X487X), GM17880 (T158M)	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*)	bFGF/KOSR MEFs	Pre- and post-XCI
Colman (Pomp et al., 2011)	GM11272 (1155del32), GM17880 (T158M)	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*, ±*TERT*)	bFGF/KOSR MEFs	Post-XCI
		Lentiviral (*OCT4*, *SOX2*, *NANOG*, *LIN28*)	2i + LIF	Pre-XCI
Chang (Ananiev et al., 2011)	GM17880 (T158M), GM07982 (V247X), GM11270 (R306C), RS0502 (R294X)	Retroviral (*OCT4*, *SOX2*, *KLF4*, *c-MYC*) Lentiviral (*OCT4*, *SOX2*, *NANOG*, *LIN28*)	bFGF/KOSR MEFs	Post-XCI

All fibroblasts reprogrammed are from female RTT-patients carrying a heterozygous mutation (indicated in parenthesis) in MECP2 unless specified. Nomenclature of cell lines beginning with "GM" are from the Coriell Cell Bank.

H3K27me3 IF (Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011), while other studies cannot conclusively place their RTT-hiPSCs within Class II or III (Amenduni et al., 2011; Ananiev et al., 2011). On the other hand, the pre-XCI RTT-hiPSCs reported by Park and colleagues lacked H3K27me3 marks and expressed *MECP2* twofold compared to male hESCs, consistent with a Class I assignment (Kim et al., 2011). However, this approach does not exclude the possibility of Class III hiPSCs that have lost XCI marks resulting in reactivation of X-linked genes (Silva et al., 2008). Likewise, the undifferentiated pre-XCI RTT-hiPSCs reported by Muotri and colleagues lacked XCI marks, such as *XIST* RNA and H3K27me3, and expressed MECP2 in a biallelic manner (Marchetto et al., 2010). Upon differentiation, the RTT-hiPSCs initiated XCI by upregulating XCI marks and exhibited a mosaic expression of WT and mutant MECP2 consistent with a Class I hiPSC. However, the apparent non-random skewing revealed by the AR assay in the RTT-hiPSC-derived neurons revealed a pattern characteristic of a Class II/III hiPSC. Overall, these data remain difficult to interpret. Further experiments to conclusively demonstrate the isolation of pre-XCI RTT-hiPSCs equivalent to Class I are required.

EVALUATION OF XCI IN RTT-hiPSCs

Given the epigenetic fluidity that exists in hPSCs in the context of XCI (Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Dvash et al., 2010; Lengner et al., 2010; Tchieu et al., 2010; Pomp et al., 2011; Diaz Perez et al., 2012), it is critical to review how the XCI status was determined in RTT-hiPSCs and review the strengths and weaknesses of different technical approaches to accurately evaluate XCI (**Tables 2** and **3**). The most common method of determining the XCI status in RTT-hiPSCs was the evaluation of XCI markers such as *XIST* by RNA-FISH and repressive chromatin marks and their mediators such as H3K27me3 and EZH2 by IF (**Table 2**). However, the evaluation of XCI based solely on *XIST* RNA and/or chromatin marks and their mediators is insufficient to define the XCI status of hPSCs (**Table 3**). Another method that has been used for evaluation of XCI in RTT-hiPSCs is the AR assay, a method that measures the DNA methylation status of the X-linked *AR* gene (**Table 2**; Allen et al., 1992). This method has two advantages, (1) it measures DNA methylation, which is a likely epigenetic candidate that maintains the Xi in a transcriptionally repressed state in the absence of *XIST* RNA and other repressive chromatin marks, and (2) it measures the skewing of XCI (**Table 3**). Although not demonstrated, it may be possible to identify pre-XCI hPSCs with the AR assay, as they will display no signal indicative of two unmethylated Xa.

The importance of XCI is its functional outcome on the expression status of X-linked genes. For these reasons, expression analyses must be performed (**Table 3**). Preferably, expression analysis is done in a quantitative and allele-specific manner such as direct sequencing of cDNA or qRT-PCR using allele-specific primers at the population level. More stringently, allele-specific expression can be done at the single cell level by using antibodies (via IF) or probes (via RNA-FISH) that can distinguish between the WT or mutant allele. Whenever possible, it is preferable to measure the expression of both WT and mutant alleles although, in some cases, it may be difficult to design primers/antibodies/probes that can distinguish the two. Allele-specific expression analysis at the population level is important to distinguish non-random monoallelic expression of post-XCI RTT-hiPSCs from biallelic pre-XCI RTT-hiPSCs. Although biallelic expression may represent pre-XCI RTT-hiPSCs, the mix of two clones of post-XCI RTT-hiPSCs that express alternative parental X-chromosomes would also show the same expression profile. This mixture of two clones could be revealed by the presence of two different-sized peaks in the AR assay. The clonality of pre-XCI RTT-hiPSCs could also be confirmed by subcloning multiple sublines to examine whether they

Table 2 | Summary of RTT-hiPSCs generated to date and their XCI status as determined by different methods.

Group	Techniques used for XCI evaluation					XCI status of RTT-hiPSCs
	***XIST*-RNA (FISH)**	**H3K27me3 (IF)**	**AR assay**	**Expression analysis**	**Other**	
Muotri (Marchetto et al., 2010)	hiPSCs – absent Neurons – present	hiPSCs – absent Neurons – present	hiPSCs – N/A Neurons – extreme skewing	hiPSCs – biallelic Neurons – monoallelic		Pre-XCI*
Ellis (Cheung et al., 2011)	hiPSCs – mix Neurons – N/A	hiPSCs – mix Neurons – N/A	hiPSCs – extreme skewing Neurons – extreme skewing	hiPSCs – monoallelic Neurons – monoallelic		Post-XCI
Renieri (Amenduni et al., 2011)	hiPSCs – N/A Neurons – N/A	hiPSCs – N/A Neurons – N/A	hiPSCs – extreme skewing Neurons – N/A	hiPSCs – monoallelic Neurons – N/A		Post-XCI
Park (Kim et al., 2011)	hiPSCs – N/A	hiPSCs – absent (pre-XC)/present (post-XCI)	hiPSCs – N/A	hiPSCs – biallelic (pre-XC)/monoallelic (post-XCI)	hiPSCs – EZH2 (qRT-PCR) – low (pre-XCI)/high (post-XCI)	Pre- and post-XCI
	Neurons – N/A	Neurons – N/A	Neurons – N/A	Neurons – N/A		
Colman (Pomp et al., 2011)	hiPSCs – present Neurons – N/A	hiPSCs – N/A Neurons – N/A	hiPSCs – N/A Neurons – N/A	hiPSCs – monoallelic Neurons – N/A		Post-XCI**
Chang (Ananiev et al., 2011)	hiPSCs – N/A Neurons – N/A	hiPSCs – N/A Neurons – N/A	hiPSCs – extreme skewing Neurons (progenitors) – extreme skewing	hiPSCs – monoallelic Neurons – monoallelic	hiPSCs – *XIST* RNA SNP	Post-XCI

**Muotri and colleagues isolated both pre- and post-XCI RTT-hiPSCs but focused on the former. **Colman and colleagues were able to isolate pre-XCI RTT-hiPSCs after naïve conversion. N/A, experiment not performed.*

Table 3 | Commonly used techniques to evaluate XCI status in hPSCs.

Method	Description	Advantages and disadvantages
XIST RNA (FISH)	*XIST* RNA is required for the initiation of XCI and coats the Xi. This can be detected by RNA-FISH as a punctate signal on the Xi.	Its absence (in hPSCs) is not directly correlated with the XCI status.
Chromatin marks and mediators	Repressive chromatin marks (H3K27me3, H4K20me1, MacroH2A) and their mediators (EZH2, EED) accumulate on the Xi during XCI. These marks can be detected via IF and appear as a punctate signal on the Xi.	Its absence (in hPSCs) is not directly correlated with XCI status.
AR assay	The X-linked *AR* gene contains a trinucleotide polymorphism in its first exon, which allows the two parental X-chromosomes to be distinguished by PCR. Digestion with methylation sensitive enzymes prior to PCR digests the unmethylated Xa. Genetic analyzers can be used to quantitate the relative proportions of Xi.	Allows XCI skewing to be calculated. Does not read out the unmethylated (active) X-chromosome. Only a single locus (Xq12) is being measured and may not reflect the entire X-chromosome. Requires polymorphism at the *AR* gene.
Allele-specific expression analysis	Determine the expression of the gene of interest in an allele-specific manner. This can be done at a population level via direct sequencing of cDNA or qRT-PCR using allele-specific primers. This can also be done at a single cell level via antibodies (IF) or probes (RNA-FISH) that can distinguish WT and mutant proteins or transcripts, respectively.	Functional read out of XCI. Only a single locus is being measured and may not reflect entire X-chromosome.
Cot1 RNA FISH	*Cot1* RNA-FISH to visualize hnRNA transcription throughout the nucleus. The Xi will be devoid of *Cot1* RNA indicating a transcriptionally silent compartment.	Functional read out of XCI. Allows global transcription to be assessed. May not detect small scale reactivation of X-linked genes. Conclusion of Xi presence is based on absence of signal.
X-chromosome wide expression analysis	Determines the expression status of the entire set of genes on the X-chromosome.	Functional read out of XCI. Visualize transcriptional activity of entire set of X-linked genes. Allows identification of three classes of hPSCs – pre-XCI, post-XCI, and partial-XCI.

contain identical or distinct retroviral transgene integration sites by southern blot analysis.

Allele-specific expression analysis of MECP2 only measures the XCI status of that particular locus and may not represent the status of the entire the X-chromosome. Therefore, it may be worthwhile to use other approaches to measure the transcription level of the entire X-chromosome. This includes *Cot1* RNA-FISH which highlight areas of actively transcribed heterogeneous nuclear RNA (hnRNA; van den Berg et al., 2009). The Xi is devoid of *Cot1* RNA indicative of a transcriptionally silent compartment. This technique has been most widely used in the hESC literature (Hoffman et al., 1987; Hall et al., 2008; Silva et al., 2008). However, *Cot1* RNA-FISH may not detect small scale reactivation of X-linked genes. Therefore, a more stringent method will be to perform X-chromosome wide expression analysis via microarray to measure the transcriptional activity of all X-linked genes (**Table 3**). It is important to note that the detection of twofold differences from the microarray inferred by male:female expression comparisons could be difficult and that allele-specific expression analysis (mentioned above) should be performed as independent validation. Given that these X-chromosome wide expression analyses have not been reported for RTT-hiPSC and their neuronal derivatives, we conclude that the methods employed to date on RTT-hiPSCs cannot unambiguously exclude the possibility of partial-XCI in either the post- or pre-XCI RTT-hiPSCs.

Finally, it is informative to investigate the XCI status of RTT-hiPSCs not only in their undifferentiated state but also upon differentiation. Pre-XCI RTT-hiPSCs should initiate XCI upon differentiation and exhibit a random mode of XCI revealed by the AR assay, accompanied by the upregulation of XCI marks such as *XIST* RNA and H3K27me3 (Dhara and Benvenisty, 2004; Hall et al., 2008; Silva et al., 2008; Dvash et al., 2010). Upon differentiation, X-linked genes should be expressed in a random monoallelic fashion (Dhara and Benvenisty, 2004) at the single cell level, which could be identified by RNA-FISH or IF. Differentiation of post-XCI RTT-hiPSCs, regardless of the presence or absence of XCI marks, will show a continued presence or absence of XCI marks, respectively, resulting in non-random XCI skewing and monoallelic expression pattern of X-linked genes (Hoffman et al., 1987; Hall et al., 2008; Shen et al., 2008; Silva et al., 2008; Liu and Sun, 2009; Dvash et al., 2010; Lengner et al., 2010; Tchieu et al., 2010; Cheung et al., 2011; Pomp et al., 2011; Diaz Perez et al., 2012). Altogether, the analysis of XCI upon differentiation of RTT-hiPSCs into neurons will be critical to conclusively demonstrate the isolation of pre-XCI RTT-hiPSCs in comparison to post-XCI RTT-hiPSCs.

PROS AND CONS OF POST- AND PRE-XCI RTT-hiPSCs

The generation of both post- and pre-XCI RTT-hiPSCs offers investigators two different cell types to study RTT (Marchetto et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011). Post-XCI RTT-hiPSCs are advantageous as they allow the generation of isogenic control and mutant RTT-hiPSCs from the same patient (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011). For disease phenotyping, healthy control hiPSCs are essential and isogenic cells from the same patient eliminate genetic background effects that exist between individuals that may have an affect on subsequent phenotyping. Furthermore, isogenic RTT-hiPSCs may respond to directed differentiation cues in a more uniform manner compared to hPSCs generated from different individuals (Osafune et al., 2008; Hu et al., 2010). Directed differentiation of post-XCI RTT-hiPSCs will yield homogeneous cultures of neurons that express either WT or mutant MECP2 allowing simpler analysis of a population of cells without influence from the opposite allele being expressed as in pre-XCI RTT-hiPSCs (Cheung et al., 2011). Finally, the generation of isogenic control and mutant RTT-hiPSCs allow the mixing and matching of WT and mutant expressing cells in different proportions which provides an opportunity to study the effects of XCI skewing as observed in RTT patients (Archer et al., 2007). Furthermore, it will also allow the mixing and matching of different cell types such as neurons and glia to study the non-cell autonomous effects of non-neuronal cell types in RTT as has become apparent in the recent RTT literature (Ballas et al., 2009; Maezawa et al., 2009; Maezawa and Jin, 2010; Lioy et al., 2011).

However, there are also potential disadvantages to post-XCI RTT-hiPSCs. From a biological standpoint, one could question whether post-XCI RTT-hiPSCs are fully reprogrammed given that the Xi did not reactivate. This is likely in part due to suboptimal culture conditions and reprogramming methods required to capture and stabilize RTT-hiPSCs in a pre-XCI state (Nichols and Smith, 2009; Hanna et al., 2010; Lengner et al., 2010; Diaz Perez et al., 2012). Although post-XCI RTT-hiPSCs have been shown to exhibit non-random monoallelic expression of MECP2, the possibility that they have partial-XCI cannot be excluded (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011). Additionally, it is important to note that although isogenic control and mutant post-XCI RTT-hiPSCs are described as *isogenic*, they are not truly isogenic by the strictest definition as illustrated by the following two points. First, all post-XCI RTT-hiPSCs generated to date were reprogrammed by integrating viral vectors (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011). Given that each RTT-hiPSC line is expected to be an independent clone, they will harbor unique vector integration sites. Therefore, methods of reprogramming such as non-integrating viral vectors (Stadtfeld et al., 2008b; Seki et al., 2010), non-replicative episomal vectors (Yu et al., 2009), and/or mRNA- (Warren et al., 2010), miRNA- (Anokye-Danso et al., 2011; Miyoshi et al., 2011), or protein- (Kim et al., 2009) based reprogramming methods will be required to overcome this limitation. Secondly, the process of reprogramming itself leads to the accumulation of diverse abnormalities in the genome of hiPSCs (Mayshar et al., 2010; Gore et al., 2011; Hussein et al., 2011; Laurent et al., 2011; Lister et al., 2011). Therefore, each hiPSC line will carry unique abnormalities that may affect the subsequent phenotype of each hiPSC line. It is thus imperative to study more than one hiPSC line per individual, affected and/or unaffected, to ensure the validity of any phenotypes.

Pre-XCI RTT-hiPSCs are advantageous because their differentiation initiates random XCI allowing generation of a mosaic culture of neurons expressing WT or mutant MECP2 (Marchetto et al., 2010; Kim et al., 2011). This provides a clinically relevant system as RTT patients are mosaic with respect to WT and mutant MECP2 expression. However, such a mosaic culture may introduce a new

variable as one needs to ensure that all differentiation experiments yield similar XCI skewing ratios. Furthermore, the mosaic culture creates difficulty when interpreting results, as any population analysis reads out both WT and mutant MECP2 expressing cells. One needs to perform single cell analysis and visualize WT and mutant cells separately, such as by using C-terminus specific antibodies of MECP2 that will not detect truncation mutations (i.e., 1155del32; Marchetto et al., 2010). However, this may be difficult on the more common point mutations of *MECP2* due to the lack of mutation-specific antibodies (Percy et al., 2007; Marchetto et al., 2010; Cheung et al., 2011). Similar to the case of post-XCI RTT-hiPSCs, although pre-XCI RTT-hiPSCs have been shown to exhibit biallelic expression of MECP2, it cannot be excluded that pre-XCI hiPSCs may have partial-XCI (Marchetto et al., 2010; Kim et al., 2011). Nevertheless, Muotri and colleagues have extensively studied RTT phenotypes in pre-XCI RTT-hiPSC-derived neurons, and reported defects in glutamatergic synapse numbers, spine density, soma size, calcium signaling, and electrophysiological properties (Marchetto et al., 2010). Interestingly, Park and colleagues found a defect in neuronal differentiation from pre-XCI RTT-hiPSCs (Kim et al., 2011), which is novel in comparison to the RTT literature (Bauman et al., 1995; Chen et al., 2001; Kishi and Macklis, 2004; Marchetto et al., 2010; Ananiev et al., 2011) and warrants further investigation. Reassuringly, this phenotype was also observed in their mutant post-XCI RTT-hiPSCs (Kim et al., 2011). Both these studies demonstrate the potential of using pre-XCI RTT-hiPSCs for disease modeling (Marchetto et al., 2010; Kim et al., 2011).

IMPACT OF XCI IN OTHER DISEASES

XCI in female hiPSCs has implications in other X-linked diseases including Fragile X Syndrome (*FMR1*), α-thalassemia (*ATRX*), Coffin-Lowry Syndrome (*RSK2*), DMD (*DYSTROPHIN*), Lesch-Nyhan Syndrome (*HPRT*), and Wiskott Aldrich Syndrome (*WASP*). In fact, many mental-disorder related genes are concentrated on the X-chromosome (Skuse, 2005; Gecz et al., 2009). DMD-hiPSCs have been generated and were shown to be post-XCI in a non-random pattern allowing the generation of isogenic control and mutant hiPSCs (Tchieu et al., 2010). HiPSCs have also been generated from Fragile X Syndrome patients although they were from male patients (Urbach et al., 2010). Perhaps more intriguing is the possibility of generating isogenic control and mutant post-XCI hiPSCs from carriers of X-linked diseases who otherwise may be apparently healthy. This will be particular advantageous in cases in which the proband is a male but has an unaffected mother and/or sisters who are carriers. Finally, the generation of post-XCI hiPSCs poses a unique opportunity for cellular therapy as a mosaic population of cells from a female patient can be reprogrammed into post-XCI hiPSCs in which hiPSCs can expressing the WT allele be used as a source for normal cells for cellular therapy purposes (Tchieu et al., 2010).

In the case of autosomal disorders, although XCI would not affect the primary gene of interest, female post-XCI hiPSCs generated from these patients may have a completely different X-chromosome transcriptome as each hiPSC line will carry either a paternal or maternal Xa. Therefore any heterozygous X-linked genes will be differentially expressed. It may be important to screen post-XCI hiPSCs, generated from female patients carrying an autosomal disorder, as well as unaffected females, by the AR assay to ensure that multiple post-XCI hiPSC lines used for downstream applications carry the same parental Xi.

CONCLUSION AND FUTURE REMARKS

The generation of hiPSCs from RTT patients represents an inexhaustible source for *in vitro* derived patient-specific neurons, assuming that RTT-hiPSCs can be expanded indefinitely with a normal karyotype and stable genome. These neurons are useful for investigating the pathogenesis of RTT and have potential for use in drug screens and identification of novel compounds for therapy (Marchetto et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Pomp et al., 2011). For this potential to be realized, efficient protocols that direct differentiation into adult stage neurons of defined subtypes may be required (Hansen et al., 2011). The X-linked nature of *MECP2* has prompted the extensive investigation of the XCI status of RTT-hiPSCs. Both post- and pre-XCI RTT-hiPSCs have been reported each with their own advantages and disadvantages for subsequent use. However, given the variation in XCI status seen in RTT-hiPSCs, it is imperative to accurately evaluate the XCI status in hiPSCs generated from RTT patients. Indeed, the XCI status of hiPSCs also has huge impact on other X-linked diseases, unaffected females who may or may not carry X-linked mutations, and perhaps also autosomal diseases.

To accurately evaluate the XCI status of RTT-hiPSCs, we propose the following workflow. RTT-hiPSCs should be screened first using the AR assay to distinguish potential pre-XCI RTT-hiPSCs that will show no peaks from potential post-XCI RTT-hiPSCs that retain an Xi as revealed by one peak. The AR assay is advantageous as a screening method given its relative technical ease to perform on a large number of samples. To confirm the candidate pre-XCI RTT-hiPSCs identified by the AR assay retain two Xa, allele-specific expression analysis of MECP2 by cDNA sequencing, qRT-PCR, RNA-FISH, or IF in an allele-specific manner will show a biallelic expression pattern. XCI marks will be absent as revealed by *XIST* RNA-FISH or H3K27me3 IF. It is important to differentiate candidate pre-XCI RTT-hiPSCs into neurons to demonstrate random XCI, as revealed by two equal peaks in the AR assay, accompanied by upregulation of XCI marks. Allele-specific expression analysis, preferably by RNA-FISH or IF at the single cell level, will yield a random monoallelic expression of MECP2. Only pre-XCI RTT-hiPSCs that meet these criteria will generate the desired unskewed mix of neurons expressing either the WT or mutant MECP2 allele. As these bona fide pre-XCI RTT-hiPSCs are passaged, it will be important to continue to assess their XCI status periodically, by performing AR assays for example, to ensure they are not transitioning into post- or partial-XCI RTT-hiPSCs.

The candidate post-XCI RTT-hiPSCs identified by presence of one peak in the initial AR assay could be divided into two groups with alternative parental X-chromosomes inactivated based on the different peak size of the AR assay. This will ensure that mutant and isogenic control RTT-hiPSCs can be isolated, and will facilitate identification of RTT-hiPSCs that express the X_u if the starting fibroblasts have been extensively passaged. Allele-specific MECP2 expression analysis of post-XCI RTT-hiPSCs will show non-random monoallelic expression and identify isogenic control

and mutant RTT-hiPSCs. Post-XCI RTT-hiPSCs will be either positive or negative for XCI marks as revealed by *XIST* RNA-FISH or H3K27me3 IF. Differentiation of post-XCI RTT-hiPSCs will produce neurons that maintain the non-random XCI skewing pattern in AR assays and non-random monoallelic expression pattern of MECP2 by allele-specific expression analysis by RNA-FISH or IF at the single cell level. These post-XCI RTT-hiPSCs should also be subjected to XCI analyses periodically upon passaging, for example by AR assays, to ensure they do not transition into partial-XCI that have reactivated large regions of the X-chromosome.

The possibility that reported post- and pre-XCI RTT-hiPSCs may in fact exhibit partial-XCI has not yet been tested. Both post- and pre-XCI RTT-hiPSCs do however recapitulate the expected neuronal maturation and electrophysiological defects indicative of RTT. To fully classify XCI in the RTT-hiPSCs, it would be informative to perform X-chromosome wide expression analysis using microarrays. Nevertheless, the impact of a partial-XCI state on disease phenotyping *in vitro* remains to be determined.

Finally, to facilitate the isolation and stable maintenance of pre-XCI hPSCs that carry two Xa and are capable of initiating random XCI upon differentiation, it is important to continue to identify the optimal culture conditions that can capture this naïve state (Nichols and Smith, 2009). Efforts are underway to try to capture this pristine state of hPSCs by modifying oxygen concentrations, addition of exogenous transcription factors, small molecules, and/or using the 2i regimen, in which the latter has been successfully applied in the mouse system (Ying et al., 2008; Buecker et al., 2010; Hanna et al., 2010; Lengner et al., 2010; Diaz Perez et al., 2012). Generation of pre-XCI hPSCs will provide an invaluable *in vitro* system to study XCI in humans which will be essential as the mouse may be not be quite as similar as once thought (van den Berg et al., 2009, 2011; Fan and Tran, 2011; Okamoto et al., 2011; Minkovsky et al., 2012).

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Rett syndrome: genes, synapses, circuits, and therapeutics

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Development of the nervous system proceeds through a set of complex checkpoints which arise from a combination of sequential gene expression and early neural activity sculpted by the environment. Genetic and environmental insults lead to neurodevelopmental disorders which encompass a large group of diseases that result from anatomical and physiological abnormalities during maturation and development of brain circuits. Rett syndrome (RTT) is a neurological disorder of genetic origin, caused by mutations in the X-linked gene methyl-CpG binding protein 2 (MeCP2). It features a range of neuropsychiatric abnormalities including motor dysfunctions and mild to severe cognitive impairment. Here, we discuss key questions and recent studies describing animal models, cell-type specific functions of methyl-CpG binding protein 2 (MeCP2), defects in neural circuit plasticity, and attempts to evaluate possible therapeutic strategies for RTT. We also discuss how genes, proteins, and overlapping signaling pathways affect the molecular etiology of apparently unrelated neuropsychiatric disorders, an understanding of which can offer novel therapeutic strategies for a range of autism spectrum disorders (ASDs).

Keywords: RTT, visual cortex, synapse, plasticity, development

INTRODUCTION

Rett syndrome (RTT) [**O**nline **M**endelian **I**nheritance in **M**an (OMIM) #312750], first reported by the Austrian physician Andreas Rett, is a monogenic, postnatal developmental disorder that affects normal brain development during early childhood in females, with an incidence of 1 in 10,000–15,000 live births (Rett, 1966; Chahrour and Zoghbi, 2007). Patients with RTT appear to reach developmental milestones seemingly normally until about 6–18 months after birth when signs and symptoms of the disease begin to appear. This typically includes severe mental retardation, stereotypic hand movements, motor coordination deficits, epileptic seizures, language and learning disabilities, and mild to severe cognitive impairments (Hagberg et al., 1983; Nomura, 2005; Chahrour and Zoghbi, 2007). Other clinical hallmarks include ataxia, spasticity, respiratory abnormalities, and autonomic dysfunction (Williamson and Christodoulou, 2006). Loss-of-function mutations in the *MECP2*, which encodes a transcriptional regulator and epigenetic modifier MeCP2, have been found to be the primary genetic component in 90% of patients suffering from RTT (Lewis et al., 1992; Amir et al., 1999; Guy et al., 2011). Interestingly, only one copy of *MECP2* is active in females due to random X-chromosome inactivation (Adler et al., 1995). RTT is not a heritable disorder and most mutations in *MECP2* arise *de novo* in germ cells, usually on the paternal side (Trappe et al., 2001). Growing evidence implicates alteration of *MECP2* expression in the etiology of several other neuropsychiatric disorders including Angelman syndrome, childhood schizophrenia, and congenital encephalopathy in boys (Schule et al., 2008). Originally, RTT was considered to be a disorder of early postnatal life; however, one recent study has shown that inducible deletion of *MECP2* in adults recapitulates the germ-line knock out phenotype in mice (McGraw et al., 2011).

The link between *MECP2* and RTT is fascinating because RTT is one of a small group of ASDs that gives us the opportunity to study mutations in a single gene and how they affect sequential phenotypic checkpoints of brain development leading to neuropathological endpoints in psychiatric disorders (Ben-Ari and Spitzer, 2010). In this review, we will first briefly discuss RTT and its genetic basis and the role of MeCP2 in brain development and plasticity. Second, we will provide a detailed description of mouse models of RTT and discuss their use to study molecular, synaptic, and circuit pathophysiology and to test novel therapeutic approaches to reverse neurological deficits. Finally, we will examine how overlapping signaling pathways affect the molecular etiology of apparently unrelated neuropsychiatric disorders like Fragile-X syndrome (FXS) and tuberous sclerosis (TSC) and how such an understanding can be utilized to design novel therapeutic strategies.

MECP2: MULTI-FUNCTIONAL GLOBAL REGULATOR WITH A LOCAL FUNCTION?

The human *MECP2* gene consists of four exons resulting in expression of two protein isoforms due to alternative splicing of exon 2. These splice variants differ only in their N-termini, and include the more abundant MeCP2-e1 isoform (encoded by *MECP2B* or *Mecp2α* in mice) as well as the MeCP2-e2 isoform (encoded by *MECP2A* or *Mecp2β* in mice; Mnatzakanian et al., 2004; Kriaucionis et al., 2006). Gene expression studies show that different brain regions are enriched with different splice variants; MeCP2-e2 is prevalent in dorsal thalamus and layer 5 of the cortex while MeCP2-e1 is detected in the hypothalamus (Dragich et al., 2007). Recent results suggest that MeCP2-e2 isoform is upregulated in Aβ-treated cortical neurons and promotes neuronal death in post-mitotic neurons, a pathway normally inhibited by forkhead protein FOXG1 (Dastidar et al., 2012). Furthermore, *MECP2* has a long conserved 3′UTR which contains multiple poly-adenylation sites, which can additionally generate four different transcripts.

MeCP2 is a highly conserved basic nuclear protein, initially found to be associated with methylated DNA (5′ CpG islands) regulating gene silencing and chromatin remodeling (Nan et al., 1993; Hendrich and Bird, 1998). MeCP2 is also an "intrinsically disordered" protein with long stretches of unorganized segments, without standard three-dimensional secondary structure, proposed to participate in the formation of a flexible scaffold required for multiple biological interactions (Adkins and Georgel, 2011). The genome-wide distribution of MeCP2 has consequences for global epigenetic state. While MeCP2 is expressed in various tissues throughout the body, including lung and spleen, it is abundant in mature, post-mitotic neurons in the adult brain. Expression of neuronal MeCP2 in mice is developmentally regulated. Initially, MeCP2 expression is high during embryonic development, with a low level at birth during neuronal maturation and synaptogenesis. This is followed by a consistent increase over the first three postnatal weeks reaching a plateau with a subsequent increase later in adult life (Shahbazian et al., 2002b; Kishi and Macklis, 2004). The role of MeCP2 in the development and maturation of the nervous system compared to the maintenance of adult neurons is not yet fully understood.

Structurally, MeCP2 is known to have two important functional domains: methyl-CpG binding domain (MBD) and the transcriptional repressor domain (TRD; Nan et al., 1993, 1997). There is also an additional C-terminal nuclear localization signal (NLS) which helps traffic the protein into the nucleus. One way to analyze MeCP2 function is to identify partner proteins which interact with these defined protein structural domains. The MBD specifically binds to methylated CpG di-nucleotides in DNA and also to unmethylated four-way DNA junctions suggesting a role for MeCP2 in regulating higher-order chromatin structure (Galvao and Thomas, 2005). The TRD is involved in transcriptional repression via the recruitment of transcriptional co-repressors and chromatin remodeling proteins. MeCP2's role as transcriptional repressor has been suggested based on the observation that MeCP2 specifically inhibits transcription from methylated promoters (Nan et al., 1997) while TRD recruits co-repressors like SIN3A and histone deacetylases (HDACs) 1 and 2 causing global compaction of chromatin by promoting nucleosome clustering. There is also evidence that MeCP2 through its TRD binds to Y-box binding protein-1 (YB1), a protein implicated in transcriptional activation regulation of splicing in reporter constructs (Young et al., 2005). Although precise protein–protein interaction mechanisms and consequences are yet unknown, it is clear that MeCP2 plays an important role in globally regulating chromatin structure and transcription of its immediate downstream targets. The abundance of MeCP2 and its binding sites in the brain makes it an unlikely candidate to be a gene-specific classical transcriptional regulator. It is possible that specificity of MeCP2 function can be significantly controlled post-translationally in a cell-type specific manner (Cohen et al., 2011). Our knowledge of the set of neuronal MeCP2 target genes is incomplete, however, such knowledge would be of great benefit in determining the molecular basis of pathology and for potential therapeutic measures.

MOUSE MODELS FOR RTT

To understand the detailed mechanism of RTT, several animal models have been generated (Calfa et al., 2011b). MeCP2 null and conditional mutant mouse models with cell-type or area-specific loss of MeCP2 in the brain show phenotypic features that resemble some features of RTT patient symptoms. All these models have been generated by mutating the mouse endogenous *Mecp2* gene or by the introduction of the human *MECP2* gene with a representative RTT mutation (see **Table 1**). These diverse approaches have produced mouse lines with gene products that range from a complete absence of the protein (Guy et al., 2001) to expression of a truncated but non-functional MeCP2 protein (Chen et al., 2001; Shahbazian et al., 2002a; Goffin et al., 2012). In addition, mouse models have been created with germ-line or conditional deletions upon recombination with different tissue, cell-type, or developmental stage specific Cre-lines (Chen et al., 2001). Behavioral face validity of these mouse models, or how well they recapitulate the usual RTT symptoms, is varied. Several of the mouse models show symptoms common to those present in RTT patients such as stereotypies, ataxia, akinesia, and breathing irregularities. Nevertheless, behavioral phenotypes, specifically anxiety and socialization are more heterogeneous across lines (**Table 1**). This behavioral heterogeneity can be attributed to variability in mouse line genetic backgrounds and the levels of expression of the MeCP2 protein due to the type of mutation. Social choice paradigms such as the social approach and social recognition tests show different results when comparing common inbred strains. Some strains (C57BL/6J, FVB/NJ, AKR/J) show a high preference for socializing with new stranger animals, whereas other strains show low preference or even direct avoidance (A/J, BALB, 129S1). Similarly the time they spend in the closed arms of the plus-maze test, a measurement that positively correlates with the anxiety level of the animal, is also variable. There seems to be an association between higher degree of anxiety and reduced social interest so mouse background has to be taken into account when comparing behavior phenotypes (Moy et al., 2007, 2008, 2009). Interestingly, the recent literature has started to address whether different translation types and levels of MeCP2 lead to variation in the anxiety and social phenotype: the complete lack of protein might produce a stronger phenotype with less anxiety-like behavior whereas a truncated protein could generate mouse lines with increased stress (Chao and Zoghbi, 2012). Understanding the correlation between functional interaction of the different *Mecp2* transcripts produced by these models with other anxiety-related genes like corticotrophin releasing hormone (*Crh*) will be capital, not only to explain the mice phenotypic variance but for clinical applicability (De Filippis et al., 2010; Kerr et al., 2010; Goffin et al., 2012).

RTT AS A DISORDER OF SYNAPTIC AND NEURAL CIRCUIT MATURATION

In many RTT patients and in mouse models of RTT, *MECP2* is absent in nearly half (female heterozygous) or every cell (male hemizygous) throughout the body. However, experiments with targeted brain-specific deletion of *Mecp2* (using Nestin-cKO mice) showing similar phenotype as that of Mecp2 null in all tissues support a crucial role for brain-specific *Mecp2* defects in the

Table 1 | Molecular, physiological, defects and behavioral abnormalities found in various mouse models of RTT.

Mouse model	Molecular/signaling	Behavior	Synaptic transmission/plasticity
***Mecp2*$^{tm1.1Bird}$ (Guy et al., 2001)** Deletions spanning exons 3 and 4, starting in early embryonic development **No detectable protein**	**TH** decrease at P55 in substantia nigra pars compacta (Panayotis et al., 2011) **NMDA** receptors expression significantly decreased from 2 to 7 weeks in several areas (Blue et al., 2011) **PSD95** and **Glur6/7** decrease in primary culture **GABA** decrease in brainstem (Medrihan et al., 2008; Maezawa and Jin, 2010)	***Anxiety behavior*** 5-week-old male mice spent more time in the open arms (Kerr et al., 2010) No anxiety in 4 week males and heterozygous females (Santos et al., 2007) ***Motility*** Decreased motility (Panayotis et al., 2011). Diminished balance from 5 weeks with weekly trials (Pratte et al., 2011) ***Social approach*** No social deficits. Higher interaction with a second stranger (Kerr et al., 2008)	Hippocampus CA1 and CA3: ↓ LTP and ↓ LFS–LTD (Asaka et al., 2006)
***Mecp2*^{T158A} (Goffin et al., 2012)** Missense point mutation at aa158 that substitutes a threonine for Alanine in the MBD region **Truncated protein**	NA	***Anxiety behavior*** Less degree of anxiety as compared to wildtypes ***Motility*** Reduced locomotor activity at 9 weeks. Decreased coordination and deficits in motor learning. ***Learning*** Deficits in context and cued (auditory) learning (Goffin et al., 2012)	NA
***Mecp2*^{2loxJaenisch} (Chen et al., 2001)** Deletions of Mecp2 exon 3 including most of the MBD but an intact C-terminus **Truncated protein**	**PSD95** in motor cortex decreases (Tropea et al., 2009) **PSD95** and **CamKII** mRNA is reduced. **Akt/mTOR** pathway activity reduced (Ricciardi et al., 2011) Decreased **NGF, IGF, BDNF** expression (Schaevitz et al., 2010)	***Anxiety behavior*** 8-week-old males and heterozygous females spent more time in open arms (Stearns et al., 2007) ***Motility*** Decreased total motility (Tropea et al., 2009) ***Social behavior*** Increased social approach. They also lost interest more quickly. Spent more time in close proximity to stranger mouse than WTs. Hemizygous males had a preference for close proximity contact (Schaevitz et al., 2010)	S1: ↓ TBS induced LTP Lonetti et al. (2010)

Continued

Table 1 | Continued

Mouse model	Molecular/signaling	Behavior	Synaptic transmission/plasticity
***Mecp2*^{308} (Shahbazian et al., 2002a)** Premature STOP codon at aa308 generates a truncated protein with conserved MBD and a portion of TRD **Truncated protein**	**CRH** increase in paraventricular nucleus of hypothalamus, central amygdala, and stria terminalis Shahbazian et al. (2002a), McGill et al. (2006) **ChAT** levels decreased in striatum. **NGF** protein levels in hippocampus upregulated (Ricceri et al., 2011)	***Anxiety behavior*** Null male mice (60 days old) spent less time in the open parts of zero maze (De Filippis et al., 2010) ***Motility*** 60 days old null male mice had less activity in dark but same activity in light (De Filippis et al., 2010). Null male mice had less total activity at night but higher fine movement activity in light (24 h test). No differences in rotarod performance (Moretti et al., 2005) ***Social behavior*** Less interaction with stimulus mouse (Shahbazian et al., 2002a; Moretti et al., 2005) ***Learning*** Not altered contextual or cued (sound) learning in 3–5 months old mice Contextual learning deficits in males (not cued learning tested; Moretti et al., 2006)	M1 and S1, L2/3: ↓ TBS induced LTP (late; Moretti et al., 2006) Hippocampus: ↓ LTP, ↓ LTD ↑ Basal transmission Moretti et al. (2006)
CaMKII-*Mecp2* (Chen et al., 2001) Cell-specific knock out of Mecp2	NA	***Motility*** No locomotor deficits but worse performance in rotarod ***Anxiety behavior*** Increased anxiety ***Social behavior*** Decreased interaction with stimulus animal ***Learning*** Impaired cue-dependent fear conditioning (Gemelli et al., 2006)	NA
Viaat-*Mecp2* (Chao et al., 2010) Cell-specific knock out of Mecp2	Decreased levels of **GAD1**, **GAD2**, and **GABA** levels (Chao et al., 2010)	***Motility*** Less motility and worse performance in rotarod ***Anxiety behavior*** Increased anxiety when measured with open field but not in plus maze ***Social behavior*** Increased interaction with stimulus animal ***Learning*** Similar rates of learning but had difficulties locating the platform in Morris water maze paradigm (Chao et al., 2010)	S1: ↓ Inhibitory quantal size (mIPSCs) ↓ Amplitude and charge but not in frequency No change in mEPSCs SC-hippocampus: no change in PPR and I–O curve Impaired LTP Chao et al. (2010)

***Mecp2*^{Tg1} (Collins et al., 2004)** Introduction of human *MECP2* using artificial chromosome	**VGLUT** and **PSD95** over-expression (Chao et al., 2007)	***Motility*** Increased time to start crossing in dowel test.but increased performance in the rotarod at 20 weeks ***Anxiety behavior*** No anxiety phenotype ***Learning*** Impaired context-dependent fear conditioning Collins et al. (2004)	Hippocampus, CA1: ↑ LTP (Collins et al., 2004) ↑ PPF (Collins et al., 2004)

Table 1*. Molecular, physiological defects and behavioral abnormalities found in various mouse models of RTT. For detailed description and abbreviations, please see the text.*

pathogenesis and profound neurological phenotype of RTT (Chen et al., 2001; Guy et al., 2001). Autopsy studies on post-mortem human brain shows an overall decrease in the size and reduction in weight and volume of the brain (Armstrong, 2005). However, the RTT brains does not show any obvious sign of degeneration, atrophy, or cell death.

STRUCTURAL CHANGES

Although there is no gross structural change in RTT brain, mouse models of RTT show delayed neuronal maturation and synaptogenesis (Fukuda et al., 2005). Several recent studies have shown functional, morphological, and molecular alterations in different mouse models of RTT. At the cellular level, neuronal soma size is reduced in the absence of MeCP2 with increased packing density (Armstrong, 2005). Synaptic structural and morphological defects include reduced dendritic branching, spine density, and reduced spine morphology (Kishi and Macklis, 2004; Belichenko et al., 2009). Presynaptically, loss of MeCP2 affects the number of axonal boutons and axonal arborization in general and their targeting (Belichenko et al., 2009), which suggests a decrease in the number of synapses in RTT brains. However, in line with the structural changes, many subtle alterations in synaptic transmission lead to the neuronal maturation defects that impact circuit-level plasticity in these mouse models of RTT.

EXCITATORY: INHIBITORY SYNAPTIC BALANCE

Analysis of spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs respectively) in MeCP2 KO mice indicates a change in the excitatory/inhibitory (E/I) balance as revealed by increased excitatory and reduced inhibitory synaptic transmission in the hippocampus and cortex (Dani et al., 2005; Nelson et al., 2006; Chao et al., 2007), whereas E/I balance from *Mecp2*^{Tg1} (with human MeCP2 over-expressing mice) shows the opposite effect (Collins et al., 2004). Whole-cell patch-clamp recordings from thick-tufted layer 5 pyramidal neurons in primary somatosensory cortex (S1) show that spontaneous EPSC and spontaneous action potential firing are reduced in MeCP2 KO male mice (Tropea et al., 2009). Additionally, quadruple whole-cell patch-clamp recordings from layer 5 pyramidal neurons of four-week-old MeCP2 KO mouse slices show that excitatory synaptic connectivity is also reduced with weakening of individual connections (Dani and Nelson, 2009). The evidence for functional deficits supported by immunofluorescence staining of PSD95 in MeCP2 KO mice showing a significant reduction in layer 5 neurons in motor cortex (M1; Tropea et al., 2009). On the other hand, extracellular field EPSPs recorded from layer 2/3 with evoked stimulation in layer 4 in primary sensory and motor areas are unaffected by the expression of a truncated MeCP2 protein (Moretti et al., 2006). Lastly, inhibitory synaptic transmission might also be altered in mouse models of RTT, as evidence indicates that there are both pre- and postsynaptic defects of GABAergic neurotransmission in the brainstem (Medrihan et al., 2008). Developmentally, MeCP2 deficiency might have a distinct developmental effect on circuit-level function. Initial phases of synaptic development and pruning are normal at retino-geniculate synapses [from postnatal day (P)9–21 in MeCP2 KO mice compared to wild-type (WT) control], whereas the circuit becomes abnormal and immature in

a subsequent experience-dependent phase (P27–34; Noutel et al., 2011).

PLASTICITY DEFECTS

Several studies have shown that long-term potentiation and depression (LTP and LTD respectively), cellular mechanisms of long-term synaptic plasticity and learning and memory, are impaired in MeCP2 KO animals. LTP evoked by theta-burst stimulation in L2/3 of S1 is reduced in magnitude in eight-week-old MeCP2 KO mice compared to wild-type controls, an impairment that can be reversed by environmental enrichment (Lonetti et al., 2010). Also, LTP is impaired in hippocampal Schaffer's collateral (SC)–CA1 synapses despite basal synaptic transmission being intact (Asaka et al., 2006). Hippocampal slices from adult MeCP2 KO mice show a clear decrease in the magnitude of EPSP with high frequency stimulation (HFS) but not with more physiological theta-burst stimulation (TBS). Interestingly, these experiments reveal a decrease only in the maintenance phase and not in the induction phases of LTP. Considering that distinct protein machinery and signaling pathways are involved in the induction and maintenance phase of LTP, it is possible that MeCP2 KO animals have selective impairment in plasticity induction. In contrast to HFS and TBS, a more physiologically plausible plasticity paradigm studied between paired L5 cortical pyramidal neurons using spike-timing-dependent protocols revealed no change in LTP in MeCP2 KO mice (Dani and Nelson, 2009). However, in this case, fewer synaptically-coupled connections were found in MeCP2 KO mice and individual connections were weaker. This suggests that loss of *Mecp2* function reduces connectivity of excitatory synapses which precedes deficits in plasticity (Dani and Nelson, 2009). In support of this stance, a recent study using *in utero* injection of short hairpin RNA to knock down *Mecp2* and glutamate uncaging by laser-scanning photostimulation to map neocortical circuit found that *Mecp2* deficiency leads to significant reduction of mainly local excitatory input strength in superficial cortical layers (Wood et al., 2009). Along with LTP deficits, LTD, on the other hand, is also abolished in SC–CA1 synapses in adult MeCP2 KO animals (>6 weeks) with no obvious changes in 3–5 week old KO animals (Asaka et al., 2006). Furthermore, mouse model (MeCP2^{308}, expressing a truncated form of MeCP2 protein showed similar impairments in synaptic plasticity with reduced LTP and LTD at SC–CA1 synapses in mice at 5–6 months of age, and exhibits increased basal synaptic transmission and decreased paired-pulse facilitation (PPF; Shahbazian et al., 2002a; Moretti et al., 2006). Conversely, a mouse model with mild *Mecp2* over-expression (MeCP2^{Tg1}) shows an enhanced LTP with normal basal synaptic transmission (Collins et al., 2004).

The molecular composition of synaptic NMDA receptor subunits showed an interesting trend in these MeCP2 KO animals: GluN2B-to-GluN2A subunit switching, which regulates the channel kinetics and biophysical properties of excitatory synapses in the developing brain, shows a delayed postnatal maturation and may be responsible for the molecular pathology of synaptic defects in RTT (Asaka et al., 2006).

Recent evidence points to the hypothesis that the neurological deficits found in RTT arise from a recoverable failure of synaptic and circuit development in the brain (Tropea et al., 2009). A plausible hypothesis, complementary to that of reduced excitatory synapse maturation, is that the level or nature of inhibition is altered in mouse models of RTT, so that cortical circuits persist in an immature state. Consistent with this hypothesis, it has been shown that the potential to trigger visual cortical plasticity by closing one eye persists into adulthood in MeCP2 KO mice, well past the critical period for such plasticity in wild-type mice (Tropea et al., 2009).

ROLE OF MeCP2 IN HOMEOSTATIC SYNAPTIC SCALING

Synaptic scaling is a form of homeostatic plasticity in which average neuronal activity levels are modulated to allow for dynamic adjust of synaptic strength to promote stability of neuronal circuits (Turrigiano and Nelson, 2004). Recent evidence shows how MeCP2 mediates activity-dependent synaptic scaling in rat hippocampal cultures (Qiu et al., 2012). Increase in neuronal activity upon bicuculline treatment leads to an increased level of MeCP2 expression, which in turn binds to the GluR2 promoter and recruits a repressor complex to inhibit its expression and availability of these molecules at the synapse. Regulating AMPA receptor GluR2 subunit expression is one direct way to mediate an adaptive response that regulates synaptic strength and prevents recurrent circuit excitation.

INHIBITION AND RTT

Epilepsy is often seen in RTT patients and often difficult to treat (Steffenburg et al., 2001). Since most cases of RTT are caused by mutations in the *MECP2* gene, it is assumed that convulsions are based on genetic mechanisms, however, balance of excitation and inhibition is also believed to play a critical role in the progression of the disease during early development. A new study has looked at dysfunctions of neuronal and network excitability using a combination of voltage-sensitive dye imaging and electrophysiology in hippocampal slices from symptomatic *Mecp2* mutant animals (Calfa et al., 2011a). They found that CA1 and CA3 regions of the hippocampus are highly hyper-excitable and network excitability in CA3 may contribute to the hippocampal dysfunction and limbic seizures observed in *Mecp2* mutant mice and RTT patients.

Whereas the loss of MeCP2 is known to alter excitatory synaptic transmission and plasticity (Nelson et al., 2006), little is known about how MeCP2 regulates the development and plasticity of inhibitory GABAergic circuits and how they are altered in RTT. In a recent study, using Viaat-Cre/MeCP2$^{-/y}$ (vesicular inhibitory amino acid transporter) mouse model, it has been shown that loss of MeCP2 from a subset of forebrain GABAergic neurons also recapitulates many features of RTT (Chao et al., 2010). MeCP2-deficient GABAergic neurons show reduced inhibitory quantal size, consistent with a presynaptic reduction in glutamic acid decarboxylase 1 (*Gad1*) and glutamic acid decarboxylase 2 (*Gad2*) levels, and GABA immunoreactivity. However, in this study, the effect of *Mecp2* deletion from particular interneuron subclasses on inhibitory circuit plasticity *in vivo* has not been assessed.

GLIA AND RTT

Since its discovery, RTT has been regarded primarily as a neuropathophysiological disorder. This conclusion was mainly based on the finding that selective re-expression of *Mecp2* in postmitotic neurons (using neuron-specific *Tau* locus) is sufficient

to rescue RTT (Luikenhuis et al., 2004). However, recent studies have shown that MeCP2 in glial cells plays a very important role in neuropathology of RTT. Unlike previous reports, it is now clear that MeCP2 is also expressed in astrocytes and *in vitro* co-culture studies have shown that MeCP2 null astrocytes can have non-cell-autonomous effects on both wild-type and MeCP2 null neurons (Ballas et al., 2009). Interestingly, a recent study has shown that re-expression of *Mecp2*, preferentially in astrocytes, significantly improves several of the hallmark mouse behavioral phenotypes of RTT mice (locomotion, anxiety levels, respiratory abnormalities, and lifespan) compared to globally null mice (Lioy et al., 2011).

Microglia may also influence the onset and progression of RTT. Aberrant microglial activity has been found in mouse models of RTT. Elevated levels of glutamate, released from microglia, may cause abnormal stunted dendritic morphology, microtubule disruption, and damage of postsynaptic glutamatergic components making microglial glutamate synthesis or release a potential therapeutic target for RTT (Maezawa and Jin, 2010; Derecki et al., 2012).

MOLECULAR PATHOPHYSIOLOGY OF RTT AND THERAPEUTIC APPROACHES

Using mouse genetic models of RTT, key molecular signaling pathways that contribute to the deficits in synaptic function and maturation have been studied. Once identified, these mouse models have also been used to experimentally validate possible therapeutic avenues using genetic, pharmacological, and behavioral approaches. So far, three major approaches have been investigated: pharmacological treatment aimed to restore signaling pathway activity, supplementary diets and reinforcement therapies, and genetic manipulation that re-establish *Mecp2* gene expression.

MOLECULAR PATHOPHYSIOLOGY AND EFFECT ON SIGNALING PATHWAYS

The specific role of MeCP2 in transcription and translational control might vary depending on the different molecules recruited and protein–protein interactions. This complexity, for example, is shown in the regulation of one of the most important targets of MeCP2 in the central nervous system: brain-derived neurotrophic factor (BDNF). MeCP2 regulates BDNF expression by binding to promoter IV and repressing its transcription until MeCP2 is phosphorylated and released via a neuronal activity-dependent mechanism. This mechanism might explain the activity-dependent increase of BDNF, however it is unclear how it would cause the lower levels of this growth factor observed in RTT patients and mouse models when compared with healthy individuals or WT animals (Chang et al., 2006). A recent study (Abuhatzira et al., 2007) has suggested that BDNF protein down-regulation may be mediated by dis-inhibition of complex repressor REST/Co-REST. The translation of this complex is suppressed in the presence of MeCP2; in MeCP2 null mice the complex is overexpressed. The REST/Co-REST complex binds to a position between BDNF promoters I and II, located upstream of promoter IV, thereby overriding the direct effects of MeCP2 on the downstream promoter. BDNF is critical for neuronal development, synaptic maturation, and plasticity through the activation of specific neurotrophic tyrosine kinase receptor type 2 (TrkB), which, in turn, activates signal transduction pathways such as PLCγ, PI3K/Akt, and MAPK/ERK that regulate protein synthesis and neural function by activating PSD95 (Yoshii and Constantine-Paton, 2007). This pathway is of central importance to the expression and amelioration of the RTT phenotype. Similarly the PI3K-Akt-PSD95 pathway is known to drive the up-regulation of PSD95 by insulin (Lee et al., 2005), and this pathway is also stimulated by IGF-1 (Zheng and Quirion, 2004). Although there is not yet a clear picture of how cellular and molecular changes in RTT neurons in turn regulate their physiological properties, there is increasing evidence showing that the molecular signaling pathways and aberrant neuronal protein synthesis at the synapses are a possible explanation for several features in the pathology of RTT. The regulation of protein synthesis via the PI3K pathway has been proven to be crucial in synaptic function, dendrite structure, and plasticity (Jaworski et al., 2005; Kumar et al., 2005; Cuesto et al., 2011). All of these functions have been shown to be compromised in RTT (Belichenko et al., 2009; Noutel et al., 2011). Recently, direct evidence has demonstrated the dysregulation of the entire Akt/mTOR axis in MeCP2 null mice, giving a molecular theoretical framework for the mechanism of action of genes regulated by MeCP2, like BDNF (Ricciardi et al., 2011).

The importance of the PI3K pathway is reflected in the number of therapies designed for RTT that aim to restore its activity through the direct application or augmented endogenous synthesis of growth factors such as BDNF or IGF-1 (Tropea et al., 2009; Kline et al., 2010; Lonetti et al., 2010; Castro et al., 2011). These therapies target the tyrosine kinase receptors and hyper-activation of their subsequent downstream cascade that will cause increased protein synthesis that in the end impact synaptic maturation and function (Yoshii and Constantine-Paton, 2007).

GENETIC RESTORATION OF MeCP2

All evidence indicates that RTT has an extremely subtle pathophysiology compared to other severe neurodegenerative disorders. Genetic intervention and pharmacological treatments have been shown to rescue certain phenotypes of RTT, indicating that some deficits are reversible. Several genetic manipulation approaches have been tested for the reversal of behavioral impairments in RTT mouse models (see **Table 2** for a summary of the major findings). One recent unbiased, high-content, small molecule screen in primary cortical neurons derived from the Angelman syndrome mouse model revealed a possible new therapeutic avenue that may be applicable to RTT (Huang et al., 2012). This study showed inhibitors of topoisomerase I and II, enzymes that regulate DNA supercoiling, could be used to unsilence the dormant paternal copy of the *Ube3a* gene (the primary genetic cause of Angelman Syndrome) in several regions of the nervous system. The resulting unsilencing lead to the expression of a functionally normal Ube3a protein, the level of which remained elevated in a subset of spinal cord neurons weeks after drug treatment. These finding highlight a remarkable potential for a short-term treatment that could lead to long-term effects on gene expression and possible reestablishment of proper neuronal UBE3A function. Whether a similar high-throughput screen can identify compounds that can be used to unsilence and reactivate X-inactivated copy of *Mecp2* is an open question and an area of active research by several laboratories.

Table 2 | Current therapeutic approaches employing RTT mouse models.

Treatment type	Drug/therapy	Mouse model	Description
Genetic manipulations	Rescue by Mecp2 overexpresion in the tau (post-mitotic neuron) population	Jaenisch (male)	Mecp2 heterozygous females were crossed with males carrying an additional copy of Mecp2 expressed under Tau promoter Total body and brain weight increased and locomotor activity improvement (Luikenhuis et al., 2004)
	Mecp2 expression at different developmental stages	Jaenisch (male)	Crossing between Mecp2 heterozygous females carrying an additional copy of Mecp2 regulated with a loxP–STOP–loxP cassette with males expressing cre under embryonic (Tau and Nestin) or postnatal (CaMKII 93,159) expressing promoters Extended lifespan and locomotor activity improvements (Giacometti et al., 2007)
	Tetracycline depending expression of Mecp2	Bird (female)	Crossing between Mecp2 heterozygous females expressing tetracycline transactivator in the CamKII population and a Mecp2 male expressing the operator Improvement in total activity and movement speed. No improvements in rotarod (Jugloff et al., 2008)
	Rescue by BDNF over-expression	Jaenisch (male)	Crossing between Mecp2 heterozygous females expressing cre with males carrying an additional copy of BDNF regulated with a loxP–STOP–loxP cassette Increased lifespan, locomotor activity (6 week), and firing rate in pyramidal neurons Chang et al. (2006)
	Tamoxifen-induced re-expresion of Mecp2	FLOX-stop (male and female)	Tamoxifen-induced Mecp2 re-expresion in pre- (only male) and symptomatic (male and female) stages Extended lifespan and improvement of a phenotype observational score that encompassed movement, breathing, hind-limb clasping, and general condition (Guy et al., 2007)
Pharmacological treatment	Desipramine	Bird (male)	Norepinephrine reuptake blocker desipramine. Dose of 10 mg/kg intraperitoneally from PND 40 (Roux et al., 2007) or 0.25 mg/ml orally from PND 30–86 (Zanella et al., 2008) Both treatments prolonged lifespan and improved breathing patterns
	Ampakine (CX546)	Jaenisch (male)	A family of AMPA receptor allosteric modulator CX546 from PND 31–35 (40 mg/kg i.p.) Decrease of hyperventilation and breathing pattern irregularities (Ogier et al., 2007)
	BDNF	Jaenisch (female)	Small analog of BDNF that acts as TrkB agonist (LM22A-4) from week 8 to 13 in female mice in (50 mg/kg i.p.). Recovery of breathing frequency by increasing the expiratory time and total breath duration. Restore of Akt/ERK activation (Schmid et al., 2012) Direct application reversed synaptic dysfunction in brainstem slices (Kline et al., 2010)
	IGF-1	Jaenisch (male)	(1–3)IGF-1 (tripeptide fragment of IGF-1) injected daily (10 mg/kg i.p.) Improvements in locomotor function, breathing frequency, PSD95 reactivity, ocular dominance plasticity (Tropea et al., 2009)
Non-pharmacological	Environmental enrichment	Jaenisch (male)	Pre-symptomatic animals (P10 or P21) are assigned to an enriched environment consisting of several toys, platforms, and ladders changed weekly. Improvement in activity but not coordination or contextual or cued fear memory (Nag et al., 2009). Improvement of rotarod performance and space memory Increased expression of BDNF in the cortex and number of excitatory but not inhibitory synapses (Lonetti et al., 2010)
	Dietary choline supplement	Jaenisch (male and female)	Tests were conducted on the offspring from choline-treated mothers (50 mM in drinking water). Slight improvements in locomotor activity and motor coordination (Nag and Berger-Sweeney, 2007) Increased NGF level in the striatum and *N*-acetylaspartate content suggesting improving neuronal proliferation and survival (Ward et al., 2009)

Genetic, pharmacological, and non-pharmacological methods have been utilized on mouse models of RTT revealing avenues for potential therapy.

CONVERGENCE OF EVIDENCE FOR A SUBSET OF NEURODEVELOPMENTAL DISORDERS

Rett syndrome, Fragile-X syndrome (FXS), and TSC are examples of monogenic disorders that show compromised synaptic plasticity (Tavazoie et al., 2005; Moretti et al., 2006; Talos et al., 2008). Although these diseases have diverse genetic origin and phenotypes, they share common intermediates in the signaling pathways that will influence the availability of new proteins for functional and structural changes at the synapse. It is widely accepted that protein synthesis, including local protein synthesis at synapses, is required for several forms of synaptic plasticity (Sutton and Schuman, 2006). This local protein synthesis enables synapses to control synaptic strength independently of the cell body via rapid translation from pre-existing mRNAs and therefore, the mechanisms and the signaling pathways regulating translation are likely to be intimately involved in modulating synaptic strength (**Figure 1**). The PI3K/Akt/mTOR axis, ERK, and PKC γ,ζ signaling pathways have been shown to be heavily involved in controlling all the steps of the protein synthesis process (Lin et al., 1994; Beretta et al., 1996; Dufner et al., 1999; Hou and Klann, 2004) and are dysregulated at different levels in RTT, FXS, and TSC (Inoki et al., 2002; Manning et al., 2002; Gross et al., 2010; Ricciardi et al., 2011).

FXS gene product FMRP may play several roles not associated directly with protein translation like trafficking and half-life stability of mRNA, but its major function is to act as a brake of protein translation by attaching to poly-ribosomes through ERK-mediated phosphorylation signaling (Mazroui et al., 2002; Gallagher et al., 2004; Qin et al., 2005). The FXS mouse model, a knock out of *Fmr1* gene, is characterized by de-repression of protein translation but interestingly also has elevated PI3K/Akt/mTOR and ERK (Hou et al., 2006) activity levels through a negative feedback loop with PIKE (Gross et al., 2010; Sharma et al., 2010). *TSC1* and *2* gene products (Hamartin and Tuberin respectively) form a complex that are more directly implicated in the protein synthesis pathway as a downstream target of PI3K that in turn becomes activated upon the binding of growth factors (e.g., IGF or BDNF). Activated PI3K leads to the recruitment of PDK1 and the serine/threonine protein kinase Akt, and subsequent phosphorylation/activation of Akt by PDK1. Activated Akt negatively regulates TSC complex by directly phosphorylating TSC2.

It is thus remarkable that although they work in opposite directions (FXS and TSC by protein synthesis up-regulation and RTT through down-regulation), all of these syndromes share at least certain common signaling pathways and a pathophysiological point of convergence in the synapse (**Figure 1**), where tight regulation is necessary for proper function. This convergence opens up the possibility that these three disorders and maybe other ASDs

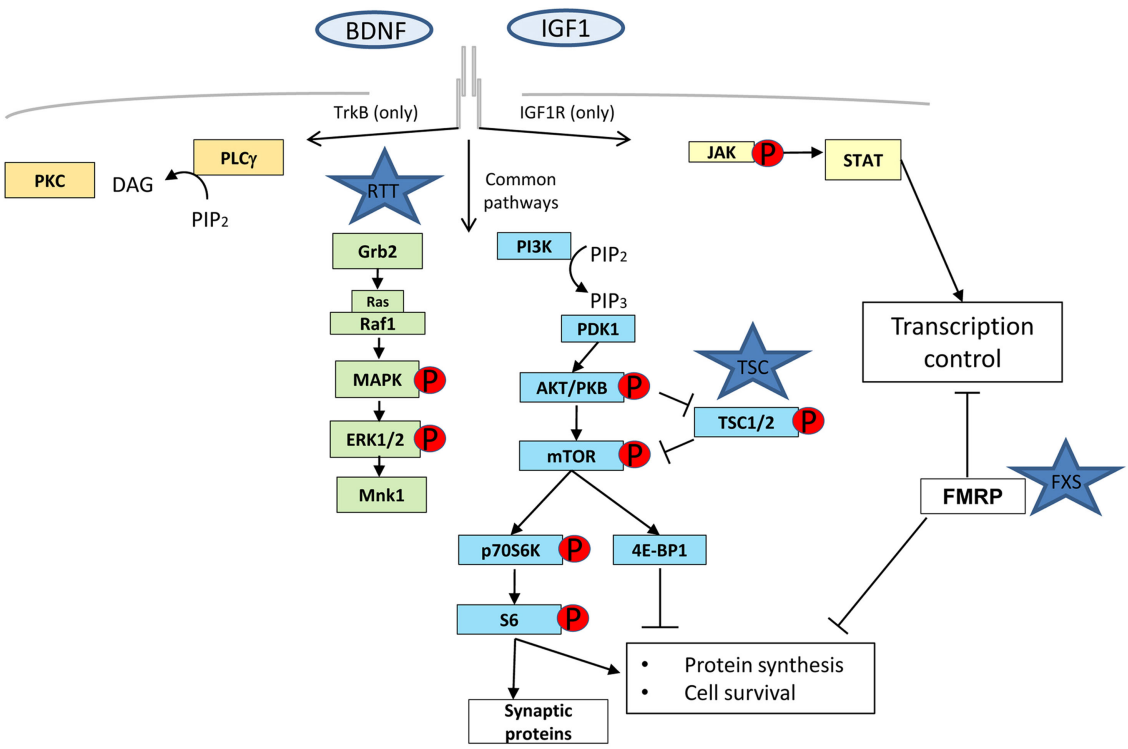

FIGURE 1 | The four major molecular pathways activated by tyrosine kinase receptor B (TrkB) and IGF-1 receptor (IGF-1R). Phospholipase gamma (PLCγ) in orange, mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) in green, Phosphatidylinositol-3 kinase (PI3K)/Akt in blue, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) in yellow. MAPK and PI3K are common transduction pathways for these two receptors whereas PLCγ is only specific for TrkB and JAK/STAT for IGF-1R. FMRP, although not directly involved in any signaling pathway is represented to show its influence in the different stages of protein synthesis: transcription, through mRNA stability control and translation.

(such as Angelman disorder with a disrupted protein synthesis control caused by a defective UBE3A) have related biological foundations which can be exploited in the design of new therapeutic strategies (Auerbach et al., 2011).

NEW AVENUES IN RTT RESEARCH

Almost two decades of research on RTT has led to the development of an intriguing story of how a single transcription factor can play a crucial role in neuronal development, synaptic maturation, and plasticity. Although the primary function of MeCP2 in normal brain development remains unclear, it is becoming increasingly evident that there is a complex interplay of genes and environment which results in the synaptic and circuit-level defects in brain function. Restoration of MeCP2 expression in three-week-old brain resulted in improvements in LTP and neuroanatomical parameters (Guy et al., 2007), illustrating that it is possible to improve the symptoms of this neuropsychiatric disorder. Disease models using induced pluripotent stem cells (iPS) from RTT patients' fibroblasts have opened up a new avenue of drug discovery for therapeutic treatment of RTT (Kim et al., 2011; Marchetto et al., 2011). Recent data also suggests that immune system, whether it is adaptive (T cells) or innate (microglia), profoundly impact normal brain function and plasticity (Derecki et al., 2010; Graeber and Streit, 2010; Tremblay et al., 2011). Therefore, bone marrow transplant from healthy animals into mutant *Mecp2* male animals is being investigated as an approach for amelioration of RTT symptoms (Derecki et al., 2012). Interestingly, several new functions of MeCP2 are only beginning to be understood including their role in dynamic genome regulation in neurons (Muotri et al., 2010). With the exciting new discoveries of genome-editing techniques using zinc-finger nucleases, an interesting new possibility would be to generate and use rat models to study RTT. Future studies with cell-type specific manipulation of MeCP2 to identify and examine the circuit-level contributions to function promise to elucidate further mechanisms of disease progression and provide new potential therapeutic targets for RTT.

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DISC1 pathway in brain development: exploring therapeutic targets for major psychiatric disorders

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Genetic risk factors for major psychiatric disorders play key roles in neurodevelopment. Thus, exploring the molecular pathways of risk genes is important not only for understanding the molecular mechanisms underlying brain development, but also to decipher how genetic disturbances affect brain maturation and functioning relevant to major mental illnesses. During the last decade, there has been significant progress in determining the mechanisms whereby risk genes impact brain development. Nonetheless, given that the majority of psychiatric disorders have etiological complexities encompassing multiple risk genes and environmental factors, the biological mechanisms of these diseases remain poorly understood. How can we move forward to our research for discovery of the biological markers and novel therapeutic targets for major mental disorders? Here we review recent progress in the neurobiology of disrupted in schizophrenia 1 (DISC1), a major risk gene for major mental disorders, with a particular focus on its roles in cerebral cortex development. Convergent findings implicate DISC1 as part of a large, multi-step pathway implicated in various cellular processes and signal transduction. We discuss links between the DISC1 pathway and environmental factors, such as immune/inflammatory responses, which may suggest novel therapeutic targets. Existing treatments for major mental disorders are hampered by a limited number of pharmacological targets. Consequently, elucidation of the DISC1 pathway, and its association with neuropsychiatric disorders, may offer hope for novel treatment interventions.

Keywords: DISC1, cerebral cortex development, genetic risk factors, major mental disorder, immune responses

INTRODUCTION

Disrupted in schizophrenia 1 (DISC1) was initially discovered at the breakpoint in a balanced chromosomal translocation t (1; 11) segregating with major mental conditions, such as schizophrenia, bipolar disorder, and major depression in a Scottish pedigree (Millar et al., 2000). Since then, accumulating evidence from genetic studies indicated that DISC1 is not only associated with schizophrenia and mood disorders, but also other psychiatric disorders of neurodevelopmental origin, such as autism, Asperger syndrome, and agenesis of the corpus callosum (Hennah et al., 2003; Hodgkinson et al., 2004; Callicott et al., 2005; Kilpinen et al., 2008; Song et al., 2008, 2010; Osbun et al., 2011). Although recent genome wide association studies (GWAS) have not found DISC1 as a key genetic risk factor for patients met the current diagnostic criteria for schizophrenia (Purcell et al., 2009; Stefansson et al., 2009; Mathieson et al., 2011), it is noted that variations of DISC1 influence anatomical and functional endophenotypes even in control subjects (Thomson et al., 2005; Di Giorgio et al., 2008; Prata et al., 2008; Tomppo et al., 2009b). Collectively, genetic variation of DISC1 may confer vulnerabilities to a wide range of neurodevelopmental psychiatric conditions by affecting brain maturation, thereby modifying brain function.

Consistently, extensive biological studies indicate that DISC1 plays a role in multiple cellular processes during and after brain development (Chubb et al., 2008; Brandon and Sawa, 2011). In fact, many protein binding partners of DISC1 are associated with various molecular pathways that regulate fundamental cellular processes for brain development and function (**Table 1**). Nonetheless, it is still unknown which functional aspects of DISC1 directly affect molecular mechanisms underlying disease susceptibility. How can we utilize accumulating biological data of DISC1 to discover novel therapeutic targets and biological markers for major mental conditions? Here, we will review DISC1-associated molecular pathways which have the potential to be novel therapeutic targets, with particular focus on well documented DISC1 pathways involved in cerebral cortex development and function (**Figure 1**). We will also discuss the potential link of DISC1 pathways and environmental factors, such as immune/inflammatory responses, to explore therapeutic interventions based on understanding disease mechanisms of genetic and environmental interaction.

DISC1 IN CEREBRAL CORTEX DEVELOPMENT

Disrupted in schizophrenia 1 plays a critical role for the regulation of cell proliferation in the developing cerebral cortex via the canonical Wnt signaling pathway (Mao et al., 2009). The data suggested that DISC1 inhibits the activity of glycogen synthase kinase 3 beta (GSK3β) via protein interaction, thereby stabilizing β-catenin which is required for proper progenitor proliferation through Wnt pathway. The same group later reported that DIX domain containing-1 (DIXDC1), a homolog of the Wnt signaling genes

Table 1 | DISC1 interacting proteins and functions.

DISC1 interactor	Function	Risk gene	Reference
CENTROSOME/CYTOSKELETON			
NDEL1	Neurite extension, migration	+	Morris et al. (2003), Ozeki et al. (2003), Kamiya et al. (2005), Taya et al. (2007), Burdick et al. (2008)
NDE1	Proliferation	+	Burdick et al. (2008), Bradshaw et al. (2009)
PCM1	Microtubule organization	+	Kamiya et al. (2008)
BBS4	Migration, primary cilia function	–	Kamiya et al. (2008), Ishizuka et al. (2011)
KIF5A	Neuronal transport	–	Taya et al. (2007)
14-3-3ε	Migration axon growth	+	Taya et al. (2007)
FEZ1	Neurite extension	+	Miyoshi et al. (2003)
Kendrin	Centrosome function	–	Miyoshi et al. (2004)
MAP1A	Microtubule associated	–	Morris et al. (2003)
MIPT3	Microtubule associated	–	Morris et al. (2003)
SYNAPSE			
Kalirin-7	Dendritic spine/synapse function	–	Hayashi-Takagi et al. (2010)
TNIK	Dendritic spine/synapse function	+	Wang et al. (2011)
Citron	Rho signaling, synapse function	+	Ozeki et al. (2003)
NUCLEUS			
ATF4	Transcription factor	–	Morris et al. (2003), Sawamura et al. (2008)
N-CoR	Corepressor for gene transcription	–	Sawamura et al. (2008)
OTHER			
PDE4B	cAMP signaling	+	Millar et al. (2005)
Girdin	AKT signaling	+	Enomoto et al. (2009), Kim et al. (2009)
Grb2	Tyrosine kinase mediated signal transduction	–	Shinoda et al. (2007)
DBZ	PACAP signaling	–	Hattori et al. (2007)
Mitofilin	Mitochondrial function	–	Park et al. (2010)

Many protein binding partners of DISC1 have been reported. DISC1 may function as an anchoring molecule to regulate various molecular pathways via interaction with said protein interactors in a context dependent manner.

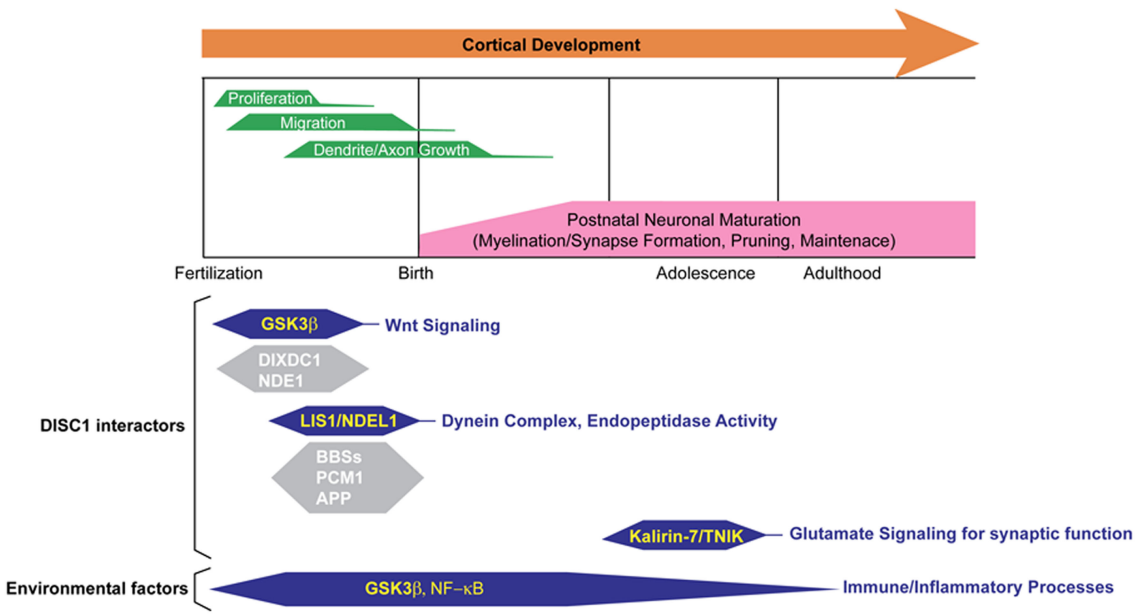

FIGURE 1 | Multiple roles for DISC1 in cerebral cortex in the developmental trajectory. Various DISC1-mediated pathways with many binding partners and environmental factors synergistically affect proper cerebral cortex development and function. For reviews of the other DISC1 interactors, see Brandon and Sawa (2011), Porteous et al. (2011), Soares et al. (2011).

Disheveled axin, interacts with DISC1 to co-modulate GSK3β/β-catenin signaling for proper cell proliferation (Singh et al., 2010). Accumulating evidences have shown that GSK3β signaling may be involved in various neuropsychiatric disorders, such as schizophrenia, autism, and Alzheimer's disease, suggesting that GSK3β appears as a prominent therapeutic target for mental disorders (Bachmann et al., 2005; Hur and Zhou, 2010). In fact, lithium, the mood stabilizer which is commonly used for the treatment of bipolar disorder, is known to inhibit GSK3 activity (Stambolic et al., 1996). The other psychoactive drugs, such as clozapine, risperidone, and valproic acid, have also been reported to affect GSK3β activity (Stambolic et al., 1996; Kang et al., 2004; Li et al., 2007; Rowe et al., 2007). Nonetheless, since GSK3β regulates various downstream effectors, which are not only implicated in the Wnt pathway, but also other signaling required for cellular development, such as sonic hedgehog and Notch signaling pathways (Hur and Zhou, 2010), it is important to examine specific GSK3β-mediated pathways relevant to disease mechanisms to find novel therapeutic strategies. In this regard, it may be ideal to focus on DISC1-mediated GSK3β pathways, especially those in association with other genetic risk factors, to explore disease-associated molecular mechanisms. For instance, collapsin response mediator protein-2 (CRMP-2)/dihydropyrimidinase-like-2 (DPYSL2), a susceptibility gene for schizophrenia (Nakata et al., 2003), is reported to be a potential protein interactor of DISC1 by yeast-two-hybrid screening (Camargo et al., 2007). Interestingly, CRMP-2/DPYSL2 is known to be phosphorylated by GSK3β for the regulation of axon outgrowth (Yoshimura et al., 2005).

Neuronal migration is a fundamental cellular process that is required for proper cortical organization. Many groups have consistently reported that knockdown of DISC1 using RNA interference (RNAi) impaired radial neuronal migration in the developing cerebral cortex (Kamiya et al., 2005, 2008; Kubo et al., 2010; Singh et al., 2010; Young-Pearse et al., 2010; Ishizuka et al., 2011). Findings from these studies suggest that DISC1, along with many protein binding partners, regulate neuronal migration via centrosome and microtubule-dependent mechanisms. Of note, some of these binding partners are known as risk or causative genes for various neuropsychiatric disorders. These include nuclear distribution element-like (NDEL1) and pericentriolar material 1 (PCM1), risk genes for schizophrenia, and BBS4, a causative gene for Bardet–Biedl syndrome that frequently accompanies impaired cognition, mental retardation, and psychosis (Burdick et al., 2008; Kamiya et al., 2008; Tomppo et al., 2009a). Amyloid precursor protein (APP) also interacts with DISC1 to recruit DISC1 to the centrosome for regulation of neuronal migration (Young-Pearse et al., 2010). Furthermore, DISC1 is a component of the LIS1/dynein motor complex (Kamiya et al., 2005). Mutations in human LIS1 gene cause classical lissencephaly resulting in mental retardation (Pilz et al., 1998). Consistently, LIS1 heterozygous knockout mice in which LIS1 expression is reduced, display disorganization of proper cortical layer formation and behavioral abnormalities, such as impaired spatial learning and motor function, indicating that this is a good animal model for human lissencephaly caused by LIS1 haploinsufficiency (Hirotsune et al., 1998). Interestingly, the prenatal administration of ALLN, a calpain inhibitor which prevents the degradation of LIS1, is effective to ameliorate neuronal migration defect and improve motor coordination in this animal model (Yamada et al., 2009).

Although mental disorders undoubtedly have genetic complexities and could not be explained by the simple "haploinsufficiency" model as the case of lissencephaly, elucidation of risk genes, and/or molecules in their interactome, specifically ones with enzymatic activity, may offer hope for novel treatment interventions for neuropsychiatric disorders. In this regard, endo-oligopeptidase activity of NDEL1 is quite interesting from a drug discovery viewpoint (Hayashi et al., 2005). As a matter of fact, inhibitors of angiotensin-converting enzyme (ACE), an exopeptidase, are currently being used to treat hypertension and renal disease (Izzo and Weir, 2011), making peptidase activity an attractive drug target. Although endogenous substrates for NDEL1-oligopeptidase in brain development remain unknown, *in vitro* experiments identified several oligopeptides, such as neurotensin and bradykinin, as potential targets for NDEL1 (Camargo et al., 1983). Interestingly, neurotensin has a modulatory effect on neurotransmitter systems, including dopaminergic neurons, which may be involved in the pathophysiologies of schizophrenia (Boules et al., 2007).

Posttranslational modifications, which affect the functional diversity of target proteins, could also have potential as novel drug targets and biological markers in the DISC1 pathways. We have recently reported that phosphorylation of DISC1 at Serine 710 is a molecular switch signaling from cell proliferation to neuronal migration in the developing cerebral cortex (Ishizuka et al., 2011). By utilizing *in utero* electroporation, this study has shown that a phosphor-dead mutant DISC1 can rescue only the proliferation defect elicited by DISC1 knockdown, whereas a phosphor-mimic mutant of DISC1 can exclusively recover impaired migration. The question arises whether the phosphorylation of DISC1 at Serine 710 may be involved in the pathophysiologies of major mental disorders, such as schizophrenia. It is obviously impractical to investigate the phosphorylation status of DISC1 in the developing human brain from subjects at risk of developing schizophrenia. Nonetheless, recent progress in induced pluripotent stem (iPS) cell technology will open new avenues to characterize such findings from preclinical studies using patient-derived neuronal cells, which might in turn identify biological markers for major mental disorders.

DISC1 AND GLUTAMATE SIGNALING FOR SYNAPTIC FUNCTION

Disrupted in schizophrenia 1 impacts upon brain development may be a challenge for treatment intervention. However, synaptic deficits revealed by the DISC1 pathway offer some potential for development of targeted pharmacologic intervention. Early reports suggested a role for DISC1 in neurite outgrowth (Miyoshi et al., 2003; Ozeki et al., 2003). Subsequent findings underline roles for DISC1 in regulating dendritic spines of the glutamate synapse (Hayashi-Takagi et al., 2010). Rac1 is activated by Karilin-7, leading to increased spine size following NMDA glutamate receptor activation. However, DISC1 appears to interact with Karilin-7, preventing access to and activation of Rac1 until NMDA receptor activation promotes release of Kal-7 and spine enlargement. Pharmacologic tools to modulate the Karilin-7/DISC1 interaction might be a means to regulate spine maintenance.

TRAF2- and NCK-interacting kinase (TNIK) represents another potential pharmacological target in the DISC1 protein interaction network. TNIK is found in postsynaptic densities and regulates c-Jun kinase, the actin cytoskeleton and a number of Wnt pathway effectors (Fu et al., 1999; Taira et al., 2004; Mahmoudi et al., 2009). Genetic association studies have found single-nucleotide polymorphisms of TNIK associated with schizophrenia (Potkin et al., 2009; Shi et al., 2009). TNIK mRNA expression was increased in the dorsolateral prefrontal cortex of schizophrenia subjects (Glatt et al., 2005) and in lymphoblasts of monozygotic twins discordant for bipolar disorder (Matigian et al., 2007). A yeast-two-hybrid screen using DISC1 as "bait" identified TNIK as an interactor (Camargo et al., 2007). Subsequently, TNIK and DISC1 were shown to interact in mouse brain (Wang et al., 2011). DISC1 was found to inhibit the kinase activity of TNIK, an action that could be reproduced by a small peptide derived from the DISC1 interaction site. This DISC1 peptide led to increased actin polymerization and decreased expression of a number of postsynaptic density proteins, including PSD95, stargazin, AMPA receptor subunit GluR1 and TNIK, itself (Wang et al., 2011).

DISC1 AND NEUROIMMUNE/INFLAMMATORY PROCESSES

Microbial infections have been recognized as environmental factors responsible for the increased incidence of schizophrenia and associated disorders (Brown and Derkits, 2010; Sham et al., 1992; Torrey and Yolken, 2003). These reports have been supported by the epidemiological findings of an association between elevated cytokines in maternal serum and schizophrenia in the offspring (DeLisi and Wyatt, 1982; Patterson, 2007; Miller et al., 2009). Subsequently, it has been demonstrated that it is the maternal immune response to a microbe that may contribute to the increased risk of schizophrenia. The role of cytokines in innate immune response makes them promising candidates for studying their functions in disruption of fetal brain development in vulnerable individuals (Dantzer et al., 2008). Most studies with prenatal immune activation have thus far used wild-type mice and rats. However, recently, there have been several reports on developing and characterizing animal models based on combining prenatal immune activation with genetic mutations relevant to schizophrenia (Ibi et al., 2010; Ehninger et al., 2012).

We have been studying possible roles for DISC1 in modulation of poly I:C-induced immune activation in pregnant mice to mimic prenatal *in utero* exposure to viruses as a model of gene–environment interactions relevant to schizophrenia (Abazyan et al., 2010). Our findings have suggested that DISC1 may be involved in mediating neuroimmune interplay in this mouse model. Given the extended interactome of DISC1, it is not surprising that this protein is at the crossroads of the signaling transduction pathways activated by immune factors.

One can envision multiple interactions between the pathways impacted by mutant DISC1 and activated by cytokines and/or bacterial lipopolysaccharide (LPS) and poly I:C itself via cytokine receptors or toll-like receptors (TLR) expressed by neurons or glia cells, respectively. One of the major common pathways is the phosphoinositide-3 kinase/AKT-signaling network (PI3K/AKT) that is activated by cytokines and poly IC and has been demonstrated to interact with DISC1 partners (Camargo et al., 2007). Another example is interactions with GSK3β, a key regulator of the host inflammatory response and the production of pro- and anti-inflammatory cytokines (Hayden et al., 2006). As described above, DISC1 inhibits GSK3β activity through a direct interaction (Mao et al., 2009). We also found altered poly I:C-induced phosphorylation of GSK3β in mutant DISC1 newborn mice that might at least in part explain altered basal and poly I:C-induced production of cytokines in fetal brains and resultant affective behaviors in adult offspring (Abazyan et al., 2010). These observations are consistent with an emerging role for GSK3β in inflammation-associated depression and anxiety (Jope, 2011).

Many immune effects of GSK3β are related to its regulation of critical transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; Hayden et al., 2006). A family of TLRs acts as primary sensors that detect a wide variety of microbial components and elicit innate immune responses. All TLR signaling pathways culminate in activation of NF-κB, which controls the expression of an array of inflammatory cytokine genes. Stimulation with TLR ligands triggers the rapid phosphorylation of specific serine residues of inhibitor of κB (IκB) proteins by the IκB kinase (IKK) complex. Phosphorylated IκB proteins are subsequently polyubiquitinated and degraded, allowing NF-κB to move into the nucleus. This so-called "canonical pathway" is involved in TLR-mediated induction of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6; Hayden et al., 2006). Prior studies with DISC1 have demonstrated that DISC1, particularly a nuclear isoform of the protein, can play an important role in regulation of transcription activity in the nucleus (Sawamura et al., 2008). Our pilot *in vitro* experiments demonstrated that DISC1 may impact NF-κB signaling. We found that expression of mutant DISC1 in N2 a neuronal cells led to delaying a recovery of IκBα after TNF-α-induced phosphorylation and ubiquitination of IκBα. This prolonged degradation due to expression of mutant DISC1 seems to suggest that perturbation in functions of DISC1 could also affect (e.g., stimulate) pro-inflammatory signaling transduction cascades in neurons.

In addition to immune signaling pathways, DISC1 and perhaps other candidate genes can play a significant role in the cellular processes utilized by microbes during their life cycles (Carter, 2009). It has been proposed that the involvement of DISC1 in the control of the microtubule network might be important both in viral traffic and in the rerouting of microtubules to the vacuoles formed by *T. gondii* (Carter, 2009).

Recent clinical trials of anti-inflammatory add-on therapy in schizophrenia have demonstrated superior beneficial treatment effects when antipsychotics were co-administered with anti-inflammatory compounds, as compared with treatment outcomes using antipsychotics alone (Meyer et al., 2011). However, a broad non-specific anti-inflammatory or immunosuppressive treatments that may have several unwanted effects such as increased sensitivity to infections (Meyer et al., 2011). Ultimately, future therapeutic approaches will result from deciphering intracellular pathways that underlie convergence of environmental influences and genetic predisposition and their influence on neurodevelopmental processes.

CONCLUSION REMARKS

Disrupted in schizophrenia 1-mediated pathways play multiple roles for critical cellular processes through many protein binding partners in a context dependent manner. Nonetheless, it is still unknown which functional aspect of DISC1 directly affects molecular mechanisms underlying disease susceptibility. Are all DISC1 functions in such cellular events implicated in disease processes or are only some specific functional aspects critical? This is a tremendously difficult question, because the molecular disposition of DISC1 is complex as reflected by multiple isoforms at both mRNA and protein levels (Ishizuka et al., 2006; Nakata et al., 2009). Nonetheless, biological functions of DISC1 are currently being explored without waiting for the complete identification of DISC1 isoforms, resulting in the identification of multiple roles of DISC1 in various functional contexts. In fact, in addition to the roles in cerebral cortex we reviewed here, DISC1 also contributes to brain development and function in other brain regions, such as hippocampal regions (Enomoto et al., 2009; Kim et al., 2009; Meyer and Morris, 2009). Further investigations with advanced genetic engineering techniques, which allow us to dissect region and cell type-specific DISC1 functions in a temporal manner, might contribute to more clearly elucidate DISC1 functions relevant to psychiatric disorders.

As complete functional recovery is unlikely for neurodevelopmental disorders, such as schizophrenia, developing preventive strategies is particularly important. Indeed, if the findings on microbial etiologies and resultant immune dysfunction are replicated, simple public health measures may prove beneficial in diminishing the incidence of infections during pregnancy to prevent an appreciable proportion of schizophrenia cases. For example, influenza vaccination, improved hygiene to prevent *T. gondii* infection, and antibiotics to treat genital/reproductive infections are feasible strategies already employed (Brown and Derkits, 2010).

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The emerging role of microRNAs in schizophrenia and autism spectrum disorders

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MicroRNAs (miRNAs) are small non-coding RNAs conserved throughout evolution whose perceived importance for brain development and maturation is increasingly being understood. Although a plethora of new discoveries have provided novel insights into miRNA-mediated molecular mechanisms that influence brain plasticity, their relevance for neuropsychiatric diseases with known deficits in synaptic plasticity, such as schizophrenia and autism, has not been adequately explored. In this review we discuss the intersection between current and old knowledge on the role of miRNAs in brain plasticity and function with a focus in the potential involvement of brain expressed miRNAs in the pathophysiology of neuropsychiatric disorders.

Keywords: microRNA, schizophrenia, autism, synaptic plasticity, neuropsychiatric disorder

INTRODUCTION

When Victor Ambros (Lee et al., 1993) and Gary Ruvkun (Wightman et al., 1993) first discovered that a small non-coding RNA lin-4 was able to repress the translation of lin -14, a gene important for developmental timing of *C. elegans*, the scientific community accepted the findings as an "oddity" of worm biology. Since then a plethora of studies have solidified the notion that these small non-coding RNAs, which are named microRNAs (miRNAs), are evolutionary conserved, are responsible for regulating the expression of the majority of protein coding genes, and play a critical role in diverse cellular functions.

The biogenesis of miRNAs starts when they are transcribed from intergenic or intronic genomic regions into primary miRNA precursor molecules, known as pri -miRNAs (reviewed in Bartel, 2004; Krol et al., 2010a). Pri -miRNAs are cleaved inside the nucleus by the components of the microprocessor complex, Drosha and Dgcr8, to generate hairpin structured RNA hairpins called pre-miRNAs (Bartel, 2004; Krol et al., 2010a). Pre-miRNAs are exported into the cytoplasm and undergo further cleavage by RNaseIII enzyme Dicer, thereby forming a complementary duplex of two miRNA strands (Bartel, 2004; Krol et al., 2010a). The miRNA duplex is unwound and one of the strands, the mature miRNA, is incorporated into a large miRNA-induced silencing complex (miRISC), which serves to detect and bind into complementary sequences inside messenger RNAs (mRNAs). As a result of an efficient binding of the mature miRNA to its target, which is usually in the 3′ untranslated region (3′ UTR) of the mRNA, translational inhibition and/or mRNA cleavage occur with mechanisms that are still not fully understood (Bartel, 2004; Pillai et al., 2007; Krol et al., 2010a). Through this miRNA-mediated control of gene expression, miRNAs are known to not only restrict the expression of their target genes in certain cell types or during specific developmental periods, but also to "fine tune" the levels of co-expressed targets so that a desired biological response can occur.

The mammalian brain is characterized by a notable abundance of miRNAs. Targeted deletion studies of the miRNA processing enzyme Dicer in different cellular populations in the mouse brain have provided strong evidence for the significance of miRNAs in the development and maturation of both neuronal and glial cells, with consequent abnormalities in brain morphology and connectivity (Cuellar et al., 2008; Davis et al., 2008; Shin et al., 2009; Dugas et al., 2010; Konopka et al., 2010; Tao et al., 2011). However, the fact that Dicer can process other types of small non-coding RNAs does not allow us to exclude miRNA-independent mechanisms behind these observed phenotypes. In addition, deletion of another miRNA processing enzyme Dgcr8 in neurons, results in alterations in dendritic branching, excitatory synaptic transmission, and short term plasticity (Stark et al., 2008; Fenelon et al., 2011; Schofield et al., 2011).

miRNAs IN BRAIN MATURATION AND FUNCTION

A large number of studies have identified miRNAs that are important for brain development and neuronal differentiation, which have been described in previous reviews (Li and Jin, 2010; Bian and Sun, 2011). However the focus of this review is on miRNAs that have been proven to influence brain maturation and plasticity, mechanisms that are known to be perturbed in psychiatric diseases (**Table 1**). Dendritic spines are protrusions at branches of a neuron's dendritic tree that form the post-synaptic end of a synapse. Their structural properties are known to reflect the degree of brain maturation and their dynamics are an important component of brain plasticity. Moreover, alterations in dendritic spine density have been reported in numerous neuropsychiatric disorders, including schizophrenia and autism spectrum disorders (ASD; Penzes et al., 2001). Interestingly, a loss of dendritic spines has been reported to occur in schizophrenia (Glantz and Lewis, 2000; Kolomeets et al., 2005; Sweet et al., 2009), whereas an increase in the number of spines has been proposed for ASD

Table 1 | MicroRNAs involved in brain plasticity and maturation with links to schizophrenia and autism spectrum disorders.

miRNA	Validated target(s)	Links to schizophrenia (or autism)	Reference
miR-21	PTEN	Altered expression in blood cells (altered in cerebellum in autism)	Abu-Elneel et al. (2008), Iliopoulos et al. (2010), Meng et al. (2006), Gardiner et al. (2011)
miR-23a	Lmnb1	Altered in cerebellum and blood cells in autism	Talebizadeh et al. (2008), Sarachana et al. (2010), Abu-Elneel et al. (2008), Lin and Fu (2009)
miR-30a	Bbnf	Altered expression in PFC	Perkins et al. (2007), Mellios et al. (2008)
miR-30d	Bdnf	Altered expression in PFC	Perkins et al. (2007), Mellios et al. (2008)
miR-34a	Grm7	Altered expression in PFC and blood cells	Kim et al. (2010), Lai et al. (2011)
miR-128	Creb1, Src, Ppp1cc, Sp1	Altered expression in blood cells, affected by antipsychotic treatment (altered in cerebellum in autism)	Lin et al. (2011), Perkins et al. (2007), Abu-Elneel et al. (2008), Gardiner et al. (2011)
miR-132	p250gap, Mecp2	Altered expression in PFC (altered in cerebellum and blood cells in autism)	Vo et al. (2005), Wayman et al. (2008), Klein et al. (2007), Kim et al. (2010), Miller et al. (2012), Talebizadeh et al. (2008), Sarachana et al. (2010), Abu-Elneel et al. (2008)
miR-134	Creb1	Altered expression in PFC and blood cells	Gao et al. (2010), Santarelli et al. (2011), Gardiner et al. (2011)
miR-137	Mb1, Lsd1, Ezh2	GWAS significance for schizophrenia	Smrt et al. (2010), Ripke et al. (2011), Sun et al. (2011), Szulwach et al. (2010)
miR-138	Apt1	Altered expression in PFC	Siegel et al. (2009), Moreau et al. (2011)
miR-181b	Vsnl1, Gria2	Altered expression in PFC/STG and blood cells (altered in blood cells in autism)	Mellios et al. (2009), Beveridge et al. (2008), Beveridge et al. (2010), Seno et al. (2011), Gardiner et al. (2011), Shi et al. (2012)
miR-195	Bdnf, Vsnl1, Reln, Nr3a, HTr2a, Rgs4, Plexna2	Altered expression in PFC/STG and blood cells, associated with changes in BDNF, SST, and NPY (altered in blood cells in autism)	Beveridge et al. (2008), Beveridge et al. (2010), Mellios et al. (2008), Wu et al. (2010), Sarachana et al. (2010), Gardiner et al. (2011), Shi et al. (2012)
miR-212	Spred1, Mecp2	Altered expression in PFC (altered in cerebellum and blood cells in autism)	Hollander et al. (2010), Im et al. (2011), Perkins et al. (2007), Talebizadeh et al. (2008), Abu-Elneel et al. (2008)
miR-219	CamKIIγ, Plk2	Altered expression in PFC/STG (altered in blood cells in autism)	Beveridge et al. (2010), Kocerha et al. (2009), Sarachana et al. (2010)
miR-346	Grid1	Altered expression in PFC and blood cells (altered in blood cells in autism)	Zhu et al. (2009), Sarachana et al. (2010), Shi et al. (2012)
miR-381	Bdnf	Altered in cerebellum in autism	Wu et al. (2010), Abu-Elneel et al. (2008)
miR-495	Bdnf	Altered in blood cells in autism	Wu et al. (2010), Sarachana et al. (2010)

Links to altered expression for autism are showed in parenthesis and in blue, all other descriptions refer to schizophrenia and are shown in red.

(Hutsler and Zhang, 2000). In both diseases, however, the changes in dendritic spine density are limited to specific brain regions and cortical layers (Glantz and Lewis, 2000; Hutsler and Zhang, 2000; Kolomeets et al., 2005; Sweet et al., 2009).

The first demonstration that miRNAs are localized in the synapse and can regulate dendritic spine structure came when miR-134 was found to be expressed in hippocampal dendritic spines and shown to be capable of reducing spine width by targeting spine growth-promoting kinase Limk1 (Schratt et al., 2006). Since then, a number of miRNAs such as miR-132 (Vo et al., 2005; Wayman et al., 2008; Edbauer et al., 2010; Impey et al., 2010), miR-125b (Edbauer et al., 2010), miR-138 (Siegel et al., 2009), and miR-137 (Smrt et al., 2010) have been shown to regulate dendritic spine structure and morphology. In the case of miR-138 it reduces spine size by targeting depalmitoylation enzyme acyl-protein thioesterase 1 (Apt1; Siegel et al., 2009), whereas miR-125b reduces spine width potentially by targeting NMDA receptor 2a (NR2a; Edbauer et al., 2010). A negative impact on spine maturation was also shown for miR-137, with the proposed mechanism of action being its effect on ubiquitin ligase Mind Bomb 1 (Mb1; Siegel et al., 2009). Neuronal activity and cAMP response binding protein (CREB) signaling was found to regulate the expression of miR-132, which has been shown to increase dendritic spine density and size and promote mature spine morphology by targeting spine inhibitor GTPase p250GAP (Vo et al., 2005; Wayman et al., 2008; Impey et al., 2010). In newborn hippocampal neurons miR-132 plays an additional role by influencing dendritic branching and synaptic integration (Magill et al., 2010; Luikart et al., 2011). Notably, growth factors such as brain-derived neurotrophic factor (BDNF) are known to interfere with miRNA expression and function. For example, BDNF can induce the expression of miR-132 and co-transcribed miR-212 (Remenyi et al., 2010) and

inhibit the actions of miR-134 (Schratt et al., 2006), both in an activity-dependent manner.

Despite the fact that most of the initial discoveries pertaining to the role of miRNAs in brain maturation were based in neuronal cultures or slices, recent studies have employed new methods for *in vivo* manipulation of miRNA levels in mouse brain, and have provided important insights into the interplay between miRNA-mediated structural effects and their impact in brain plasticity and function. The most well studied miRNA family is that of miR-132/miR-212, two miRNAs of the same family that share sequence similarities, and are expressed from the same genomic location. Both miR-132 and miR-212 have been demonstrated to respond to cocaine treatment and *in vivo* manipulation of miR-212 expression was found to modulate cocaine-induced plasticity and cocaine seeking behavior by indirectly promoting CREB signaling (Hollander et al., 2010). A follow-up study revealed that miR-132/212 were involved into a complex activity-dependent loop with BDNF and Methyl-CPG-binding protein (Mecp2), that resulted in the observed effects in cocaine plasticity (Im et al., 2011). A similar interplay between Mecp2 and miR-132 expression, that involves BDNF, has been previously shown to occur in neuronal cultures (Klein et al., 2007).

Furthermore, two independent studies have recently shown that miR-132 is an experience-dependent miRNA in mouse visual cortex, which regulates dendritic spine morphology *in vivo*, and whose balanced expression is needed to maintain visual cortex plasticity (Mellios et al., 2011; Tognini et al., 2011). In the first study miR-212 was included together with miR-132 in the subset of miRNAs that showed significant changes in their expression following visual deprivation, an established paradigm for studying cortical plasticity (Mellios et al., 2011). *In vivo* inhibition of miR-132 function in led to increased levels of spine inhibitor p250GAP and reduced dendritic spine density and immature morphology (Mellios et al., 2011). In the second study, on the other hand, *in vivo* upregulation of miR-132 expression promoted spine maturation (Tognini et al., 2011). Notably, both of these manipulations abrogated ocular dominance plasticity, the ability of neurons of the visual cortex to adjust their synaptic weights in response to altered visual drive following visual deprivation during a specific sensitive period of plasticity known as the critical period. These finding suggest that balanced levels of miR-132 are required for brain plasticity, and that too much or too little miR-132 expression can result in precocious or delayed cortical maturation, respectively. The effect of miR-132 in experience-dependent cortical maturation is very likely to not be limited to the visual cortex, since multiple studies have shown that miR-132 expression is induced in other brain regions in an experience-dependent manner (Nudelman et al., 2010).

Furthermore, a number of recent studies have shown that a subset of brain expressed miRNAs can affect learning and memory. Specifically, miR-134 expression in the hippocampus of Sirtuin 1 (Sirt1) knockout mice, which display memory deficits during fear conditioning and aberrant long term potentiation (LTP), was shown to be increased, and *in vivo* inhibition of miR-134 expression in hippocampus, was able to rescue both the memory and plasticity impairments (Gao et al., 2010). The novel mechanism proposed by this study was that SIRT1 normally inhibits miR-134 transcription, and miR-134 targets CREB, which in turn increases BDNF expression (Gao et al., 2010). Two more studies described CREB-targeting miRNAs as being important for plasticity and memory, with miR-124a shown to block serotonin-induced long term plasticity in *Aplysia* (Rajasethupathy et al., 2009), and miR-128b proposed to regulate the formation of fear extinction memory in mice (Lin et al., 2011). The latter study also demonstrated that miR-128b, which is an intronic miRNA, targets its host gene, regulator of calmodulin signaling (*Src*, or *Rpp21*), along with a number of other plasticity-related genes such as Reelin (*Reln*), protein phosphatase 1c gamma (*Ppp1cc*), and trans-acting transcription factor 1 (*Sp1*). Of note, miR-124 is an example of a brain enriched miRNA that acts in a temporal and cell-specific manner, since it can also affect neuronal development (Cheng et al., 2009), differentiation, including neurite branching (Makeyev et al., 2007; Visvanathan et al., 2007; Yu et al., 2008), and is able in parallel to influence the activation of brain microglia (Ponomarev et al., 2011), in which it is also abundantly expressed (see also **Figure 1**). Finally, transgenic mice overexpressing miR-132 were also shown to have impairments in novel object recognition memory (Hansen et al., 2010). Surprisingly, the conditional and transient deletion of Dicer1 in adult mouse forebrain caused an unexpected improvement in learning and memory (Konopka et al., 2010).

miRNAs IN SCHIZOPHRENIA

The first psychiatric disease to be linked to miRNA expression and function was Tourette's syndrome, following the report that mutations in the 3′UTR of *SLITRK1* were affecting the targeting by miR-189 in a limited subset of patients (Abelson et al., 2005). However, a plethora of postmortem studies using subjects diagnosed with schizophrenia provided the strongest support for aberrant miRNA expression in psychiatric diseases (Perkins et al., 2007; Beveridge et al., 2008, 2010; Mellios et al., 2009, 2010; Zhu et al., 2009; Kim et al., 2010; Moreau et al., 2011; Santarelli et al., 2011; Miller et al., 2012). Among the reported dysregulated miRNAs are those described above as being important regulators of brain maturation and plasticity (**Table 1**). The known limitations of postmortem studies, however, and differences in miRNA quantification techniques, have not allowed for a comprehensive description of which miRNAs are reliably affected in schizophrenia, and more importantly which might be important for the pathogenesis of this highly heterogeneous psychiatric disorder. In addition to individual miRNA expression the possibility that miRNA processing might be perturbed as a whole in a subset of subjects of schizophrenia has been proposed (Beveridge et al., 2010; Santarelli et al., 2011): although debatable, the idea deserves to be further investigated, especially in light of the fact that patients suffering from DiGeorge 22q11,2 deletion syndrome, which harbor microdeletions that affect miRNA processing gene Dgcr8, display a 30-fold increase in the prevalence of schizophrenia and schizoaffective disorder (Lindsay et al., 1995; Murphy et al., 1999; Stark et al., 2008).

A subset of miRNA altered in the brains of subjects diagnosed with schizophrenia, such as miR-132/212 (Perkins et al., 2007; Kim et al., 2010; Miller et al., 2012), miR-219 (Beveridge et al., 2010), and miR-195 (Beveridge et al., 2008, 2010) deserves special

attention. In the case of miR-132 and miR-212, their prementioned links to activity-dependent synaptic plasticity, and neuronal maturation, are of interest given the known abnormalities in synaptic plasticity and connectivity observed in schizophrenia. The fact that NMDA inhibition is reported to affect the stability of mature miR-132 in neuronal cultures (Krol et al., 2010b) is of additional importance since NMDA hypofunction is hypothesized to be linked to the core of schizophrenia pathophysiology. On a similar note, miR-219, whose expression is downregulated in response to acute pharmacological blockade of NMDA receptors with diclozapine and in NMDA receptor 1 (*Nr1*) knockout mice, can target calcium/calmodulin-dependent protein kinase IIγ subunit (*CamKIIγ*; Kocerha et al., 2009). However, both miR-219 and CamKIIγ levels are increased in the prefrontal cortex (PFC) and superior temporal gyrus (STG) of patients with schizophrenia (Beveridge et al., 2010), suggesting an additional level of complexity. Another notable similarity between miR-219 and miR-132 is that they were both shown to influence genes important for circadian clock entrainment (Cheng et al., 2007), which is of potential relevance to schizophrenia given the observed deficits in circadian synchronization (Lamont et al., 2010).

Another miRNA that deserves notice is miR-195 which was found to be increased in the brain of subjects with schizophrenia (Beveridge et al., 2010), and which has been proven to regulate numerous schizophrenia-related genes, such as *Bdnf* (Mellios et al., 2008), *Reln* (Beveridge et al., 2010), Visinin-like 1 (*Vsnl1*; Beveridge et al., 2010), 5-hydroxytryptamine (serotonin) receptor 2a (*Htr2a*; Beveridge et al., 2010), and glutamate receptor, ionotropic, *N*-methyl-D-aspartate 3A (*Grin3*; Beveridge et al., 2010). The presence of two GABAergic expressed genes (*Reln*, *Vsnl1*) in the above list of validated miR-195 targets, and the fact that BDNF is known to influence GABAergic gene expression and function, position miR-195 as an intriguing candidate for schizophrenia research given the known deficits in GABAergic gene expression and inhibitory neuron function in schizophrenia (Akbarian et al., 1995; Guidotti et al., 2000; Costa et al., 2004; Hashimoto et al., 2008). The findings that disease-related changes in miR-195 are inversely correlated to those of BDNF protein levels, which in turn are associated with the observed changes in somatostatin (Sst) and neuropeptide Y (Npy) mRNA levels in human PFC of subjects with schizophrenia (Mellios et al., 2009), adds value to the above hypothesis. Last, but not least, an *in silico* study of schizophrenia-related miRNAs and transcription factors, depicted miR-195 as the core component of the predicted schizophrenia regulatory gene networks (Guo et al., 2010).

Despite the known neuronal-specific targets of schizophrenia-related miRNAs it is tempting to speculate that disease-related changes in the expression of a subset of miRNAs in schizophrenia might not be limited to neurons but could be also prevalent in glial cell populations. For example, miR-219 expression is abundantly expressed in oligodendrocytes and regulates their maturation (Dugas et al., 2010), and miR-132, miR-195, and many members of the miR-30 family are also expressed in astrocytes (Moser and Fritzler, 2010; Mor et al., 2011; Numakawa et al., 2011; **Figure 1**). In addition, the findings of the existence of microvesicles secreted by astrocytes called exosomes that contain miRNAs and could be taken up by neurons introduce a novel unexplored mechanism between cell to cell communication in the brain (Faure et al.,

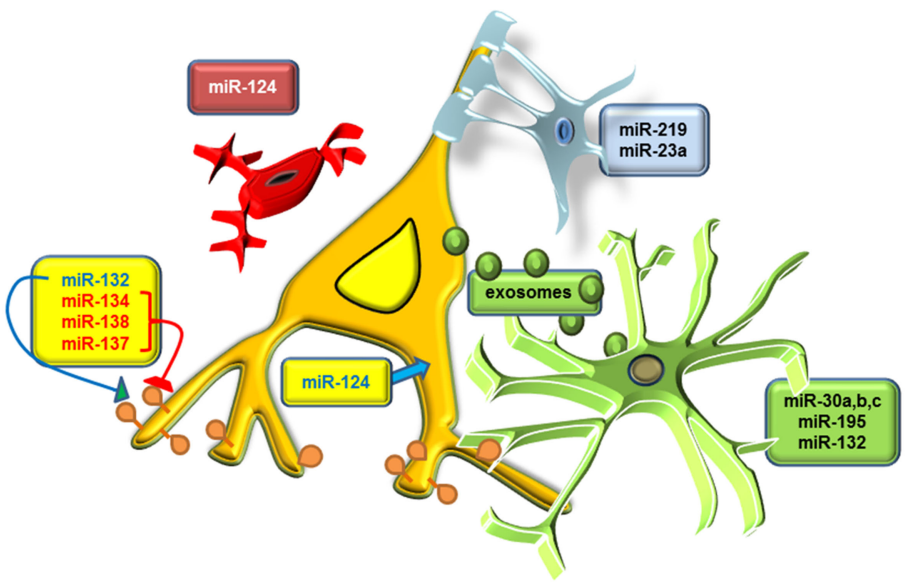

FIGURE 1 | Cell-specific expression of miRNAs regulates neuronal and glial functions. Drawing showing examples of miRNAs enriched in different cellular populations in the central nervous system. A neuron is shown in yellow–orange with miR-132 activating and miR-134, miR-137, and miR-138 inhibiting spine growth. Expression of miR-132 is present (albeit in lower levels) in astrocytes (shown in green), which also express high levels of miR-195 and miR-30a, miR-30b, and miR-30c. Secretion of microvesicles that are known to contain miRNAs (exosomes) by astrocytes is also depicted. Expression of miR-124 is shown in both neurons, where it promotes neurite outgrowth, and microglia (shown in red), where it suppresses their activation. Oligodendrocytes (shown in light blue) express high levels of miR-219 and miR-23a, which regulate their differentiation.

2006; Smalheiser, 2007; Valadi et al., 2007; **Figure 1**). Differential expression of miRNAs within different neuronal populations was recently discovered in mouse neocortex, with the example of miR-34a, a miRNA altered in the PFC of subjects diagnosed with schizophrenia (Kim et al., 2010), being particularly enriched in parvalbumin expressing interneurons (He et al., 2012). Future studies using similar cell-specific methods for miRNA detection, including exosome profiling, are needed to elucidate the role of non-neuronal miRNAs in schizophrenia.

In addition to the alterations in miRNA expression in schizophrenia a growing number of genetic studies has provided intriguing links between miRNAs and schizophrenia. Initially, single nucleotide polymorphisms (SNPs) within miR-206 and miR-198 were shown to be nominally associated with schizophrenia (Hansen et al., 2007). Furthermore, another SNP in pre-miR-30e was strongly linked to the disease (Xu et al., 2010). Notably, the expression of miR-30e is known to be altered in the PFC of subjects with schizophrenia, together with other members of the miR-30 family (Perkins et al., 2007), including miR-30a and miR-30d, which have been proven to target BDNF (Mellios et al., 2009; Wu et al., 2010). In addition, another member of the miR-30 family, miR-30b, was shown to be predominantly affected in female subjects with schizophrenia, and to be influenced by a disease-related SNP in estrogen receptor alpha (Mellios et al., 2010). However, the most robust genetic evidence came from a recent meta-analysis of genome-wide association studies (GWAS) for schizophrenia that reported that a SNP within intronic miR-137 displayed the most significant association with the disease (Ripke et al., 2011). This impressive finding was accompanied by the observation that four more significant loci contained miR-137 targets (Ripke et al., 2011). Other than the pre-mentioned reported effect of miR-137 in dendritic spine maturation (Smrt et al., 2010), it is of interest that Mecp2 inhibits miR-137 expression, which in turn can target other chromatin modifying genes, such as Lysine (K)-specific demethylase 1a (*KDM1A*, also known as *LSD1*; Sun et al., 2011), and Histone-lysine *N*-methyl-transferase, Enhancer of zeste (*Drosophila*) homolog 2 (*EZH2*; Szulwach et al., 2010). Notably, a recent finding demonstrated that miR-137-mediated regulation of LSD1 can also regulate neuronal differentiation and migration by affecting neural stem cell renewal (Sun et al., 2011). Furthermore, the interplay between miR-137 and genes important for chromatin remodeling is of importance given the hypothesized role of epigenetics in schizophrenia (Akbarian, 2010), and especially since a large number of other schizophrenia-related miRNAs, such as miR-132/212, miR-195 are known to be sensitive to epigenetic regulation (Li et al., 2011; Tognini et al., 2011; Zhang et al., 2011). Interestingly, recent work has uncovered that the deficits in miR-132 in schizophrenia are accompanied by an increase in miR-132 target DNA methyl-transferase alpha (DNMT3a; Miller et al., 2012), thus creating an epigenetically regulated double negative feedback loop. Further work is warranted to determine whether epigenetic regulation of miRNA expression could play an important role in the pathophysiology of the disease.

miRNAs IN AUTISM SPECTRUM DISORDERS

Unlike the large number of studies examining the role of miRNAs in schizophrenia, little is known about the potential importance of miRNAs for ASD, and especially for the non-syndromic ASD subtypes. So far two studies have determined the expression of miRNAs in lymphoblastoid cell cultures of ASD patients (Abu-Elneel et al., 2008; Talebizadeh et al., 2008; Sarachana et al., 2010; Seno et al., 2011), and one study has identified dysregulated miRNAs in the cerebellum of ASD patients (Abu-Elneel et al., 2008). Among the most promising results (**Table 1**) is the finding of altered expression of miR-132/212, the two miRNAs of the same family described above to be dysregulated in schizophrenia (Perkins et al., 2007; Kim et al., 2010; Miller et al., 2012) and to be important for the control of experience-dependent cortical plasticity (Mellios et al., 2011; Tognini et al., 2011). Notably, miR-132 effects on synaptic structure are modulated by FMRP (Edbauer et al., 2010), the product of the Fmr1 gene responsible for Fragile-X Mental Retardation, the leading cause of mental retardation in adults and a subtype of syndromic ASD (D'Hulst and Kooy, 2009). Intriguingly, miR-132 is known to be induced by endotoxins and regulate the immune response (Taganov et al., 2006; Shaked et al., 2009; Lagos et al., 2010), as well as to be affected by cytomegalovirus infection (Wang et al., 2008), which are known to contribute to autism pathogenesis (Stubbs et al., 1984; Onore et al., 2012). In addition, miR-132 levels are reduced in Rett Syndrome, another subtype of syndromic ASD, caused by mutations in Mecp2 (Klein et al., 2007; Wu et al., 2010). As mentioned above, Mecp2 can increase the expression of miR-132 through its effect on BDNF, and decrease miR-137 by binding to its promoter and inducing transcriptional inhibition (Klein et al., 2007; Wu et al., 2010). However, the effect of Mecp2 on miRNAs is far more complex, since Mecp2 is known to regulate a large number of miRNAs in the mouse brain, and influence a plethora of miRNA-mediated functions with potential importance for brain disease (Wu et al., 2010; de Leon-Guerrero et al., 2011).

Furthermore, miR-195, miR-219, miR-346, miR-181b, and miR-128b, all known to be altered in schizophrenia (Beveridge et al., 2008, 2010; Zhu et al., 2009), exhibit changes in ASD samples as well (Abu-Elneel et al., 2008; Talebizadeh et al., 2008; Sarachana et al., 2010; Seno et al., 2011), strengthening the argument for the existence of common molecular pathways behind the pathophysiology of both neuropsychiatric diseases. Interestingly, and as mentioned above, miR-195 is known to target BDNF (Mellios et al., 2009) and miR-128b to inhibit CREB (Lin et al., 2011), whereas miR-132/212 are induced by BDNF and positively regulate CREB signaling (Hollander et al., 2010; Remenyi et al., 2010), which, in conjunction with two more BDNF regulating miRNAs (miR-495, miR-381; Wu et al., 2010) altered in autism (Talebizadeh et al., 2008; Sarachana et al., 2010), allows us to speculate that activity-dependent miRNAs could be affected in ASD. Such abnormalities in activity-dependent BDNF/CREB-related miRNAs are expected to have an impact on synaptic connectivity and maturation, which are known to be perturbed in ASD. As in the case of schizophrenia, the cell-specificity of ASD-related alterations in miRNA expression warrants further investigation, since differentially expressed miRNAs are known to be also expressed in astrocytes and oligodendrocytes (Dugas et al., 2010; Moser and Fritzler, 2010; Numakawa et al., 2011; Mor et al., 2011; Lin and Fu, 2009. In the case of miR-23a, for example, which together with miR-132 is altered in three out of four ASD studies (Abu-Elneel

et al., 2008; Talebizadeh et al., 2008; Sarachana et al., 2010), there is strong evidence suggesting that it is an oligodendrocyte enriched miRNA that regulates their maturation by inhibiting lamin B1 (Lin and Fu, 2009; see also **Figure 1**). Another pathway that is emerging to be regulated by ASD altered miRNAs and that is also affected by BDNF signaling, is that of phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB also known as Akt), with the best example being the reported changes in miR-21, a miRNA known to target phosphatase and tensin homolog (PTEN; Meng et al., 2006; Iliopoulos et al., 2010), which is responsible for inactivating Akt. Given that patients with PTEN mutations represent a substantial proportion of syndromic ASD (Varga et al., 2009), further research is warranted to elucidate the importance of miR-21 for ASD pathogenesis.

CONCLUSION

The increasing number of studies uncovering novel miRNA-mediated mechanisms that influence brain maturation and plasticity, in conjunction with the parallel findings for the potential involvement of such miRNAs in schizophrenia and autism, elevates the importance of miRNAs for brain function and dysfunction to a new level. It is tempting to speculate that a subset of the reported dysregulated in neuropsychiatric disorders miRNAs might turn out to be integral for shedding light on the elusive pathophysiology or pathogenesis of these complex and enigmatic disorders. Moreover, given the fact that both autism and schizophrenia pathogenesis appears to be influenced by mechanisms related to neurodevelopment, priority should be given to ASD or schizophrenia altered miRNAs with specific developmental patterns. For example, the experience-dependent miR-132/212 miRNA family holds great promise for schizophrenia, since its expression in mouse PFC peaks around adolescence (Miller et al., 2012), which coincides with the age of onset of schizophrenia. In theory, if a miRNA is indeed regulating genes and pathways that could trigger the onset of the disease, it is expected that its dysregulation will have the biggest impact at the time point when its expression reaches the maximum, because this is when its targets will be most robustly affected. Moreover, in the case where alterations in specific miRNAs are only an epiphenomenon of the disease, they could be ideal biomarker candidates, given the increased stability and durability of miRNAs, even in harsh handling conditions (Chen et al., 2008). Indeed, recent studies are exploring this possibility and have already reported some aberrantly expressed miRNAs in blood cells that could be associated with schizophrenia symptomatology, including miRNAs known to be altered in the brains of schizophrenia subjects as well, or affected by antipsychotic treatment (Gardiner et al., 2011; Lai et al., 2011; Woelk et al., 2011; Shi et al., 2012; **Table 1**). Ideally, the use of blood expressed miRNAs as biomarkers can be expanded to monitor the progress and response to therapeutic treatment of a disease, such as in the case of miR-134 and bipolar disorder (Rong et al., 2011). Lastly, the emerging technologies of induced neurons (Ambasudhan et al., 2011), or induced pluripotent stem cell-derived (Pedrosa et al., 2011) neurons, from patient fibroblasts might be an alternative for uncovering novel miRNA biomarkers, as well as for discovering new treatment options.

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The effects of psychosis risk variants on brain connectivity: a review

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In light of observed changes in connectivity in schizophrenia and the highly heritable nature of the disease, neural connectivity may serve as an important intermediate phenotype for schizophrenia. However, how individual variants confer altered connectivity and which measure of brain connectivity is more proximal to the underlying genetic architecture (i.e., functional or structural) has not been well delineated. In this review we consider these issues and the relative sensitivity of imaging methodologies to schizophrenia-related changes in connectivity. We searched PubMed for studies considering schizophrenia risk genes AND functional or structural connectivity. Where data was available, summary statistics were used to determine an estimate of effect size (i.e., Cohen's *d*). A random-effects meta-analysis was used to consider (1) the largest effect and (2) all significant effects between functional and structural studies. Schizophrenia risk variants involved in neurotransmission, neurodevelopment and myelin function were found to be associated with altered neural connectivity. On average, schizophrenia risk genes had a large effect on functional (mean $d = 0.76$) and structural connectivity (mean $d = 1.04$). The examination of the largest effect size indicated that the outcomes of functional and structural studies were comparable ($Q = 2.17$, $p > 0.05$). Conversely, consideration of effect size estimates for *all* significant effects suggest that reported effect sizes in structural connectivity studies were more variable than in functional connectivity studies, and that there was a significant lack of homogeneity across the modalities ($Q = 6.928$, $p = 0.008$). Given the more variable profile of effect sizes associated with structural connectivity, these data may suggest that structural imaging methods are more sensitive to a wider range of effects, as opposed to functional studies which may only be able to determine large effects. These conclusions are limited by methodological considerations, and require further investigation involving larger samples, multiple genes, and novel analysis techniques for confirmation.

Keywords: schizophrenia, functional connectivity, structural connectivity, genotype, effect size

INTRODUCTION

EXAMINING THE FUNCTION OF SCHIZOPHRENIA (SZ) RISK VARIANTS

Schizophrenia is a complex genetic disorder affecting roughly 1% of the world's population (see Lewis and Lieberman, 2000 for a review). It is characterized by hallucinations and delusions, reduced emotion and cognitive impairment, and imposes a heavy cost on society (for example, the total cost of psychotic disorders in Europe in 2010 was recently calculated as €93.9 billion; Gustavsson et al., 2011). While there is no consensus about its exact causes, the heritability of SZ is estimated to be about 80% (Sullivan et al., 2003). Genome-wide association (GWAS) and copy number variation studies have identified several common and rare gene variants associated with the disorder (O'Donovan et al., 2008). Understanding the function of these variants could, therefore, lead to a greater understanding of disease pathogenesis, which could direct new treatments.

Schizophrenia patients present with variable symptom profiles and distinct disease trajectories. This heterogeneity may be in part due to the complex genetics of SZ, which in turn poses significant problems for understanding the mechanisms by which genetic variants confer risk for this disease. In an attempt to address this complexity, researchers have focused on so-called "intermediate phenotypes," which are measurable variations that occur on the pathway between genes and disease, and as such may be closer to the underlying genetic architecture than clinical symptoms (see Gottesman and Gould, 2003 for a review). Possible intermediate phenotypes for SZ include changes in brain chemistry, structure, function (Braff et al., 2007) and connectivity (Meyer-Lindenberg, 2009).

While changes in functional and structural connectivity may be a critical aspect of the SZ disease profile, there has been little systematic evaluation of the relative sensitivity of these different indices to genetic risk for SZ. In this meta-analysis we outline empirical investigations that have utilized functional magnetic resonance imaging (fMRI) or diffusion tensor imaging (DTI) to investigate the effects of SZ risk variants on functional and structural brain connectivity. We also consider the relative magnitude

of these effects, in order to determine the extent of the genetic impact on brain connectivity.

ALTERED FUNCTIONAL CONNECTIVITY IN SZ

In the early 20th century, German neurologist Carl Wernicke proposed that SZ arises from altered neural connectivity (or dysconnectivity) rather than from abnormalities in specific parts of the brain (see Stephan et al., 2009 for a review). One hundred years later, advances in neuroimaging technology have enabled scientists to empirically consider dysconnectivity as a key component of SZ pathogenesis.

Two or more brain regions are said to be functionally connected if they show a correlation of activity over time (Friston et al., 1993).The hypothesis that functional connectivity is altered in SZ is supported by positron emission tomography (PET; e.g., Friston and Frith, 1995; Meyer-Lindenberg et al., 2001, 2005) and fMRI studies (Lawrie et al., 2002), which reveal abnormal prefronto-temporal connectivity in SZ patients while they perform cognitive tasks. Electroencephalogram (EEG) research also demonstrates abnormal functional connectivity patterns in patients with SZ (Breakspear et al., 2003), and one genetic mouse model of SZ reveals decreased hippocampal-prefrontal connectivity during a T-maze task (Sigurdsson et al., 2010).

Support for the role of functional connectivity as an intermediate phenotype for psychiatric disorders includes a fMRI study by Pezawas et al., 2005). These authors examined the effects of a 5-HTTLPRpolymorphism that is associated with anxiety and depression, on functional connectivity between the amygdala and cingulate cortex. It was reported that variant-associated changes in connectivity predicted almost 30% of the variance in the behavioral effects of this polymorphism. Behavioral variability was also in fact better predicted by changes in connectivity than changes in regional brain activation.

Understanding the underlying biological causes of altered functional connectivity has the potential to lead to a better understanding of SZ pathogenesis, but so far the etiology of functional dysconnectivity remains unclear. However, different mechanisms have been proposed, which we will discuss in the following sections.

THE "DISCONNECTION" HYPOTHESIS OF SZ

The "disconnection" hypothesis was first proposed by Karl Friston and colleagues in the 1990s (Friston, 1998). This hypothesis postulates that SZ is primarily caused by abnormal *N*-methyl-D-aspartate (NMDA)-receptor mediated synaptic plasticity, which in turn, is caused by dysregulation of these receptors by neurotransmitters such as dopamine. Support for the role of the NMDA receptor in SZ comes from several studies. Firstly, drugs that block the NMDA-receptor, such as ketamine and phencyclidine, can induce psychotic symptoms in healthy controls (see Javitt, 2010 for a review). Similarly, ketamine administration induces sensory processing deficits in controls similar to deficits seen in patients, suggesting a role for NMDA receptors in these deficits (Umbricht et al., 2000). Activity of midbrain dopaminergic neurons is partially regulated by glutamatergic projections from the prefrontal cortex (PFC), acting via NMDA receptors, and NMDA receptor-blockade enhances amphetamine-induced increases in striatal dopamine in controls, similar to increases seen in SZ patients (Kegeles et al., 2000). Finally, genetic variants that play a role in NMDA-signaling have been associated with increased SZ risk in candidate gene studies [e.g., *G72*,*GRM3* and *RGS4*; see Harrison and Weinberger (2005) for a review].

FUNCTIONAL CONNECTIVITY MRI ANALYSIS TECHNIQUES

Functional connectivity can be measured with a variety of tools (e.g., PET, EEG), but this review will focus on papers using fMRI to measure the phenotype in healthy controls and patients with SZ. Using the blood oxygen level dependent (BOLD) response as an indirect measure of neuronal activity (Ogawa et al., 1990), there are a range of approaches to analysis. This review will focus on *seeded connectivity* and *psychophysiological interaction* (PPI).

A seeded connectivity analysis begins with the selection of a seed region, which can be a voxel, or cluster of voxels in the fMRI time-series (Nallasamy and Tsao, 2011). The mean time-course for the seed region is then correlated with all other voxels in the brain. Voxels that pass a certain threshold are considered to be functionally connected with the seed region, resulting in a functional connectivity map. While the PPI approach also measures the co-variation of the BOLD signal in voxels across the brain (Friston et al., 1997), it also measures changes in the interactions between brain regions in response to different psychological tasks.

STRUCTURAL CONNECTIVITY AND SZ

White matter (WM) contains myelinated nerve cells that connect various gray matter (GM) areas of the brain to each other, and carry nerve impulses between neurons. Compromised WM integrity is evident in SZ (Kubicki et al., 2007; Ellison-Wright and Bullmore, 2009). Moreover, WM abnormalities are apparent in individuals at high risk of SZ and also in patients during the early stages of illness, suggesting that these abnormalities may be a stable characteristic of the disease (Witthaus et al., 2008; Perez-Iglesias et al., 2010). There are two key postulations regarding the nature of WM deficits in SZ: The "*global theory*" and the "*macro-circuit theory*." The global theory of WM disruption in SZ suggests that WM is compromised uniformly throughout the brain, whereas the macro-circuit theory proposes that specific WM tracts are compromised, which may be a cause *or* consequence of abnormalities in the gray matter regions these tracts connect (Buchsbaum et al., 2006; Konrad and Winterer, 2008).

Diffusion tensor imaging

Diffusion tensor imaging is a method used to measure the diffusion of water molecules in brain WM. Healthy brain WM has a complex axonal structure and, therefore, water diffusion will be restricted along the direction of the axons. This is known as *anisotropic* diffusion. However, if brain WM is compromised water diffusion can become less restricted (i.e., *isotropic*). A common measure derived from DTI to describe the degree of anisotropy during diffusion is *fractional anisotropy* (FA). However, other measures of diffusion such as radial and axial diffusivity can also be obtained. Based on the voxel-wise information provided by DTI, fiber tracking algorithms can be implemented in regions of interest to reconstruct the underlying three-dimensional WM pathways. While caution must be exercised when interpreting measures of anisotropy (Jones, 2008, 2010; Tournier et al., 2011), such measures are thought to index structural integrity of WM tracts and,

thus, may be reasonably considered to be implicit indices of brain connectivity.

WM integrity and SZ: evidence from DTI investigations

A review by Kubicki et al. (2007) noted that the most frequent positive findings of DTI studies in SZ were decreased FA within the prefrontal and temporal lobes, as well as abnormalities within the fiber bundles connecting these regions. WM tracts within these regions that were found to be affected included: (a) the uncinate fasciculus that connects parts of the limbic system with areas in the frontal cortex; (b) the cingulum bundle; and (c) the arcuate fasciculus that connects part of the temporo-parietal junction with the frontal cortex and is thought to be part of the superior longitudinal fasciculus. Ellison-Wright and Bullmore (2009) conducted a meta-analysis of 15 DTI studies, which included a total of 407 patients with SZ and 383 comparison subjects. Results identified two regions of FA decreases in SZ subjects in comparison to controls. The first region was in the left frontal deep WM, which is traversed by WM tracts interconnecting the frontal lobe, thalamus and cingulate gyrus. The tracts include: (a) anterior thalamic radiation (ATR); (b) corticobulbar tracts; (c) inter-hemispheric fibers running through the genu of the corpus callosum; (d) the inferior fronto-occipital fasciculus; (e) the cingulum bundle. The second region was in the left temporal deep WM that is traversed by WM tracts interconnecting the frontal lobe, insula, hippocampus–amygdala, temporal and occipital lobes. These tracts include: (a) inter-hemispheric fibers running through the splenium of the corpus callosum; (b) the inferior fronto-occipital fasciculus; (c) the inferior longitudinal fasciculus; (d) the fornix/striaterminalis. These two reviews of the current DTI/SZ literature suggest that specific networks of WM are disrupted in SZ providing support for the macro-circuit theory of WM disruption in the disease.

Pathophysiological mechanisms of compromised WM

As the integrity of axons is dependent on myelination and factors influencing myelination, it is possible that myelin and oligodendroglia function also plays a role in the pathophysiology of SZ (Davis et al., 2003). Since myelination also impacts synaptic plasticity, oligodendrocyte abnormality and subsequent myelin dysfunction may contribute to the development of SZ by altering synaptic function and information processing (Fields, 2008). Conduction velocity along axons is also thought to be essential for learning processes (Fields, 2008) and disruption of this has the potential to lead to the range of cognitive impairments observed in SZ (Tanaka et al., 2009). Furthermore, oligodendrocyte and myelin dysfunction also impacts neuronal activity that is relevant to SZ, such as glutamate and dopamine signaling. Evidence from psychotic episodes of multiple sclerosis (MS) patients and experimentally induced demyelination suggests that altered myelin function leads to altered dopamine signaling (Takahashi et al., 2011). Similar analyses have also revealed increased levels of glutamate in brains of MS patients as well as increased expression of glutamate receptors on oligodendrocytes (Takahashi et al., 2011). Glutamate transporters are also present on oligodendroglia and are thought to regulate glutamate concentrations to prevent glutamate-induced excitotoxicity (Pitt et al., 2003). Over activation of oligodendroglial glutamate receptors is excitotoxic and can result in oligodendrocyte death (Davis et al., 2003).

Recently, attention has turned toward the consideration of genes that influence oligodendrocyte architecture and how these genes may also be associated with SZ risk. Hakak et al. (2001) examined the expression of 6500 genes derived from postmortem cortical tissue of SZ patients and controls. The expression levels of six myelin-related genes were significantly down regulated for SZ patients in comparison to control subjects. These genes included: myelin-associated glycoprotein (MAG), CNP, myelin and lymphocyte protein (MAL), gelosn (GSN), ErbB3, and transferring. Down regulation of these genes supports the view that oligodendrocytes, the cell type from which all these genes derive in the brain, contribute to the pathophysiology of SZ.

USING IMAGING GENETICS TO EXAMINE NEURAL CONNECTIVITY

Two previous meta-analyses have considered the magnitude of the impact of gene variants on brain function, each reporting large effect sizes. Munafo et al. (2008) examined the effect sizes of the 5-HTTLPR polymorphism and amygdala activation, while Mier et al. (2010) examined the magnitude of effect of the catechol-*O*-methyltransferase (*COMT*) Val158Met polymorphism on brain function, reporting association between this variant and activation of the PFC. However, to our knowledge no studies have specifically considered the effect size of gene variants in studies of functional and structural connectivity, or compared effect sizes between these phenotypes. Consideration of the relative impact of these two measures of brain connectivity will help us to better delineate whether or not one phenotype is more proximal to the underlying genetics, and thus preferential as an intermediate phenotype for studies of SZ. This could not only aid our theoretical understanding of the SZ disease trajectory but may also have significant practical implications for future investigations.

METHODS

We searched for relevant papers based on the criteria of studies that included genes implicated in SZ risk *and* measures of either DTI or functional connectivity. PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) was used to search for relevant functional and structural connectivity papers published until June 2011. The following search terms were included in this search: [schizophrenia OR schiz*] AND [genetic or gene*] AND [MRI OR DTI] AND [connectivity] AND [structural OR functional]. This literature search was supplemented with a review of the references from each of the papers identified. In total 24 studies meeting these search criteria were retrieved, including 12 DTI studies and 12 functional connectivity studies. Individual studies differed slightly in terms of MRI acquisition and analysis parameters (e.g., voxel size, size of Gaussian function used for smoothing). However, all studies were included regardless of these differences, due to the small number of studies available. Where the data presented were insufficient for effect size calculations, a request for supplementary data was sent to the corresponding author. This led to data being available for 19 out of the 24 studies identified (10 DTI studies and 9 functional connectivity studies).

Effect size calculations were performed using two online effect size calculators *http://www.uccs.edu/~faculty/lbecker/* and

www.lyonsmorris.com/ma1/index.cfm Estimates of effect size were calculated based upon either descriptive data (i.e., mean, SD, and *N*), or statistical data (i.e., *t, F*). The purpose of this paper was to estimate differences in effect size rather than differences in direction of effect. That is we were interested in delineating the relative sensitivity of these two indices of brain connectivity to genetic variability, rather than accounting for the overall impact of a specific variant or group of variants. Therefore, direction of effect was not included in the analysis and all effect sizes were considered positive.

A random effects meta-analyses considering the relative difference in the impact of SZ risk genes on functional and structural connectivity was carried out using the comprehensive meta analysis (CMA; software package v2; www.meta-analysis.com). For the purposes of this analysis, Hedge's *g* and its associated variance were calculated for the outcome of each significant effect in each study. As with prior estimates of Cohen's d, g was calculated using a variety of input variables including descriptive and inferential statistics. In the first analysis, the largest effect for each study was chosen so to reflect the maximal sensitivity to gene effects within each investigation. In a secondary analysis, all of the effects for each significant result in each paper were taken into account. This strategy allowed us to account for both variability in the number and range of significant effects reported across methodologies.

RESULTS

Overall, 8 fMRI and 10 DTI studies were included in the meta-analysis. Summary information from all of these studies is presented in **Tables 1** and **2**.

FUNCTIONAL CONNECTIVITY

A total of 44 effect sizes were calculated from the functional connectivity studies. Effect sizes (i.e., Cohen's *d*) ranged from medium to large ($d = 0.46$–1.65) with an average effect size of 0.76 (SD $\pm$ 0.23). The largest effect size ($d = 1.65$) was reported for the impact of a single nucleotide polymorphism (SNP) in *ZNF804A* on functional connectivity within the right PFC in SZ patients (Rasetti et al., 2011). While large effect sizes ($d > 0.7$) were also calculated in other studies examining the effects of this SNP on functional connectivity (Esslinger et al., 2009, 2011; Rasetti et al., 2011) these results were not consistent: the smallest effect size was also reported for this SNP ($d = 0.46$; Paulus et al., 2011).

STRUCTURAL CONNECTIVITY

A total of 24 effect sizes were calculated for structural connectivity investigations. Effect sizes ranged from small to large ($d = 0.38$–1.95) with an average effect size of 1.04 (SD = 0.42). The largest effect size was revealed for the impact of *NRG1* SNP on WM integrity in the left ATR (Sprooten et al., 2009). Large effect sizes were also observed for all the other studies examining the impact of *NRG1* on WM integrity (all $d > 0.80$). Similar effect sizes were revealed for studies investigating the ErbB4 gene, with Cohen's *d* for these studies ranging from 0.81 to 1.41. Both the *MTHFR* gene and the *5-HTT* gene had large effect sizes of 1.29 and 0.92 respectively. The smallest effect size of 0.38 was computed for the effect of a *COMT* haplotype on left prefrontal WM integrity. Cohen's d for the *COMT* papers ranged from 0.38 to 0.76.

META-ANALYSIS

In our first meta-analysis we considered only the largest effect sizes in each study (**Figure 1**; **Table 3**). This analysis revealed no significant difference in outcome variability between the effect sizes for functional and structural studies ($Q = 2.171$, $p = 0.141$). Our second analysis examined all of the effects for each result in each paper (**Figure 2**; **Table 3**). This analysis revealed a significant difference between effects sizes in functional and structural studies ($Q = 6.928$, $p = 0.008$).

DISCUSSION

The aim of this review was to consider the nature and magnitude of effect of SZ risk variants on functional and structural connectivity. Our focus was the overall magnitude of such effects, rather than delineating the direction of effect of a specific variant. Therefore, we focused on the size, rather than the direction of individuals results. Examining the effect of risk variants on connectivity in fMRI and DTI studies, we found that variation in genes implicated in neurotransmission, plasticity, development and myelin function are associated with altered neural connectivity. Meta-analyses of effect size data revealed that there was no significant difference between the effect sizes of functional and structural studies when the largest effect size of each study was analyzed. However, when all effects were taken into consideration, the effect sizes for the structural studies were larger than in the functional connectivity studies, and there was a significant lack of homogeneity across the modalities. Mostly likely, given the absence of difference when only the largest effects from each set of studies are considered, this difference reflects the greater variation in effect sizes in structural studies compared to fMRI studies.

FUNCTIONAL CONNECTIVITY STUDIES

To date, the effects on functional connectivity of a number of candidate SZ genes (*DISC1*, *PRODH*, *PPP1R1B*) and one gene with genome-wide significance for SZ risk (*ZNF804A*), each of which were found to be associated with altered functional connectivity. The mean effect size for the functional connectivity studies was large ($d = 0.76$), with the largest effect size reported for the impact of the rs1344706 variant in *ZNF804A*.The ZNF804A risk variant rs1344706 has become the focus of much interest in SZ research over the last 3 years, as GWAS and follow-up analyses have established strong evidence for a link between this variant and risk for the disorder (O'Donovan et al., 2008). While the function of the *ZNF804A* gene is unknown, it has been speculated to play a role in gene regulation (O'Donovan et al., 2008) and glutamate and dopamine transmission (Esslinger et al., 2009). ZNF804A represents the only SZ-implicated gene that we are aware of whose effects on functional connectivity have been investigated now on a number of occasions. Importantly, the size of effect reported for this variant has varied considerably. For example, as well as having the highest effect of the variants considered it also has the lowest effect of the variants considered ($d = 0.46$; Paulus et al., 2011), suggesting that establishing the true effect of any variant on functional connectivity will require investigation in multiple and adequately powered cohorts. Variants from the candidate SZ gene literature by comparison, while each showing large effects on functional connectivity (*d*-range: 0.73–0.87), have each only been the subject of

Table 1 | Details of the functional connectivity studies included in this meta-analysis.

First author and date	Gene of interest	Connectivity	Method	Statistic	*n*	Cohen's *d*
Meyer-Lindenberg et al. (2007)	*PPP1R1B*	L. PFC – striatum, frequent haplotype carriers > non-frequent haplotype carriers	SC	4.41[†]	126	0.79
		R. PFC – striatum, frequent haplotype carriers > non-frequent haplotype carriers	SC	4.57[†]	126	0.82
		L. PFC – striatum, frequent haplotype carriers > non-frequent haplotype carriers	SC	4.31[†]	142	0.73
Kempf et al. (2008)	*PRODH*	dlPFC – striatum, reference haplotype carriers > protective haplotype carriers	SC	3.91*	103(108 total)	0.79
		dlPFC – striatum, risk haplotype carriers > protective haplotype carriers	SC	2.88*	48(108 total)	0.87
Di Giorgio et al. (2008)	*DISC1*	R. hippocampus – R. dlPFC, Ser/Ser > Cys carriers	PPI	3.58*	80	0.81
Esslinger et al. (2009)	*ZNF804A*	R. dlPFC – L. hippocampus, AA > CA > CC	SC	3.98[§]	115	0.75
		R. dlPFC – R. dlPFC, CC > CA > AA	SC	4.05[§]	115	0.77
		R. dlPFC – L. dlPFC, CC > CA > AA	SC	3.59[§]	115	0.68
Esslinger et al. (2011)	*ZNF804A*	R. dlPFC – L. MFG, CC > CA > AA	SC	5.09[‡]	111	0.98
		R. dlPFC – R. MFG, CC > CA > AA	SC	4.68[‡]	111	0.9
		R. dlPFC – R. SFG, CC > CA > AA	SC	4.5[‡]	111	0.86
		R. dlPFC – L. MFG, CC > CA > AA	SC	3.9[‡]	111	0.74
		R. dlPFC – L. hippocampus, AA > CA > CC	SC	4.28[§]	111	0.82
		R. dlPFC – R. hippocampus, AA > CA > CC	SC	3.34[§]	111	0.64
Walter et al. (2011)	*ZNF804A*	L. TPJ – L. inferior frontal gyrus, AA > CA > CC	SC	3.77*	109	0.73
		L. TPJ – L. cuneus, AA > CA > CC	SC	3.76*	109	0.73
		L. TPJ – R. thalamus, AA > CA > CC	SC	3.68*	109	0.72
		L. TPJ – L. caudatetail, AA > CA > CC	SC	3.96*	109	0.77
		R. dlPFC – R. precentral gyrus, CC > CA > AA	SC	4.27*	109	0.83
		R. dlPFC – L. MTG, CC > CA > AA	SC	3.32*	109	0.65
		R. dlPFC – L. LG, CC > CA > AA	SC	3.83*	109	0.74
Paulus et al. (2011)	*ZNF804A*	R. dlPFC – L. HF, AA > CA > CC	SC	2.3*	94	0.48
		R. dlPFC – L. HF, AA > CA > CC	SC	2.22*	94	0.46
		R. dlPFC – R. HF, AA > CA > CC	SC	2.85*	94	0.59
		R. dlPFC – R. HF, AA > CA > CC	SC	2.19*	94	0.46
		R. dlPFC – L. dlPFC, AA > CA > CC	SC	3.42*	94	0.71
		R. dlPFC – L. dlPFC, AA > CA > CC	SC	2.26*	94	0.47
		R. dlPFC – R. dlPFC, AA > CA > CC	SC	2.39*	94	0.5
		R. dlPFC – R. dlPFC, AA > CA > CC	SC	2.33*	94	0.49
		R. dlPFC – R. dlPFC, AA > CA > CC	SC	2.33*	94	0.49
		R. dlPFC – R. dlPFC, CC > CA > AA	SC	2.43*	94	0.51
Rasetti et al. (2011)	*ZNF804A*	Controls: R. dlPFC – L. HF, CC > CA > AA	SC	2.72*	96	0.56
		Controls: R. dlPFC – L. dlPFC, CC > CA > AA	SC	3.65*	96	0.75
		Controls: R. dlPFC – R. PFC, CC > CA > AA	SC	3.21*	96	0.66
		Controls: R. dlPFC – L. hippocampus, CC > CA > AA	PPI	3.74[§]	96	0.77
		Controls: R. dlPFC – R. hippocampus, CC > CA > AA	PPI	2.89[†]	96	0.6
		Siblings: R. dlPFC – R. hippocampus, AA – abnormal coupling	SC	2.53*	83	0.57
		Siblings: R. dlPFC – L. PFC, AA < C carriers	SC	2.77*	83	0.62
		Siblings: R. dlPFC – R. dlPFC, AA – greater task-related modulation of coupling	PPI	4.36[§]	83	0.98
		Patients: R. dlPFC – R. PFC, CC > CA > AA	SC	4.58[§]	33	1.65
		Patients: R. dlPFC – L. hippocampus, AA < C carriers	PPI	3.56[§]	33	1.28
		Patients: R. dlPFC – L. PFC, CC > CA > AA	PPI	2.84*	33	1.02
		Patients: R. dlPFC – R. PFC, CC > CA > AA	PPI	3.40*	33	1.22

*n, sample size; SC, seeded connectivity; PPI, psychophysiological interaction; dlPFC, dorsolateral prefrontal cortex; MFG, middle frontal gyrus; SFG, superior frontal gyrus; TPJ, temporo-parietal junction; MTG, middle temporal gyrus; LG, lingual gyrus; IFG, inferior frontal gyrus; *p-value is uncorrected for multiple comparisons; [†] false discovery rate corrected within region of interest; [‡] false discovery rate corrected for whole brain; [§] family wise error corrected within region of interest.*

Table 2 | Details of the structural connectivity studies using DTI included in this meta-analysis.

First author and date	Gene	Connectivity	Statistic (*t* or *F*)	*N*	Cohen's *d*
McIntosh et al. (2008)	*NRG1* SNP8NRG243177	Reduced FA in ALIC	$t = 2.65$*	43	0.83
Winterer et al. (2008)	*NRG1* SNP8NRG221533	Reduced FA in MF subcortical WM	$t = 4.67$***	50	1.35
Sprooten et al. (2009)	*NRG1* SNP8NRG221533	Reduced FA in left ATR	$t = 5.52$***	28	1.95
	NRG1 SNP8NRG243177	Reduced FA in left ATR	$t = 4.69$***	28	1.66
Wang et al. (2009)	*NRG1* SNP8NRG221533	Reduced FA in anterior cingulum	$F = 5.27$*	31	0.86
	NRG1 SNP8NRG221533	Reduced FA in anterior cingulum	$F = 18$***	34	1.54
Konrad et al. (2009)	*ErbB4* rs707284	Reduced FA in temporal lobe WM	$t = 4.24$***	50	1.22
	ErbB4 rs758440	Reduced FA in temporal lobe WM	$t = 2.81$***	50	0.81
	ErbB4 rs839541	Reduced FA in temporal lobe WM	$t = 4.31$***	50	1.24
	ErbB4 rs839523	Reduced FA in temporal lobe WM	$t = 4.73$***	50	1.37
	G-T-G-T versus lower risk	Reduced FA in temporal lobe WM	$t = 3.85$***	32	1.41
	G-T-G-T versus all other	Reduced FA in temporal lobe WM	$t = 3.2$***	50	0.92
	All other versus non-risk	Reduced FA in temporal lobe WM	$t = 3.66$***	50	1.057
Zuliani et al. (2011)	*ErbB4* rs4673628	Reduced FA in right ALIC	$t = 3.48$*$^{?}$	36	1.19
	ErbB4 rs4673629	Reduced FA in left ALIC	$t = 3.98$*$^{?}$	36	1.37
Thomason et al. (2010)	*COMT* val158met	Main effect of genotype on FA, AD, RD in GCC	$F = 3.04$*	40	0.76
	COMT val158met	Main effect of genotype on FA, AD, RD in ATR	$F = 2.79$*	40	0.7
	COMT val158met	Main effect of genotype on FA, AD, RD in UF	$F = 2.47$*	40	0.6
Liu et al. (2010)	*COMT* val158met	Decreased FA in right CST for Val/Val carriers	$F = 5.197$*	79	0.51
	LPS, HPS and APS haplotypes	Association with mean FA in left PF lobe	$F = 2.79$*	68	0.38
	LPS, HPS and APS haplotypes	Association with mean FA in right PF lobe	$F = 3.58$*	68	0.47
	LPS, HPS and APS haplotypes	Association with mean FA in right UF	$F = 3.507$*	68	0.47
Roffman et al. (2011)	*MTHFR* 677T	Reduced FA in bilateral DACC	$F = 6.59$*	18	1.29
Pacheco et al. (2009)	*5-HTTLPR*	Increasing number of low expressing alleles – decreasing FA in left FUF	$t = -3.03$*	37	−0.92

*n, sample size; FA, fractional anisotropy; MF, medial frontal; WM, white matter; ALIC, anterior limb of internal capsule; AD, axial diffusivity; RD, radial diffusivity; ATR, anterior thalamic radiation; UF,uncinate fasciculus; GCC, genu of corpus callosum; CST, corticospinal tract; PF, prefrontal; DACC, dorsal anterior cingulate cortex; FUF, frontal uncinate fasciculus; *p < 0.05; ***p < 0.001; *$^{?}$p < 0.05 family wise error corrected*

single studies to date (*DISC1*; Di Giorgio et al., 2008; *PRODH*; Kempf et al., 2008; *PPP1R1B*; Meyer-Lindenberg et al., 2007).

In 10 of the 12 fMRI studies included in this review, SZ risk variants were reported to affect functional circuits that included the PFC during the performance of a variety of tasks, such as memory encoding and retrieval, working memory, emotion processing, and during rest. These findings reflect the PFC's dominant role in many processes related to higher cognitive functioning, making it consistently implicated in SZ pathogenesis (Callicott et al., 2000).There are several possibilities for why PFC function is altered in SZ as reflected in these studies. For example, the "*reduced neuropil hypothesis*" of schizophrenia suggests that reduced PFC gray matter (observed in the absence of a concomitant change in cell numbers)may reflect decreased dendritic spines/axon terminals; the inefficiencies synaptic transmission expected to result may well lead to the altered functional connectivity patterns seen here (Selemon and Goldman-Rakic, 1999).

STRUCTURAL CONNECTIVITY STUDIES

A number of candidate SZ risk genes have been investigated in terms of their effects on structural connectivity. This includes genes that are involved in myelination (*NRG1, ErbB4*) neurotransmission (*COMT, MTHFR, 5-HTTLPR*) and neurodevelopment (*BDNF* and *DISC1*). Almost all variants considered here were associated with significant variation in FA scores using DTI.

The average effect size for these studies was large, with the largest effect size computed for the impact of NRG1 on FA in the left ATR (Sprooten et al., 2009). The NRG1 gene codes for the *NRG1* protein, that is involved in growth and differentiation of neuronal and glial cells and is necessary for the normal development of the nervous system. *ErbB4* is a receptor for the *NRG1* protein. It is thought that NRG1 may mediate its effects on SZ susceptibility through functional interaction with ErbB4 (Norton et al., 2006). Interestingly, ErbB4 was also observed here to show effects on WM integrity that would be considered to be in of large magnitude. The role of these genes in myelin function suggests a mechanism by which they confer risk for SZ. The relatively large impact of these genes on structural connectivity, noted here, suggests that genetically mediated "dysconnectivity" in SZ results from macro-circuit WM abnormalities in addition to micro-circuit synaptic plasticity. However, since oligodendrocyte dysfunction may also impact synaptic function and information processing via a myelin-dependent impact on synaptic plasticity (Fields, 2008), it remains to be established if the influence of these variants is specific to structural connectivity or if they also impact upon functional connectivity.

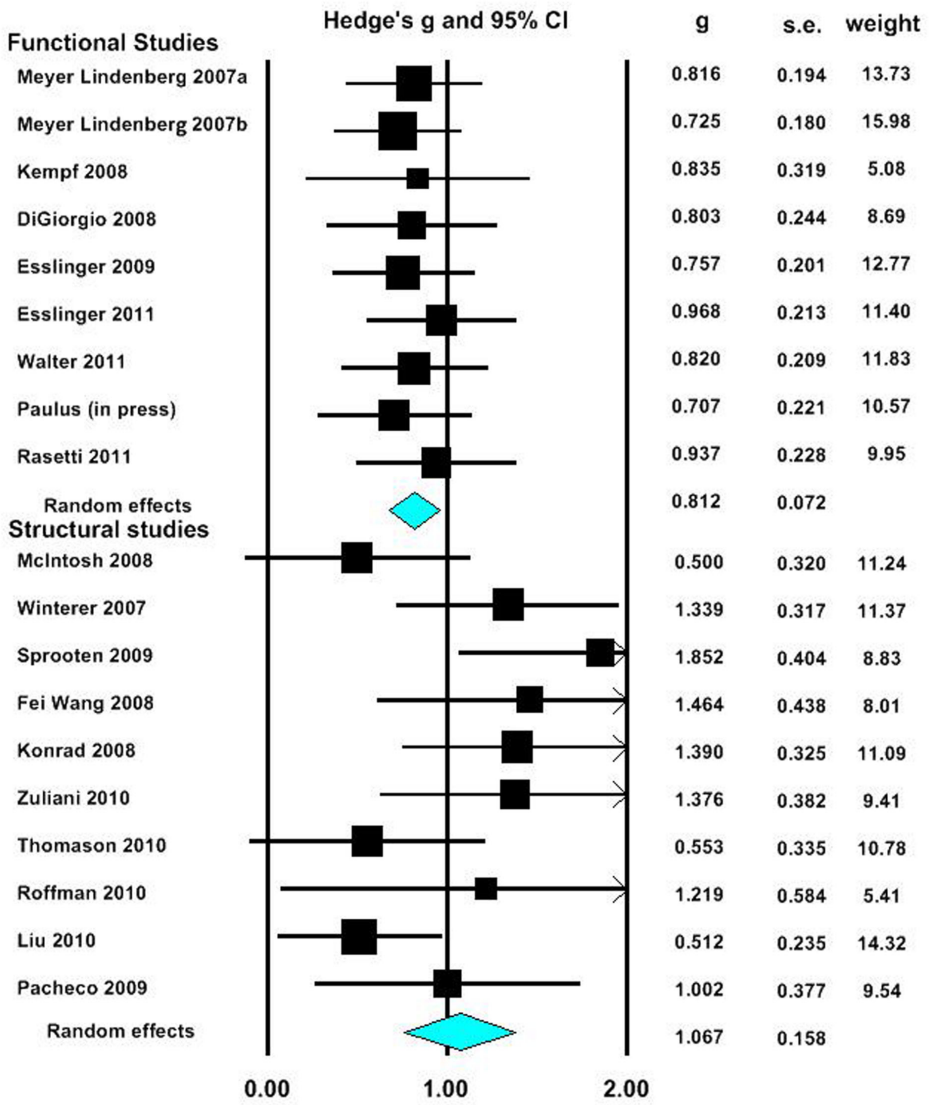

FIGURE 1 | Forest plot reporting Hedges' *g* and 95% CI for the analysis showing the largest effect size in each paper. CI, confidence interval; g, Hedges' *g*; SE, standard error.

Table 3 | Results of random-effects meta-analysis comparing the relative difference in the impact of variants on functional and structural connectivity.

	Effect size and 95% confidence interval						Heterogeneity		
	No. of studies	**Point estimate**	**SE**	**Variance**	**Lower limit**	**Upper limit**	***Q*-value**	**df (*Q*)**	***p*-value**
MAXIMUM ESTIMATE									
Functional	9	0.812	0.072	0.005	0.671	0.953			
Structural	10	1.067	0.158	0.025	0.758	0.377			
Total between							2.171	1	0.141
ALL ESTIMATES									
Functional	44	0.687	0.032	0.001	0.625	0.750			
Structural	24	0.934	0.088	0.008	0.761	1.108			
Total between							6.928	1	0.008

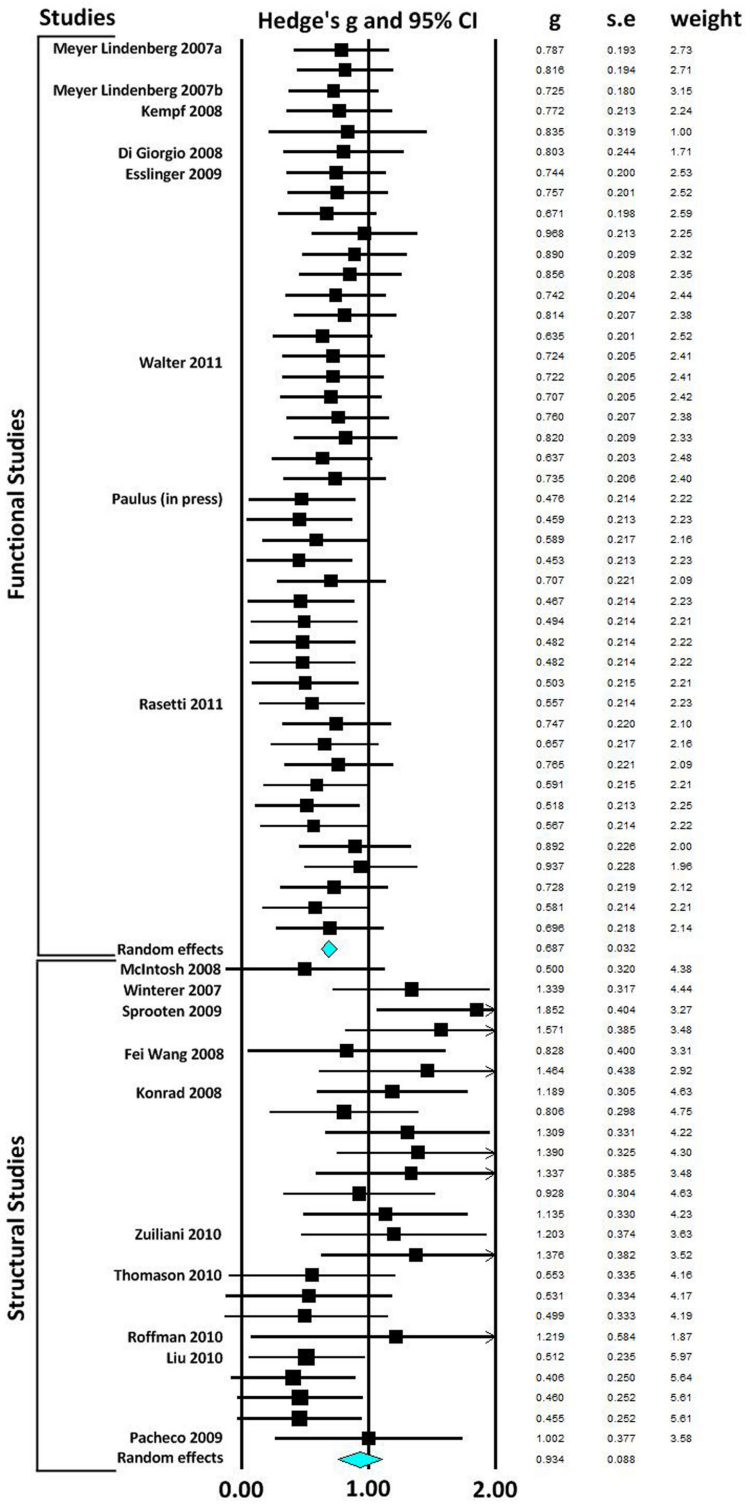

FIGURE 2 | Forest plot reporting Hedges' *g* and 95% CI for each functional and structural connectivity analysis. CI, confidence interval; g, Hedges' *g*; SE, standard error.

Other gene variants associated with variation in WM connectivity included COMT, MTHFR, 5-HTTLPR, BDNF and DISC1. Results for both the *MTHFR* gene and the *5-HTT* gene had large effect sizes. The *MTHFR* gene codes for the an enzyme that plays

a role in the regulation of intracellular methylation reactions and may influence dopamine signaling (Roffman et al., 2008). The hypofunctional 677T variant of this gene has been associated with increased SZ risk (Gilbody et al., 2007). The reuptake of serotonin to the presynaptic neuron for recycling or degradation after serotonin release is regulated by the serotonin transporter (*5-HTT*). Although the *5-HTTLPR* polymorphism has been found to be associated with SZ in a South India population (Vijayan et al., 2009), other genetic association studies have given conflicting results (Rao et al., 1998). The smallest effect size was computed for the effect of a *COMT* haplotype on left prefrontal WM integrity (Liu et al., 2010). The COMT gene codes for an enzyme that is involved in the degradation of dopamine. Therefore, the evidence from DTI studies investigating the impact of the COMT gene on WM indicates that neurosignalling processes involved in SZ may also impact structural connectivity. Finally, *BDNF* (Chiang et al., 2011) and *DISC1* (Sprooten et al., 2011) which are genes that are crucial for neurodevelopment, were also associated with WM connectivity in SZ. However, as insufficient data was available, effect sizes for these studies were not calculated.

META-ANALYSIS

The papers included in this review most commonly report a large effect of gene variants on functional and structural connectivity. This result is similar to previous meta-analyses in imaging genetics, which reported large effect sizes of gene variants (i.e., *5-HTTLPR* polymorphism and the *COMT* Val158Met polymorphism) on brain function (Munafo et al., 2008; Mier et al., 2010). This result is also consistent with the intermediate phenotype hypothesis that common SZ risk variants will show small effects on behavior and disease risk, but large effects at the level of the brain (Tost et al., 2011).

When the maximum effect size value for each paper in our meta-analysis was compared between fMRI and DTI studies, no significant difference was found between these measures. As only a small number of studies were obtained, there may be a lack of power to detect such differences. However, examination of effect sizes for all significant effects indicate that structural connectivity studies were associated with overall larger and more variable effect sizes. This suggests that measures of structural connectivity, such as DTI, may be sensitive to a wider range of effects compared to functional connectivity measures, which may only be able to accurately detect large effects. This result may also indicate that structural connectivity is closer to the level of genes than functional connectivity.

LIMITATIONS

A number of limitations need to be considered in evaluating the findings of the present study. Firstly, many of the studies included in the meta-analysis have examined the effects of polymorphisms that do not have consistent association with SZ phenotypes. This makes it difficult to determine the relevance of these genes for our understanding of SZ pathogenesis (for a review, see Meyer-Lindenberg, 2010). Secondly, it should also be noted that the sample sizes included in these studies are relatively small and thus, are under powered to detect differences in brain connectivity conferred by individual variants. Due to the interplay between sample size, power, and effect size, smaller studies generally show larger effects in meta-analyses (Sterne et al., 2000) and may lack sufficient power to detect smaller effects. Related to the general issue of sample size, it is important to note that the average sample size of the studies utilizing DTI was smaller than that for the functional studies. As a result, the effect sizes for the structural papers may be over-inflated. However, the results of our meta-analysis suggest that despite smaller samples, the structural imaging studies were associated with a wider range of effects, suggesting that sample size is not the only factor at play here.

Due to the under-representation of publications with negative results, the studies included in this review may not be representative of connectivity research in its entirety, but rather a bias toward only publish papers showing statistically significant results. Therefore, while our effect size findings are calculated on the basis of published effect sizes, it is possible that the true effect sizes are smaller, and to an extent that is unknown. Similarly, it is also unclear to what extent differences in scanning parameters between the studies included in this meta-analysis influenced results. More systematic investigation of these differences will in the future be possible with the accumulation of more studies.

An additional limitation in the studies considered here is that each investigation examined the effects of only one particular variant. However, the true function of these genes may be affected by additive or epistatic interactions with other variants. As such, the results presented in this review may be incomplete without taking these interactions into account (Nicodemus et al., 2010).

Finally, it is probable that these results could be impacted by differences in functional and structural methodological approaches. For example, a number of analysis methods can be employed to measure functional connectivity between brain regions. However, we are not currently aware of the relative strengths and weaknesses of these different approaches. There are also various approaches used to quantify WM connectivity using DTI, which also pose different strengths and limitations (see Jones, 2010 for a review).

CONCLUSIONS AND FUTURE DIRECTIONS

In a short period of time, imaging genetics has made important progress in delineating genetic effects on neural connectivity. In particular, it has established neural connectivity as a key intermediate phenotype for SZ, which can be used to explore the complex trajectory from genetic risk to clinical symptoms.

Despite the progress that has taken place, we believe important advances can be made in this research field in four key areas. Firstly, future studies should examine the effects of gene variants on neural connectivity in larger sample sizes, as this can provide the extra statistical power that may be necessary to detect smaller effects of these genes. Multi-site research projects, such as the IMAGEN project in Europe, may be particularly suited for compiling imaging and genetic databases of thousands of subjects (Schumann et al., 2010). Secondly, future studies should examine additive and epistatic effects of gene variants on neural connectivity, as these variants are unlikely to be working in isolation. Thirdly, future studies should examine the effects of risk variants in healthy controls and SZ patients as the opposite effects of these genes on connectivity can be found in these different groups (e.g., Prata et al., 2009).

Finally, future studies could benefit from the novel application of recently developed analysis techniques to imaging genetics. For example, DCM hold potential for constructing models of changing brain interactions that also take into account genetic variation (Meyer-Lindenberg, 2009). Other recent advances include the use of parallel ICA to simultaneously analyze independent components derived from fMRI and genetic data (Liu et al., 2009). For example, Meda et al. (2010) used this technique in a pilot study to identify simultaneous independent components of fMRI data and SNP data, derived from a sample of 35 controls and 31 SZ patients. The authors found correlations between different neural networks and a number of SNPs, including polymorphisms involved in altered dopamine transmission. While the authors only included a small number of SNPs and a small sample size, this research suggests a powerful new approach for future studies examining the effects of SZ risk variants on functional brain networks. Similarly, more advanced DTI techniques could be implemented that use high angular resolution to account for multiple crossing fibers within a single voxel. Such imaging techniques include Q-space approaches and mixture models (Tournier et al., 2011). These models provide mathematical alternatives to the tensor model for the characterization of diffusion processes. Furthermore, Jones (2010) recommends the integration of DTI with other measures of WM such as measures of axon density and myelination that can be acquired using techniques such as magnetization transfer or multicomponent relaxometry.

In conclusion, the present meta-analysis examined the nature and magnitude of effect of SZ risk variants on functional and structural connectivity. Gene variants impacting upon both synaptic plasticity and axonal connectivity have been associated with altered neural connectivity in patients and healthy controls. As such, it is likely that both mechanisms make important contributions to SZ pathogenesis. On average, risk variants exert a large effect on functional and structural connectivity. There is also more variability in the effects of variants on structural connectivity, compared to functional connectivity. While imaging genetics has made considerable progress in the field of neural connectivity in a short period of time, important advances are still to be made. It is hoped that this research will lead to a better understanding of the biological mechanisms mediating genetic risk for SZ, which can then be used to direct novel treatments for the disorder.

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The long and the short of it: gene and environment interactions during early cortical development and consequences for long-term neurological disease

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Cortical development is a complex amalgamation of proliferation, migration, differentiation, and circuit formation. These processes follow defined timescales and are controlled by a combination of intrinsic and extrinsic factors. It is currently unclear how robust and flexible these processes are and whether the developing brain has the capacity to recover from disruptions. What is clear is that there are a number of cognitive disorders or conditions that are elicited as a result of disrupted cortical development, although it may take a long time for the full pathophysiology of the conditions to be realized clinically. The critical window for the manifestation of a neurodevelopmental disorder is prolonged, and there is the potential for a complex interplay between genes and environment. While there have been extended investigations into the genetic basis of a number of neurological and mental disorders, limited definitive associations have been discovered. Many environmental factors, including inflammation and stress, have been linked to neurodevelopmental disorders, and it may be that a better understanding of the interplay between genes and environment will speed progress in this field. In particular, the development of the brain needs to be considered in the context of the whole materno-fetal unit as the degree of the metabolic, endocrine, or inflammatory responses, for example, will greatly influence the environment in which the brain develops. This review will emphasize the importance of extending neurodevelopmental studies to the contribution of the placenta, vasculature, cerebrospinal fluid, and to maternal and fetal immune response. These combined investigations are more likely to reveal genetic and environmental factors that influence the different stages of neuronal development and potentially lead to the better understanding of the etiology of neurological and mental disorders such as autism, epilepsy, cerebral palsy, and schizophrenia.

Keywords: neurogenesis, neuronal migration, blood brain barrier, glia, maternal inflammation

INTRODUCTION

The development of the brain is determined by a complex interplay of intrinsic genetic programs and environmental interactions and *in utero* depends additionally on numerous materno-fetal interactions. Circulation, oxygenation, metabolic interactions, and immune responses are all orchestrated between the fetus and the mother and largely work in harmony. However, when these interactions malfunction, they could contribute to developmental abnormalities. The unfolding genetic program of cortical development is influenced by these environmental factors in a highly complex fashion. While the developing brain is kept separate from the systemic circulatory system by the brain barriers, and the maternal circulation is separated from the fetal circulation in the placenta, it is clear that changes in the maternal/intrauterine and systemic fetal environments may result in modifications of normal fetal brain development. Therefore, neurological and mental disorders have an array of pathophysiological hallmarks that reflect a complex etiology. These disorders are typically diagnosed by clinical features, as the genetics and molecular basis of the diseases are still largely opaque. There is an emerging field that studies these interactions in the context of barrier biology, immunology, endocrinology, teratology, and developmental neurobiology. The challenge is to relate the genetic and molecular program of neurodevelopment to the various insults (including fetal alcohol and drug exposure, inflammation and hypoxic-ischemia) for the better understanding of the pathophysiological-mechanisms of these developmental disorders. The aim of this review is to discuss the normal cortical developmental program within the context of the developing fetal and maternal environments and give insights to some potential environmental-genetic interactions that contribute to neurodevelopmental disorders. Of particular interest is the possibility that one environmental insult could contribute to multiple diseases, depending on the stage of brain development affected. For example, maternal alcohol consumption or infection can have a specific time window when the consequences are more severe. For the purpose of this review we divide development into four stages. The first three correspond to key periods in neurogenic development: (i) proliferation, (ii) migration, and (iii) initial differentiation. In humans this corresponds to (i) 1–7 weeks, (ii) 8–15 weeks and, weeks 16–26, although the true boundaries of

these processes are not this absolute. We shall also discuss a fourth stage, which in humans is from the 26th week into the postnatal period. This stage largely represents the influence of other cell systems on neuronal functions, such as myelination and activity dependent circuit formation. **Boxes 1** and **2** give summary of the fundamentals of early cortical development for the general reader. The concomitant development of different components of the CNS will be discussed as well as the known pathological consequences of environmental insults during these periods.

MATERNAL/FETAL ENVIRONMENT

MATERNAL SIGNALING

Fetal development is greatly dependent on the maternal environment and it is clear that a spectrum of insults can produce changes in the fetus that include mild fetal growth restriction, through to severe birth defects or still birth. A number of maternal complications have been specifically related to negative fetal outcomes, including pre-eclampsia, depression, diabetes, infection/inflammation, and extremes in body weight (Barker, 2007; Hoerder-Suabedissen et al., 2008; Zammit et al., 2009).

Congenital abnormalities, including cardiovascular and neural tube defects, are more common in offspring of overweight or diabetic mothers, potentially due to the teratogenic effects of hyperglycemia and relaxin (Hawdon, 2011). These maternal conditions can also alter both the systemic and central control of metabolism in the offspring (Meaney et al., 2007; Sirimi and Goulis, 2010; Gluckman et al., 2011; Hawdon, 2011) and may even lead to epigenetic modulation of neural genes regulating

Box 1 | Fundamentals of cerebral cortical development.

PROLIFERATION

The telencephalon, which later develops into the cortex and basal ganglia, starts to generate neurons in the mouse from approximately E10.5, with neurogenic divisions occurring from E11–E17 (Rakic and Sidman, 1968). Proliferative cell division occurs first at the ventricular surface (in the ventricular zone, VZ) and then starts basal to this zone, in the subventricular zone (SVZ; **Figure 4**). As neurogenesis progresses cell division switches from a symmetric, proliferative division to an increasingly neurogenic division (Konno et al., 2008). Once neurogenesis is complete, the same pluripotent progenitors produce glia. During this period there is also vascularization of the telencephalon, invasion of microglia, and migration of GABAergic neurons from the ventral to the dorsal telencephalon (Earle and Mitrofanis, 1997; Parnavelas, 2000; Stubbs et al., 2009).

The different rounds of division in the proliferative zones are responsible for the "birth" of different cortical neuronal populations, which migrate to establish the cortical layers. A combinatorial transcription factor signaling specifies the future phenotype of cerebral cortical neurons (Guillemot et al., 2006; Hevner et al., 2006). The timing of this is largely based on intrinsic genetic programming, as cell populations grown *in vitro* will differentiate into layer populations after the same number of divisions as observed *in vivo* (Shen et al., 2006).

MIGRATION

The cortex develops in an "inside-first-outside-last" fashion, with deep cortical layers born first, and superficial cortical layers born last (Rakic and Sidman, 1968). Cells that are born in the VZ or SVZ proliferative zones migrate into the cortical plate (CP) along the basal projections of the radial glia (Métin et al., 2008). The first cell populations to migrate out of the proliferative zones are the cells of the preplate which are visible as early as E12, and which split to form the marginal zone and the subplate (Marin-Padilla, 1971). Following this, waves of division occur and differentiating neurons migrate past the subplate to form subsequent layers of cortical pyramidal projection neurons (Bystron et al., 2008). The contribution of the VZ and SVZ to the cortical layers is a subject of contention. In general it is thought that VZ progenitors contribute neurons to the lower cortical layers (V and VI) and to the SVZ, while the SVZ is the primary source of upper cortical layers (IV and II/III; Tarabykin et al., 2001; Wu et al., 2005), but there is evidence for SVZ neurogenesis for all layers (Kowalczyk et al., 2009).

There is a degree of plasticity in the genetically programmed cell fate, where extrinsic signaling factors such as neurotrophins (including nerve growth factor, brain-derived neurotrophic factor, neurotropin-3, and neurotrophin-4) can alter the differentiation of late generated neurons (Fukumitsu et al., 2006). The degree of plasticity appears to decrease, and may be restricted (Desai and McConnell, 2000). There are a number of examples where late born neurons may take on protein expression of early cortical layer subtypes, but still migrate to their later cortical layer (Fukumitsu et al., 2006).

DIFFERENTIATION

Recent studies have elucidated a large number of molecules that are associated with specific cortical layers (Guillemot et al., 2006; Molyneaux et al., 2007; Belgard et al., 2011), in some cases these genes (e.g., Satb2, Ctip2, Fezf2) appear to be involved in fate specification of neuronal precursors, and are expressed in the proliferative zones at the time of layer birth and then continue in the differentiated neuronal population (Alcamo et al., 2008; Britanova et al., 2008; Chen et al., 2008).

Full maturation of the cortex requires cellular differentiation, target connection, and strengthening. The majority of this process occurs in the late embryonic and postnatal period in rodents. In humans it can continue into adolescence. The glutamatergic CP neurons will integrate with the GABAergic interneurons into functional circuits (Fishell and Rudy, 2011).

The early steps of cerebral cortical circuit formation are orchestrated by subplate (Kostovic and Rakic, 1990). In mouse, subplate neurons are a largely transient population of neurons that sit at the base of the CP, in layer VIb in the postnatal brain (Hoerder-Suabedissen et al., 2009). The subplate forms the earliest cortical projections and appears to act as a scaffold for the establishment of connections with subcortical structures, e.g., the thalamus (Allendoerfer and Shatz, 1994; Molnár and Blakemore, 1995). It has recently been hypothesized that these cells have different roles in the brain depending on the developmental stage, and that earlier in telencephalic development the subplate is key in guiding thalamic projections, while at later stages the remaining subplate cells may integrate more into cortical networks to orchestrate the development of the ultimate cortical circuits (Kanold and Luhmann, 2010).

Box 2 | Progenitors of the developing cortex.

As the telencephalic vesicles expand from the neural tube the ventricular surface is composed of neuroepithelial progenitors. These cells undergo symmetric proliferative divisions to increase the pool of progenitor cells (Breunig et al., 2011). They are considered to be neural progenitor cells, as their daughter cells are able to produce a number of different cell types (reviewed in Pinto and Götz, 2007). However, unlike other stem cell populations, the neuroepithelial cells are heterogeneous and appear to be restricted both in number of potential divisions and the fate of daughter cells (Williams and Price, 1995; Gal et al., 2006; Lui et al., 2011). As the cortex develops, the proliferative population in the ventricular zone undergoes changes. These changes include a slight loss of the apical-basal polarity that is so characteristic of the neuroepithelial cells, and are considered to be required for the switch from proliferative to neurogenic divisions (Aaku-Saraste et al., 1996; Lui et al., 2011).

A number of factors combine over a very short period of development to allow division to switch from symmetric, proliferative division to asymmetric, neurogenic division (where one progenitor and one neuron are produced from a mitotic event; Sahara and O'Leary, 2009). The division of the cell contents, and most particularly the apical polarity and adherence components, appears to be responsible for whether division will be symmetric or asymmetric (Farkas and Huttner, 2008). Current evidence suggests that even distribution of the cell contents, characterized by vertical mitotic division in the early developing cortex, is associated with symmetric, proliferative division. In comparison, uneven distribution, visible by an angled or horizontal mitotic cleavage, is associated with asymmetric, neurogenic divisions (Zhong and Chia, 2008; Fietz and Huttner, 2011).

Another factor that appears important for the switch from proliferative to neurogenic division is the length of the cell cycle. There is clear evidence that radial glial progenitors undergoing neurogenic division have a longer G1 phase than neuroepithelial cells undergoing symmetric, proliferative divisions, and that experimentally lengthening the G1 phase in neuroepithelial progenitors will induce neurogenesis (Hartfuss et al., 2001; Lukaszewicz et al., 2002; Calegari et al., 2005; Wilson et al., 2011).

Once neurogenesis has started in the VZ, basal progenitor cells can also be observed in the SVZ. Basal progenitors have lost their apical projections and undergo division at the basal margin of the VZ. They typically undergo symmetric, neurogenic proliferation and are regulated by a different set of transcription factors, including Tbr2 (Noctor et al., 2004; Hevner et al., 2006).

behavior (Keverne, 2010). Maternal psychosocial stress has been found to result in altered fetal weight, insulin resistance, metabolic, immune and endocrine function, and decreased cognitive performance (Entringer et al., 2010) which is likely to be mediated through glucocorticoid exposure and regulation (Meaney et al., 2007; Harris and Seckl, 2011). Hypoxia, while a normal part of placental vascularization, can become pathological in pre-eclampsia (Redman and Sargent, 2005) or inflammatory conditions such as malarial infection (Rogerson and Boeuf, 2007). Chronic intrauterine hypoxia will result in reduced fetal growth and ischemic brain damage (Redman and Sargent, 2005; Duncan et al., 2006; Rogerson and Boeuf, 2007; Gunn and Bennet, 2009). Cytokines are also a normal part of the regulatory system during pregnancy (Orsi and Tribe, 2008), and like hypoxia, dysregulation of the cytokine response locally or systemically has been associated with poor fetal outcome (Orsi and Tribe, 2008). Chorioamnionitis is a well-characterized maternal inflammatory condition, which increases circulating cytokines and has been associated with developmental brain damage (Impey et al., 2001). There is clear evidence that changes in fetal, rather than maternal, inflammation correlates more reliably with severity of neurological outcome (e.g., Yoon et al., 2000). The contribution of the fetal environment to neurological damage will be discussed below.

ROLE OF THE PLACENTA

Placental tissue is of trophoblast origin, but has maternal and fetal compartments, based on the domains of circulating blood. The umbilical arteries, veins, and the fetal capillaries make up the fetal circulation, while the maternal blood enters the intervillous spaces from the spiral arteries and exit via the uterine veins (Schoenwolf and Larsen, 2009). The cells that border the intervillous space, including syncytiotrophoblasts and cytotrophoblasts, provide a barrier between the maternal and fetal circulation (**Figure 1**) that change during pregnancy to meet the increased demand of the growing fetus (Neerhof and Thaete, 2008). The placenta contributes to many aspects of normal fetal development and early life programming (Murphy et al., 2006), and has been shown to contribute directly to early brain development by providing morphogenic serotonin (Bonnin et al., 2011). Monoamine neurotransmitters start to accumulate within the cortex from E17.5 through to P30 in the mouse (Cheng et al., 2010), 5-HT in particular at the earliest stages of this period. Knockout (KO) of the monoamine oxidases has been shown to modulate proliferation in the intermediate progenitor cells within the cortex at E17.5 and in the progenitor cells within the SVZ at P2 and in the adult (Cheng et al., 2010). Studies of Bonnin et al. (2011) suggest a new, direct role for placental metabolic pathways in modulating fetal brain development. Understanding such maternal-placental-fetal interactions and 5-HT may hold the key to the understanding of the etiology of some adult mental disorders.

Placental insufficiency has generally been retrospectively diagnosed following evidence of intrauterine growth restriction (IUGR; Huppertz et al., 2006), and may partly explain the high association between IUGR and brain dysfunction (Rees et al., 2008; Raikkonen and Pesonen, 2009). Maternal inflammation, which has also been clearly linked to brain damage in the developing offspring, has been found to cause reduced placental blood flow and may cause damage through a hypoxic as well as inflammatory pathway (Girard et al., 2010). However, low-level continuous inflammation in the fetus can cause brain damage without hypoxia (Duncan et al., 2006). It is generally considered that the placenta provides a barrier that prevents direct infection of the fetus (Ashdown et al., 2006; Hutton et al., 2008). However, Dahlgren et al. (2006) and Hsiao and Patterson (2011) have shown that maternally produced IL-6 can be found in the fetal circulation and correlates with long-term behavioral deficits in the offspring, suggesting that

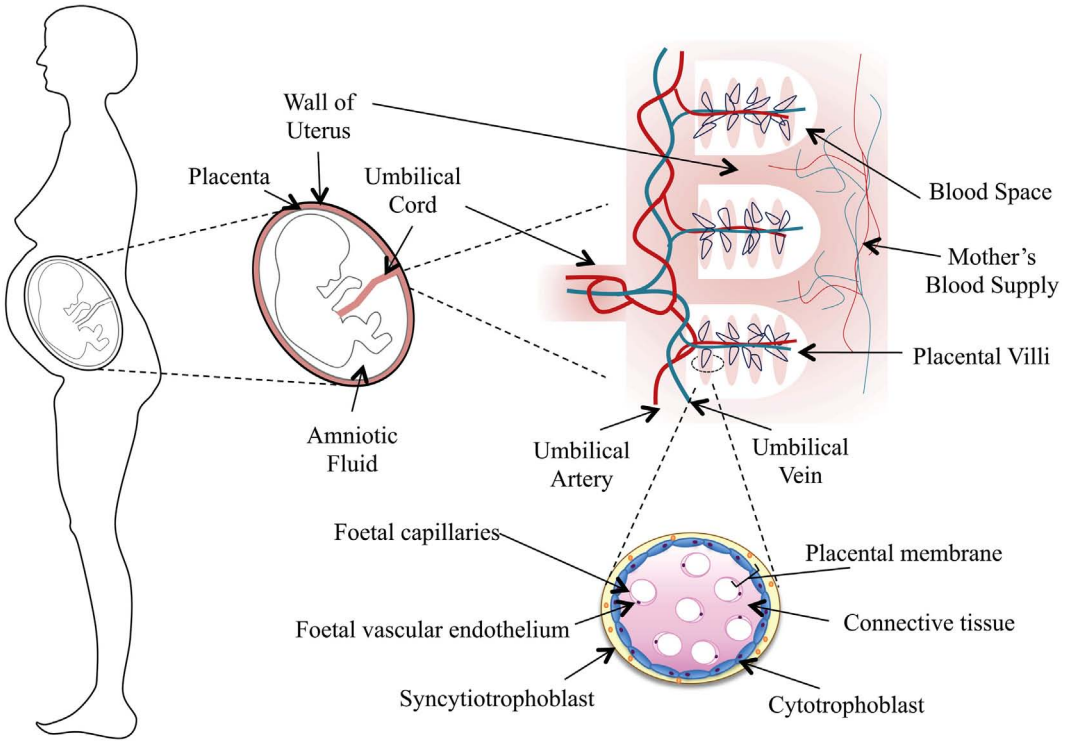

FIGURE 1 | Schematic illustration of the fetal environment and placental structure. The fetus develops in the uterus isolated from the maternal circulation through the materno-fetal barrier in the placenta. The maternal blood enters the intervillous spaces from the spiral arteries. There is transfer from the intervillous space across the placental barrier to the fetal capillary network that joins the umbilical vessels. The syncytiotrophoblasts, cytotrophoblasts, and connective tissue that make up the placental membrane are the key structures of the placental barrier.

inflammation is still able to pass from the mother to the fetus despite this barrier.

FETAL IMMUNE RESPONSE

There is extensive epidemiological and experimental evidence suggesting that the presence of inflammatory mediators in the fetal circulation, particularly those produced by the fetus, are the best predictors of fetal brain damage (Yoon et al., 2000; Badawi et al., 2006; Elovitz et al., 2011). The maturity of the fetal immune response may therefore contribute to the outcome of maternal/intrauterine insults. Circulating inflammatory cells, including T-cells, B-cell, and macrophages, are produced as early as 7 weeks in humans (approximately E10 in mice; Melchers, 1979; Solvason and Kearney, 1992; Holt and Jones, 2000). However, T-cell precursors, in particular, are functionally immature at this stage, which may contribute to abnormally strong innate immune responses to inflection (Haynes et al., 1988; Michaelsson et al., 2006; Zhao et al., 2008). Antigen presentation is also impaired during development, as major histocompatibility complex (MHC) class II expression is reduced on antigen presenting cells (Jones et al., 2002) although some accessory proteins may be expressed at adult levels.

The liver bud is responsible for the production of acute phase proteins, which occurs from E16 onward in the rat liver in response to pro-inflammatory cytokines such as IL-6 (Thomas et al., 1990), and some acute phase proteins (e.g., α_2-macroglobulin) are known to be expressed already at E12 in the rat liver (Fletcher et al., 1988). C-reactive protein (CRP) in humans is expressed, and developmentally regulated, in preterm neonates with or without placental inflammation (Leviton et al., 2011). CRP levels are specifically associated with the severity of cortical growth retardation in preterm children (Kaukola et al., 2009); other acute phase proteins are associated with white matter damage (Leviton et al., 2011), but the evidence for direct pathological effects of acute phase proteins in cortical development is scarce. However, peptides homologous to CRP and other acute phase proteins are known to play a role in synaptic refinement in the developing cortex (Bjartmar et al., 2006).

ENDOCRINE INTERACTIONS BETWEEN FETUS AND MOTHER

CNS glucocorticoid receptors and thyroid hormone receptors are expressed from very early in development (Kitraki et al., 1996, 1997); although their level of expression is considerably less than in adults. Cortical effects of both under- and overexposure to glucocorticoids have been described in animal models, including changes in CP size and maturity in a model of maternal adrenalectomy on day 1 of pregnancy (Trejo et al., 1995), and stress during pre- and postnatal life is associated with a wide variety of neurological disorders (Cirulli et al., 2009; Harris and Seckl, 2011). Glucocorticoids are intertwined with immune system function as well, inhibiting inflammatory responses and cytokine expression. Antenatal steroid use has been associated with reduced mortality

and complications in preterm children (Whitelaw and Thoresen, 2000). However, multiple courses of steroids have also been associated with reduced brain growth, impaired myelination, and other abnormalities (Whitelaw and Thoresen, 2000). There is substantial epidemiological evidence to implicate hypothyroidism in developmental cognitive/behavioral deficits (Zoeller and Rovet, 2004; Berbel et al., 2009) including impaired visuomotor skills, low IQ, ADHD, cerebellar dysfunction, and hearing impairment (Ahmed et al., 2008; Berbel et al., 2009; Patel et al., 2011). Estrogens have also been implicated in embryonic neurogenesis, (Brinton, 2009), with an E2 synthesizing enzyme found in the embryonic cortex and the estrogen receptor-alpha is present on progenitor cells during cortical neurogenesis (Martínez-Cerdeño et al., 2006). Furthermore, E2 administration rapidly promotes proliferation, and *in utero* blockade of estrogen receptors decreases proliferation of embryonic cortical progenitor cells (Martínez-Cerdeño et al., 2006).

VASCULAR DEVELOPMENT AND THE BRAIN BARRIERS

The cardiovascular system is the first to become functional in the embryo (Brand, 2003). Due to its high metabolic demands, the cortex receives profuse blood supply, which initiates as two plexi, which progressively become less discrete and vascular density is much more uniform in late gestation (Virgintino et al., 1998; Javaherian and Kriegstein, 2009; Stubbs et al., 2009; Liebner et al., 2011; **Figure 2**).

Angiogenesis is controlled by numerous soluble ligands and their receptors, with some ligands (notably VEGF) playing different roles depending on the stage of vessel development (for extensive reviews see Hanahan, 1997; Gaengel et al., 2009; Quaegebeur et al., 2010). Pericytes are recruited to the vessels via endothelial platelet-derived growth factor (PDGF). The loss of pericytes leads to abnormal capillary morphology and microaneurysms (Lindahl et al., 1997). The developing vasculature is closely associated with early neurogenic compartments in the telencephalon (Javaherian

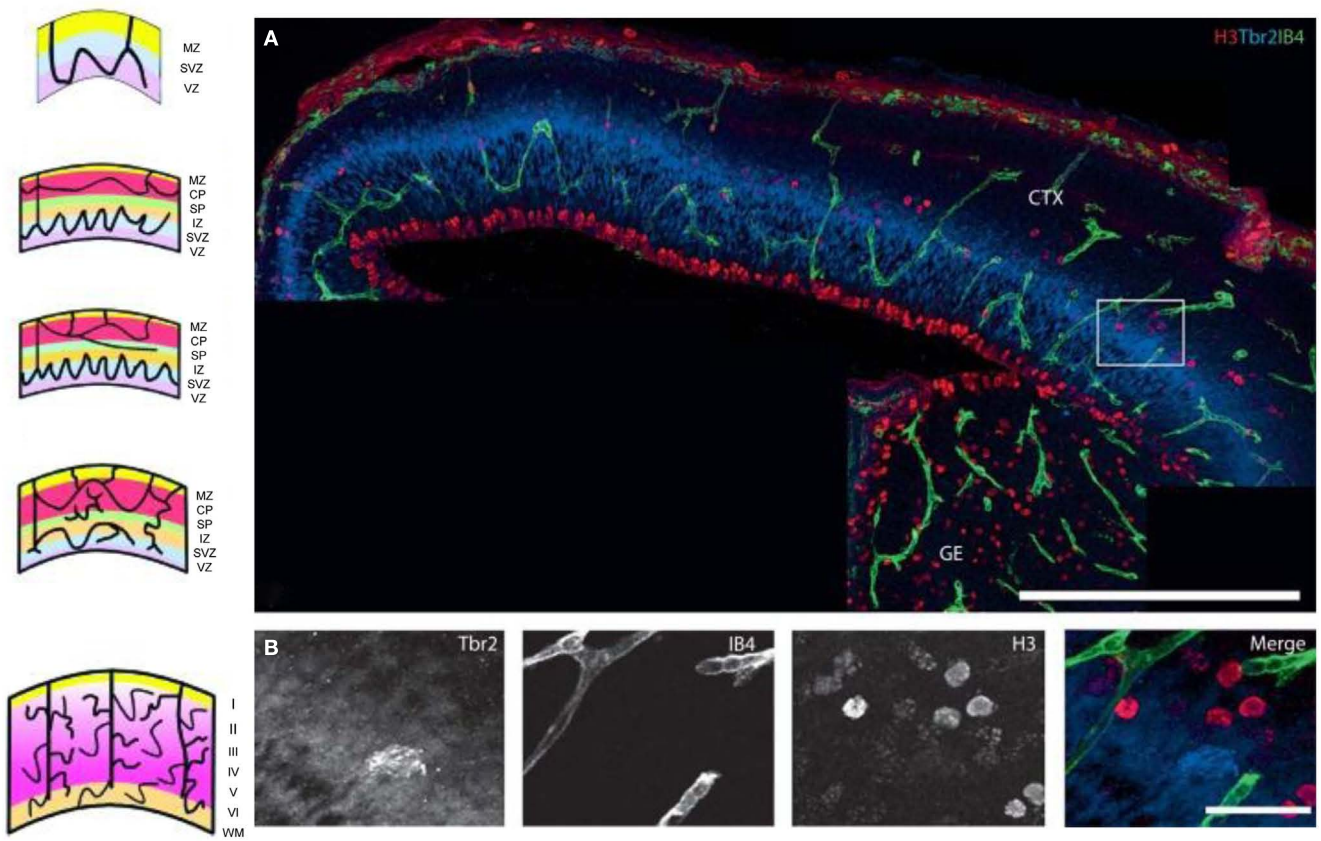

FIGURE 2 | Development of vasculature in relation to cortical layering and cell proliferation in the embryonic cerebral cortex. Left panel show generalized schematic drawings of the developing cortical blood vessel plexi at various embryonic (E14, 15, 18, P8) and adult stages. Initially, there are two dense plexi in the germinal zone (VZ and SVZ) and CP, connected by tangential blood vessels. By E15, a few blood vessels tangential to the pial surface start to appear in the intermediate zone (IZ), but this region remains relatively less vascularized compared with the plexi in the VZ/SVZ and the CP. At E18, toward the end of the neurogenesis, the ventricular plexus has lost much of its definition; and by P8 and adulthood, a more homogeneous structure with many small parenchymal arteries is present. MZ, marginal zone; SP, subplate; I–VI represent cortical layers in adult. **(A)** Association of SVZ blood vessels to mitotic profiles in developing cerebral cortex and ganglionic eminence in an E14 brain. Blood vessels (revealed with IB4-green) and the mitotic profiles (revealed with pH3-red immunoreactivity) on a low power image of a sagittal section through the cortex. Scale bar = 400 μm. CTX = Cerebral cortex. **(B)** Higher magnification of the area indicated by the rectangle in A, illustrate examples of dividing intermediate progenitor cells ($pH3^+Tbr2^+$) that are in close proximity to the cortical vascular plexi ($IB4^+$). Scale bar = 50μm. Modified from Stubbs et al. (2009) and Nie et al. (2010).

and Kriegstein, 2009; Stubbs et al., 2009; Nie et al., 2010; **Figure 2**), and many signaling molecules are shared between these developing systems (discussed below).

The CNS vasculature features the added complexity of the blood-brain barrier (BBB) (**Figure 3A**), which is required for ionic and neurotransmitter homeostasis, protection from neurotoxic agents, and selective provision of nutrients (Neuwelt et al., 2011). The BBB involves both tight junction formation (the failure of which critically impairs BBB function; Nitta et al., 2003), as well as reduced vesicular transport and a specific asymmetric polarity of endothelial transporter expression, e.g., GLUT-1 (Dobrogowska and Vorbrodt, 1999). There is evidence that the first penetrating intraneural capillary (at E10) showed no fenestrations and junctional complexes between endothelial cells (Bauer et al., 1992) and are impermeable to even very small molecules (Ek et al., 2006). The brain is also separated from the external environment by the arachnoid barrier on the surface of the brain and the epithelial barrier at the choroid plexus (**Figure 3C**), which are also present early in development (Ek et al., 2001, 2003, 2010; Johansson et al., 2005) suggesting a functional barrier and protective system. The composition of the cerebrospinal fluid (CSF) is different in the developing brain compared to the adult (Dziegielewska et al., 1981), which appears to be due to a developmentally regulated specific transfer mechanism across the choroid plexus epithelial cells (Habgood et al., 1992; Liddelow et al., 2009, 2011) that is important for normal brain development (Johansson et al., 2008, discussed in more detail below).

Disruption of the BBB has been identified in an age-specific manner following both systemic and centrally induced inflammation. Interestingly, the window of susceptibility appears to be different for these, depending on the site of inflammatory stimuli. Systemic inflammation caused by LPS injection produces

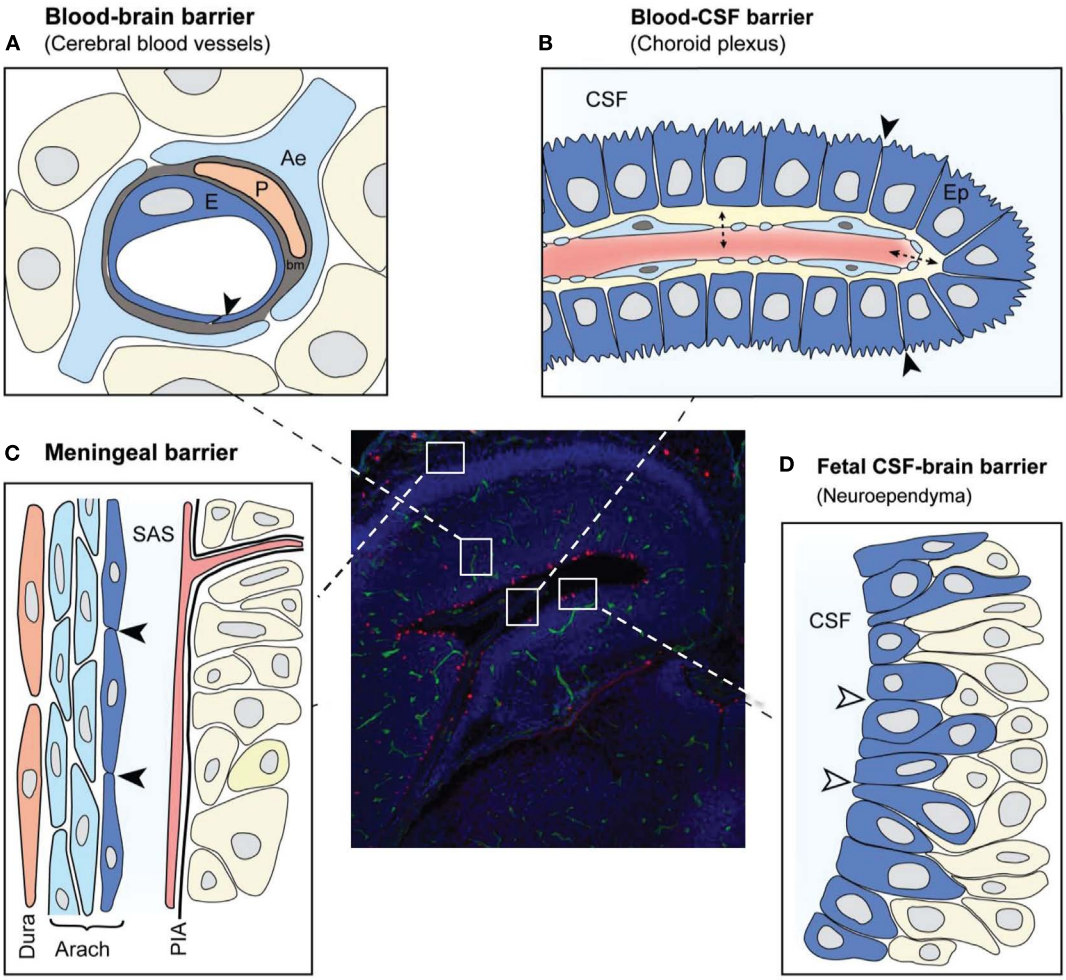

FIGURE 3 | Schematics of the brain barriers. Barrier interfaces in the brain are indicated in blue in the schematic diagrams of the blood-brain barrier, blood-CSF barrier, meningeal barrier, and fetal CSF-brain barrier. The primary site of the blood-brain barrier **(A)** is the endothelial cells of the cerebral blood vessels (E), supported by pericytes (P) and astrocytic endfeet (Ae). In the choroid plexus **(B)** the epithelial cells (Ep) are the barrier interface of the blood-CSF barrier, as the endothelial cells in the stroma are fenestrated. The meningeal barrier **(C)** is at the boarder of the arachnoid membrane (Arach) and the subarachnoid space (SAS). The fetal CSF-brain barrier **(D)** exists during early development, at the endfeet of the radial glial progenitors. This forms a barrier between the CSF and the developing parenchyma. Modified from Saunders et al. (2008).

disruption of the BBB in white matter tracts in the first postnatal week in rats (Stolp et al., 2005), whereas IL-1β in the striatum produces a substantial increase in barrier permeability in juvenile animals, but not neonates or adults (Anthony et al., 1997). Previous work (Stolp et al., 2005) suggests that the blood-CSF barrier (**Figure 3B**) does not have an altered permeability following systemic inflammation, however it has been hypothesized that the function of the choroid plexus epithelial cells may change, altering the composition of the CSF (discussed below).

TELENCEPHALIC ENVIRONMENT DURING PROLIFERATION, MIGRATION, AND DIFFERENTIATION

The environment surrounding the developing brain is, therefore, complicated and the different developmental timetables for organs and regulatory systems can interact to cause variation in brain development. The environment within the developing brain can be described in a similar manner. Many different cell types are produced within the brain and need to differentiate, migrate, and integrate effectively to produce normal brain function.

VASCULAR REGULATION AND NEUROGENIC NICHE

The developmental control of the vasculature goes hand-in-hand with neurogenesis in the brain. There is clear evidence that angiogenesis follows morphogenic factors such as Dlx1/2, Nkx2.1, and Pax6 to produce a ventricular vascular plexus that develops in a ventral-dorsal gradient (Vasudevan et al., 2008). This plexus extends in the dorsal telencephalon as the SVZ is established (Vasudevan et al., 2008), and it appears that progenitors within the SVZ exist within a neurogenic vascular niche (Javaherian and Kriegstein, 2009; Stubbs et al., 2009; Nie et al., 2010) that has also been well established in the adult brain (Shen et al., 2008). There are many similarities and interconnections between cardiovascular and cortical development. For instance, the outgrowth and alignment of blood vessels relies on molecular signals such as semaphorins and netrins, cues also used within the developing nervous system (reviewed by Larrivee et al., 2009). One clear example is VEGF, which is known to be crucial in vascular development (Carmeliet et al., 1996) but some isoforms also regulate proliferation in the neuroepithelium (Darland et al., 2011).

CEREBROSPINAL FLUID

The CSF in the developing brain plays an important role in cerebral expansion (Gato and Desmond, 2009). CSF is secreted by the choroid plexus, which develop from as early as E12 in the rat (Dziegielewska et al., 2001). The primary role of the choroid plexus is to secrete and control the composition the CSF and the cellular mechanisms for CSF secretion, such as aquaporin expression, are established from the day of choroid plexus appearance (Johansson et al., 2005). The choroid plexus regulates the protein composition in the CSF by either production (e.g., TTR, Igf2; Dickson et al., 1986; Hynes et al., 1988; Southwell et al., 1993) or specific transfer from the blood (Habgood et al., 1992; Johansson et al., 2006; Liddelow et al., 2009), which result in their high concentration within the CSF during early development (Dziegielewska et al., 2001; Johansson et al., 2008). It has been well established that the high protein content of developmental CSF is important for cortical expansion (Gato and Desmond, 2009), but initial experiments only assessed this in terms of providing an osmotic force for ventricular, and therefore cortical, expansion. However, recent experiments have confirmed that proteins within the CSF interact with receptors on the ventricular surface and regulate proliferation within the VZ (Martin et al., 2006; Lehtinen et al., 2011). The generally high CSF protein concentration decreases in the rat at approximately E17 (Johansson et al., 2008), but it is likely that specific proteins within in the CSF still occur at high concentrations during the embryonic period and continue to influence neurogenesis (e.g., Igf2; Lehtinen et al., 2011).

MICROGLIA

Linage tracing has been used to demonstrate that microglia are derived from the systemic progenitors and migrate into the brain around E9.5 in the rodent (Ginhoux et al., 2010). Microglia slowly increase in number within the brain, with higher distribution density within the meninges and the VZ/SVZ border followed by increasing density in the CP as gestation continues (Antony et al., 2011). Microglia are considered to play a role during these early stages of development in supporting the proliferation of progenitors within the cortex. While depletion of microglia from the developing brain in PU.$1^{-/-}$ mice (McKercher et al., 1996) is associated with grossly normal cortical development up to E16 (when animals die due to hematopoietic dysfunction) there is some evidence that there is decreased cortical proliferation in the absence of microglia, which ultimately leads to a reduction in astrocyte numbers (Antony et al., 2011). At an equivalent stage of development, altered microglia function, by a loss-of-function mutation in the DAP12 gene, also provides evidence that microglia affect neuronal maturation and later synaptic formation and function (Roumier et al., 2008).

In the fourth phase of telencephalic development there is a massive increase in the number of microglia (up to 20-fold) that can be specifically observed with an amoeboid phenotype within white matter tracts (Prinz and Mildner, 2011). Microglia slowly spread through the entire CNS throughout the postnatal period and take on a "resting," ramified phenotype (Hristova et al., 2010). During this period, when microglia have previously been considered to be static, there is evidence that they play a role in synaptic pruning and may be important for aspects of structural reorganization associated with LTP and LTD (Schlegelmilch et al., 2011). Recently, microglia have been shown to engulf and eliminate synapses during development (Paolicelli et al., 2011). Microglia activation is considered a natural step in elimination of juvenile connections, e.g., in the corpus callosum or during normal formation of the barrel cortex (Berbel and Innocenti, 1988; Maki Hoshiko, Nobuhiko Yamamoto and Etienne Audinat et al., unpublished), and has been demonstrated in pathological neural activity (Avignone et al., 2008) both are highly suggestive of functional involvement in cortical circuit remodeling. There is evidence that the chemokine fractalkine (CX3CR1) is important in mediating microglial synaptic pruning. In KO mice unable to produce the fractalkine receptor (Cx3cr1KO) there is a decrease in microglial densities in developing brain regions and newborns had an excess of dendritic spines and immature synapses, although these changes were found to be transient (Paolicelli et al., 2011). Interestingly, mice lacking functional Hoxb8 (a transcription factor that is involved in the development of the hematopoietic system) exhibit

obsessive grooming behavior, which is linked to the loss of the Hoxb8 microglial population in the brain (Chen et al., 2010). This provides a novel link between microglia and behavioral evidence of neuropathology.

ASTROGLIAL DEVELOPMENT

During stage 3 of telencephalic development, there is a switch from neurogenesis to gliogenesis (**Figure 4**). Experiments using fluorescence-activated cell sorting and time-lapse microscopy show radial glia can generate astrocytes as well as neurons (Malatesta et al., 2000; Noctor et al., 2004; **Box 2**). The neuron to glia switch is reliant on both cell-intrinsic and extracellular cues. Early in forebrain development the promoters of GFAP and S100B are methylated, ensuring astrocyte development is repressed (Juliandi et al., 2010). Demethylation increases the sensitivity to extracellular cues such as developmental cytokines (Molne et al., 2000; Juliandi et al., 2010). Neurons are known to secrete cytokines involved in gliogenesis, particularly members of the IL-6 family: LIF, CNTF, CT1 (Juliandi et al., 2010). Embryonic cortical neurons can regulate the onset of cortical gliogenesis via cardiothrophin-1 (Barnabe-Heider et al., 2005). This is a neural feedback mechanism whereby newly born neurons can instruct precursors to generate astrocytes via the secretion of cytokines, ensuring that gliogenesis does not occur until after neurogenesis. The use of cytokines in regulating the development of glia leaves the possibility that gliogenesis can be disrupted by additional cytokine production following an environmental insult such as perinatal inflammation.

Following astrocyte specification, precursors migrate to their final positions where they begin the process of terminal differentiation. Astrocytes initially migrate tangentially along white matter tracts and then move in a radial direction in the gray matter (Jacobsen and Miller, 2003). The major waves of rodent CNS synaptogenesis occur during the first 2–3 weeks of postnatal life and astrocytes are known to secrete molecules that induce synapse formation (Christopherson et al., 2005; Kucukdereli et al., 2011). Their influence on synaptic function might be stage-specific and modulate well described periods of developmental plasticity. The early experiments by Müller and Best (1989) demonstrated that the transplantation of astrocytes from the visual cortex of newborn kittens into the visual cortex of adult cats reinstalls ocular-dominance plasticity in adult animals.

OLIGODENDROCYTES

Oligodendrocyte precursors are produced from three different waves of division, from different progenitor populations, primarily in the third and fourth phases of telencephalic development. The majority of oligodendrocyte precursors originate from the

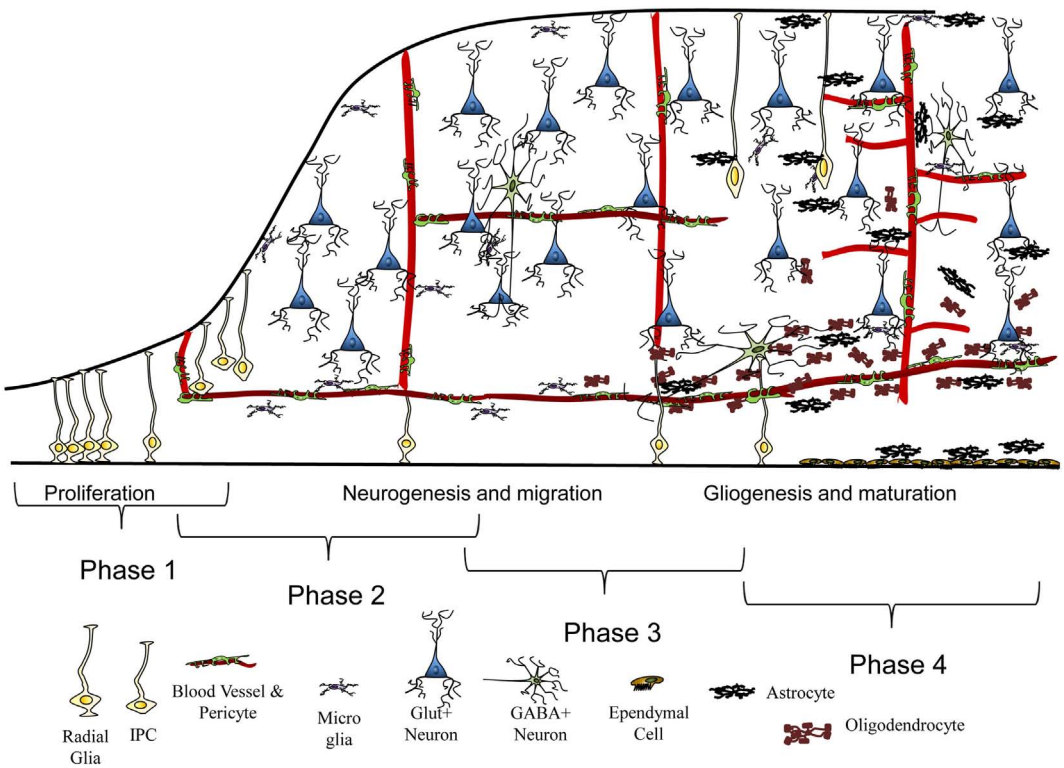

FIGURE 4 | Pattern of telencephalic development. Schematic indicating the increasing complexity of the brain over time. Initially proliferation occurs to increase the size of the progenitor pool (phase 1 of telencephalon development). As neurogenesis starts, vascular plexi, supported by pericytes, form and microglia infiltrate the brain. A second proliferative zone in the SVZ forms. Neurons migrate and differentiate into cortical layers in an inside out manner during phase 2 and 3 of development. In phase 4 of development, gliogenesis occurs in the dorsal and ventral telencephalon. GABAergic interneurons increase their migration into the cortex. There is vascular remodeling and a maturation and integration of connections between neurons, supported by the micro and macroglia.

ventricular zones in the ganglionic eminences of the ventral telencephalon, a brain region that is also responsible for the production of interneurons (see below). Two prenatal waves of production occur from this zone in the rodent, with the oligodendrocyte precursors migrating to the cortex before full maturation and myelination occurs. A third, postnatal, wave of production occurs in the dorsal ventricular zone after the completion of neurogenesis (Kessaris et al., 2006). In both proliferative zones oligodendrocyte precursors are produced as part of a fate restriction of the neural stem cells that occurs by a largely epigenetic regulation (Liu and Casaccia, 2010). The cross-talk that occurs between transcription factors, which is regulated by DNA methylation or acetylation, provides an initial support for astroglial genesis, that switches to support oligodendrogenesis over the developmental period (Liu and Casaccia, 2010; Rivera et al., 2010). In both the ventral and dorsal telencephalon, Wnt signaling has been implicated in activating oligodendrogenesis over the previous astroglial or interneuron proliferative fates (Langseth et al., 2010; Zhong et al., 2011).

INTERNEURONS

Approximately 25% of cortical neurons are inhibitory GABAergic interneurons, which arise from precursors in the subpallium and tangentially migrate into the developing neocortex (Wonders and Anderson, 2006). The rodent interneurons arise mainly from the ganglionic eminences (Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001; Jimenez et al., 2002; Nery et al., 2002), from approximately E12.5 until birth (Flames and Marín, 2005). There is considerable evidence that rodent models do not accurately reflect the complexity of the primate cortex, which likely receives interneurons from the ventricular zone, SVZ and subpial granular layer in addition to the GE (Letinic and Rakic, 2001; Letinic et al., 2002; Wonders and Anderson, 2006; Jones, 2009; Petanjek et al., 2009). However, this issue in human is not yet resolved (Clowry et al., 2010; Hansen et al., 2010). The establishment of interneuron networks in rodents begins a few days before the glutamatergic network and continues until postnatal life (Cossart, 2011). The origin of interneurons, both in terms of their birth date and region of origin, contributes to determining their electrophysiological properties and neurochemical characteristics (Butt et al., 2005). Birth date and phenotype also predict the laminar fate of interneurons in the rat (Rymar and Sadikot, 2007). Interneuron migration is thought to be induced by motogenic factors such as neurotrophins and hepatocyte growth factor; the pattern of interneuron migration is established by a variety of chemoattractive and chemorepulsive cues, including stromal-derived factor 1, neuregulins, slit proteins, and ephrins (Hernandez-Miranda et al., 2010). The correct integration of interneurons during CNS development is essential for normal coordinated brain function. Epilepsy and schizophrenia are two neurodevelopmental conditions that occur, at least in part, as a result of interneuron dysfunction (discussed below).

CAUSES OF TELENCEPHALIC INJURY

It is clear that many elements contribute to a fully functional central nervous system, and that dysfunction in one can have widespread consequences, however establishing etiology of neurodevelopmental disorders has been difficult (see **Box 3**). Genes that have been associated with these conditions typically have low penetrance and may confer risk for a number of distinct clinical conditions. Likewise, a wide number of environmental insults, such as inflammation and hypoxia, have been indicated by epidemiological studies to contribute a small but significant risk to many neurological and mental disorders. Gene-environment interactions or other forms of confounding insults, along with the developmental windows of susceptibility are now a focus of current research aiming to explain these observations.

TWO-HIT HYPOTHESIS AND WINDOW OF VULNERABILITY

A "two-hit" hypothesis has been put forward in a number of diseases where onset of disease cannot clearly be linked to a specific genetic or environmental insult. In diseases such as schizophrenia and autism, there is evidence for both genetic contributions and environmental insults, but neither one accurately predicts disease. Support for a two, or multiple, hit etiology for these diseases is particularly strong as limited clear neuropathology has been identified that can be linked to one specific genetic or environmental insult. Instead, a wide variety of broadly linked functional systems are effected, including genes involved in synapse formation, cell–cell signaling, and trophic pathways, and injury widely affecting GABA, dopaminergic, and glutamatergic transmission (Maynard et al., 2001). It has been hypothesized that the first hit in schizophrenia may affect neurogenic and cell specification pathways, such as Notch, while the second hit may have a greater effect on functional integration (Maynard et al., 2001).

The term two-hit hypothesis has also been used to describe the increased deficit caused by two or more low-level cytotoxic insults, which combine to produce greater long-term damage. In a basic, proof of principal study, Genetta et al. (2007) have shown an additive cytotoxic effect of low dose alcohol and hypoxia exposure in primary neuron cultures. There have also been many studies showing that inflammation combined with hypoxia can cause increased neurological injury. Inflammation induced by lipopolysaccharide injection 4, 6, or 72 h prior to hypoxic-ischemic injury has been found to significantly increase infarct size in postnatal day 7 rats (Eklind et al., 2001, 2005). Prenatal LPS exposure has also been associated with increased sensitivity to later hypoxic insult in the immature brain (Larouche et al., 2005). However, the mechanism of this sensitivity is confused, and may be due to the priming of the immune cells (Williamson et al., 2011; Bilbo et al., 2012). In comparison, inflammation as a first hit in schizophrenia is presumed to cause long-term changes in brain structure or function that occur at a subclinical level and are exposed by a second hit in later life (Meyer et al., 2005, 2006; Fatemi et al., 2008; Meyer and Feldon, 2009). Interestingly, there is also some evidence that insults during early life may contribute to neurodegenerative disease later in life. For example, inflammation during embryology (E10.5 in the mouse) results in loss of dopaminergic neurons in the basal ganglia, and increased sensitivity to the 6-hydroxydopamine neurotoxin later in life (Ling et al., 2002, 2004). This substantial decrease in dopaminergic neurons may also increase the susceptibility of affected individuals to "normal" age-induced cell loss, and may account for cases of extremely early onset Parkinson's disease.

The mechanism by which environmental insults such as inflammation/hypoxia and genetics combine in neurodevelopmental disease will be discussed below. It is also important to consider

Box 3 | Diagnosis and experimental tools.

EPIDEMIOLOGY

Epidemiological studies linking prenatal insult with later neurodevelopmental disorders are subject to many difficulties, including the limitations of retrospective assessment of possible exposure to insult, and the years to decades elapsed between gestational insult and disease diagnosis. Early studies assessing the association between influenza and schizophrenia are an example of these difficulties, as assessments were made based on potential exposure to influenza during gestation (i.e., occurrence of an influenza epidemic during the pregnancy), rather than the actual presence of maternal infection (Brown and Derkits, 2010). More recent studies, that have utilized medical records and analyzed available blood samples, have found a significant risk of schizophrenia following inflammation in the first and second trimester of pregnancy. With conditions such as cerebral palsy, the successful outcome of epidemiological studies is more attainable. Due to the relatively early age of clinical diagnosis, as well as multiple neuropathologies that can be identified with imaging technology, it is possible for studies such as the ELGANS study (O'Shea et al., 2009; Leviton et al., 2011) to determine the links between multiple environmental insults, extreme prematurity, and risk of developing cerebral palsy or mental retardation in large cohort groups with well-defined assessment criteria.

IMAGING

Imagining modalities have been utilized to provide a whole brain view of neurodevelopmental disorders. The results from these studies, while advantageous, highlight the difficulty of determining the etiology of these disorders (Anagnostou and Taylor, 2011). Changes in the brain are generally subtle, requiring extensive group comparison with carefully controlled age-matched groups, and the origins of changes in signal are generally difficult to determine. For example, progressive loss of cortical gray matter is observed in cases of child-onset schizophrenia, but from MRI images it is currently difficult to determine whether these changes are of neuronal origin (e.g., increased synaptic pruning), or are due to changes in astrocytes, vasculature, or myelination of the affected cortical regions. Histological comparisons from autopsy may partly aid this analysis, although in the case of schizophrenia samples are generally late-stage disease and confounded by a variety of treatment and life-style choices (Toga et al., 2006). However, imaging techniques such as MRI, PET, and photo-acoustic ultrasound are now available with good resolution for small animals. The use of these together with standard histological assessments may allow links to be made between the structure and function of the brain in animal models and equivalent human clinical conditions.

BEHAVIOR

Deciphering how risk factors act on brain development that result in altered circuits, and subsequently influence the behavioral symptoms, is the real challenge of psychiatric research. Endophenotype is a psychiatric concept to divide behavioral symptoms into more stable phenotypes with a clear genetic connection. However, the involvement of distinct genes, which could underlie certain endophenotypic traits, in neural development is very often not known. Systematically examining the composition, morphology, connectivity of the brain is very rarely linked up to particular structure or cell types that make up circuits underlying behavioral endophenotypes in animal models. Nevertheless, animal models of behavior will remain valuable tools for the investigation of altered brain circuits (Amann et al., 2010).

the timing of insult in relation to the stages of brain development. The term "window of vulnerability" is commonly used when considering developmental neurological injury. However, it is not yet clear if this vulnerability is intrinsic to specific periods of development (e.g., proliferation or synapse formation), and therefore may move from region to region along with the normal developmental gradients of these processes. Alternatively, it has been suggested that different cells may become selectively vulnerable to damage as a result of the different intrinsic maturation process. An example of this are subplate neurons, which are thought to have increased vulnerability to injury in the prenatal human due to an early birth date and therefore earlier maturation of glutamate receptor populations (Furuta and Martin, 1999; Talos et al., 2006; Nguyen and McQuillen, 2010). This leads to increased sensitivity to excitotoxicity at a time of development where there is an increased risk of hypoxic-ischemic injury.

Cerebral palsy provides an example of the consequence of the stage of brain maturity at the time of insult on subsequent neuropathology. Injury at term, e.g., umbilical cord asphyxia during delivery, causes cerebral palsy with a typical pattern of gray matter loss, as well as damage to the hippocampus and cerebellum. Whereas preterm injury, leading to cerebral palsy, is generally associated with white matter and subcortical gray matter damage (Gunn and Bennet, 2009; Volpe, 2009). There are a few confounding maturation steps that underlie these differences in regional damage. White matter is beginning to be myelinated in the preterm period in humans, and specific populations of precursor oligodendrocytes have been shown to be particularly susceptible to damage, which corresponds to this period. In contrast, areas of term damage are typically described as "watershed" damage, indicating that these brain regions are at the edge of the vascular zones, therefore increasing susceptibility to hypoxic damage (Gunn and Bennet, 2009; Volpe, 2009). In preterm white matter, similar watershed regions have been suggested due to the pattern of vascular development, however, it was determined that differences in vascular blood flow could not account for the different magnitude of damage in the region (McClure et al., 2008). Therefore, the different patterns of damage have been associated with selective sensitivities of developing cells at the time of insult. This is an example whereby different insults, at different stages of brain development can cause distinct, but related neuropathologies which contribute to the one clinical diagnosis.

GENETICS

The genetic regulation of brain development is extremely complicated, with a wide number of genes regulating proliferation and differentiations states, as well as more subtle aspects of neurological function, such as signaling pathways and receptor function.

Altered neurological development leads to a variety of subtle or complicated behavioral dysfunctions, from slight mental retardation to gross motor and cognitive deficits. A number of genetic variants have been associated with these general disorders, as well as specific neurodevelopmental diseases. For example, a large number of gene variants associated with increased risk of autism spectrum disorders (ASDs) have a wide range of functions in the central nervous system and are associated with a variety of other neurological disorders (Aldinger et al., 2011). Congenital neurological disorders due to genetic variation may have a subtle neuropathology, such as for autism, or may be associated with gross changes in the brain, such as for microcephaly.

Primary recessive microcephaly affects approximately 1:10,000 people, and is classified based on small brain size and altered gyrations of the cerebral cortex (either lissencephaly or polymicrogyria) that are present at birth (Abuelo, 2007). The genetic basis of this condition has been clearly linked to a number of mutations that all affect neural progenitors (Buchman et al., 2011; Mahmood et al., 2011; Poulton et al., 2011). The function of the abnormal spindle microcephaly (APSM) gene may occur directly through the regulation of the mitotic spindle and indirectly by modulating the Wnt signaling pathway (Buchman et al., 2011). Tbr2 is another gene that normally regulates proliferation within the developing cortex, where gene manipulation has been associated with microcephaly in experimental models (Arnold et al., 2008; Sessa et al., 2008) and in clinical cases of microcephaly in humans (Baala et al., 2007).

Inhibitory transmission is crucial for normal cortical function and its defects are associated with a variety of neurological and psychiatric disorders, including autism (Levitt, 2005), schizophrenia (Lewis et al., 2005), and epilepsy (Noebels, 2003). The pathobiology of schizophrenia remains poorly understood, but genetic and anatomical studies are beginning to indicate the complicated neuropathology. Recently identified epigenetic changes in GAD67 and reelin genes support the GABAergic hypothesis of schizophrenia (O'Connell et al., 2011). Post-mortem studies have also revealed reductions in inhibitory neurotransmission markers in schizophrenics (Volk et al., 2000); although this may not be the case in all subjects and modern hypotheses focus on abnormal interneuronal connectivity rather than simply insufficient numbers of cells (Marín, 2012), these observations are nonetheless suggestive of developmental abnormalities (Harrison and Weinberger, 2005). For instance, Disrupted in Schizophrenia 1 (DISC1) mutations are known to cause familial schizophrenia (Millar et al., 2000). DISC1 appears to function in cell migration among other pathways, as mouse models with DISC1 inhibition show reduced migration of both developing pyramidal cells (Kamiya et al., 2005) and MGE-derived interneurons (Steinecke et al., 2012). Neuregulin 1 (NRG1) and its receptor ErbB4, both schizophrenia risk genes (Stefansson et al., 2002, 2003), are also involved in migration: ErbB4 mutant mouse cerebellar cultures exhibit reduced granule cell migration along radial glia (Rio et al., 1997) and electroporations of dominant-negative ErbB4 into the MGE prevent migration to the cortex, whereas heterologous expression of NRG1 induces migration toward the expressing cells *in vitro* and *in vivo* (Flames et al., 2004). There are numerous other examples of development-related schizophrenia risk gene such as Tcf4 and CNTNAP2 (Blake et al., 2010). However, as most risk-conferring polymorphisms are intronic, their functions and relevance to disease mechanisms are not well characterized. Moreover, DISC1 has not been demonstrated to have significant association with schizophrenia in genome-wide association studies (GWAS). It is likely that there are numerous endophenotypes in schizophrenia with different underlying genetic causes, but these have not yet been well defined.

One of the GWAS results for schizophrenia has been the MHC region (Stefansson et al., 2009), suggesting the involvement of immune mechanisms in the pathogenesis. This in conjunction with the well-documented risk conferred by maternal infections and behavioral abnormalities in animal models (Patterson, 2009) would support an underlying immunopathology, but the GWAS results implicating MHC genes have been criticized for using intergenic single-nucleotide polymorphisms (SNPs) in many cases – although the SNPs are assumed to be in linkage disequilibrium with the encoded genes, this cannot exclude non-coding RNA involvement in schizophrenia; moreover, the genic SNPs that have shown significant associations have not been thoroughly characterized in terms of their function (Gejman et al., 2011). Associations have also been found between schizophrenia and the pro-inflammatory cytokines interleukin 1β (Xu and He, 2010), interleukin 6 (Paul-Samojedny et al., 2010), and the anti-inflammatory interleukin 10 (Bocchio Chiavetto et al., 2002), further supporting the involvement of inflammatory pathways, even if the pathomechanisms through which these alleles influence disease development are obscure.

Autism spectrum disorders share some of the problems of schizophrenia: numerous preliminary gene associations have not been replicated, and the pathobiology is uncertain and likely to be heterogeneous. In this case, the evidence for a developmental defect is much clearer, given its early onset. This is reflected in the functions of implicated genes. For instance, neuroligin 4 was identified in a family with X-linked mental retardation and autism (Laumonnier et al., 2004). Neuroligins are thought to function in presynaptic development, and they interact with neurexins to regulate postsynaptic development (Dean and Dresbach, 2006) – neurexin 1 has also been associated with ASDs (Kim et al., 2008) and schizophrenia (Rujescu et al., 2009), consistent with hypotheses of abnormal synaptic development in these disorders. Copy number variations and mutations in SHANK2 and SHANK3, synaptic scaffolding genes, have been linked to ASDs (Durand et al., 2007; Berkel et al., 2010), as have K^+ channel regulation associated genes such as DPP6 and DPP10 (Marshall et al., 2008), all indicative of synaptic dysfunction in autism. Cell migration pathways have been implicated by a genome-wide study for copy number variations, which produced significant results for cell adhesion genes with presumptive migratory functions including ASTN2 (Glessner et al., 2009). CNTNAP2, another ASD risk gene (Arking et al., 2008), has also been characterized as crucial for normal neuronal migration: CNTNAP2$^{-/-}$ mice show behavioral abnormalities, ectopic neurons in the corpus callosum (indicative of defective migration) and reduced numbers of GABAergic interneurons among other abnormalities (Peñagarikano et al., 2011). MeCP2, the gene

affected in Rett syndrome featuring autism and other developmental defects, also impairs GABAergic transmission: mice exhibit behavioral defects suggestive of ASD in both full KOs and interneuron-specific KOs of MeCP2 (Chao et al., 2010). Overall, it can be concluded that both autism and schizophrenia share developmental origins and synaptic dysfunction as their main pathways, but these are not independent of environmental influences (Harrison and Weinberger, 2005; Bill and Geschwind, 2009).

In autism and schizophrenia, immune regulating gene variants have also been heighted as disease risk factors. In particular there are multiple lines of evidence linking variants in DRB1 alleles of the human leukocyte antigen (HLA) with both of these neurodevelopmental conditions (Crespi and Thiselton, 2011). It is hypothesized that these genetic variants contribute to disease ontology through modulation of the normal immune signaling in brain development. Inflammation during pregnancy has also been linked to both these neurological disorders in epidemiology (see below), indicating that genetic and environmental variations can affect normal development, and leading to disease pathology.

INFLAMMATION

A variety of genes with known roles in the immune system have recently been found in progenitor cells in the developing cortex. Toll-like receptors are a group of pathogen recognition receptors, that respond to bacteria (e.g., TLR2 and TLR4) and viral (e.g., TLR3) presence (Medzhitov, 2007). These receptors mediate a pro-inflammatory response through NF-κB, and have also been found to be activated by tissue damage. Two recent studies have shown the constitutive expression of TLR2 and TLR3 in progenitor cells within the ventricular zone in early and intermediate telencephalic development (Lathia et al., 2008; Okun et al., 2010). In both cases activation of the receptor causes a decrease in proliferation, but in the case of TLR2 the absence of the constitutive presence, in $TLR2^{-/-}$ mice, does not affect proliferation or differentiation of progenitors (Okun et al., 2010), while in $TLR3^{-/-}$ mice there is an increase in proliferation in the VZ and SVZ (Lathia et al., 2008). The presence of these and other inflammatory mediating receptors in the neural progenitor cells provide a potential mechanism for recent observations of inflammatory induced damage to the developing brain. Many studies have shown behavioral changes following maternal immune activation during early gestation. The pathology underlying these behavioral changes has been unclear; however, our recent study has shown that maternal inflammation induced at in the early phases of telencephalic development causes decreased proliferation in the VZ (Stolp et al., 2011; **Figure 5**). Part of the mechanism for this may be the activation of the TLRs, although this interaction has not yet been described. What has been reported is a decrease in β-catenin at the ventricular surface (Stolp et al., 2011), which may also contribute to the decreased proliferation in this model, and suggests that a complex interplay of many factors that normally regulate proliferation may be affected by maternal inflammation. Interestingly, the maternal inflammatory response that produces reduced ventricular proliferation at E13.5 in the mouse does not appear to significantly affect the proliferation of basal progenitors within the SVZ (Stolp et al., 2011; **Figure 5**). This suggests that the regulation of proliferation in the SVZ may be different from the VZ, or that the mechanism of entry of inflammatory molecules into the brain may selectively affect one population and not the other. The basal progenitors in the SVZ are closely associated with the blood vessels that make up the ventricular vascular plexus in early development, while the VZ progenitors have close contact with the CSF in the ventricular system. In the adult brain, systemic inflammation has been found to alter transcription of acute phase proteins and adhesion molecules in the choroid plexus and there is an increase in interleukin-6 and the chemokine CCL2 in the CSF (Marques et al., 2009). It is possible, though currently unstudied, that systemic inflammation may also cause increased cytokine levels in fetal CSF that directly regulate proliferation in the VZ.

An alternative mechanism for inflammatory regulation of progenitor proliferation is a modification in the normal function of microglia. The work of Roumier et al. (2008) suggests that inflammatory activation has the same effect as microglial loss-of-function in the $DAP12^{KI}$ mouse. They provide evidence to suggest that the loss-of-function mutation actually causes minor activation of the microglia and cytokine production that modulates neuronal function, which can be recapitulated at E15 in the mouse by induced maternal inflammation. In the developing brain microglia have a typically amoeboid morphology associated with their entry from the systemic circulation and subsequent differentiation. It has been hypothesized that this morphology conveys a reactive phenotype and that inflammation or injury in the developing brain may be more severe than in the adult brain, where microglia need to be reactivated from a "resting" state. At later stages of brain development inflammation has been associated with interneuron dysfunction. For instance, neonatal exposure to LPS is associated with reduced parvalbumin immunoreactivity in the CA1–CA3 region (but not the prefrontal cortex) with later defects in object recognition (Jenkins et al., 2009), and viral infection at P4 can cause a gradual loss of hippocampal interneurons (Pearce et al., 2000). Such changes can have an indirect effect on the cortex: one recent study showed that neonatal intrahippocampal LPS in rat increases cytokine expression in the adult hippocampus and cortex, impairs dopaminergic modulation of prefrontal interneurons and causes a deficit in prepulse inhibition (Feleder et al., 2010). Inflammation during fetal development has been most strongly linked with brain damage leading to cerebral palsy. Many studies have shown that maternal inflammation in the later part of the second trimester, or equivalent in animal models, causes an array of white matter injury, including diffuse demyelination and cystic lesions (Rezaie and Dean, 2002), which correspond well to the periventricular leukomalacia that is a common MRI observation in cerebral palsy (Counsell et al., 2003). It seems that the susceptibility of the white matter to damage is largely due to the presence of pre-oligodendrocytes that have a low damage threshold (Back and Rivkees, 2004).

As one of the links between immune and CNS development, there is a plethora of evidence for various cytokines regulating CNS development. For instance, CSF-1 is a cytokine involved in monocyte survival and proliferation, but null mutations also cause defects in cortical circuitry (Michaelson et al., 1996). The gp130 family cytokines such as LIF and CTNF promote radial glial cell renewal and ventricular zone proliferation in rodents (Deverman

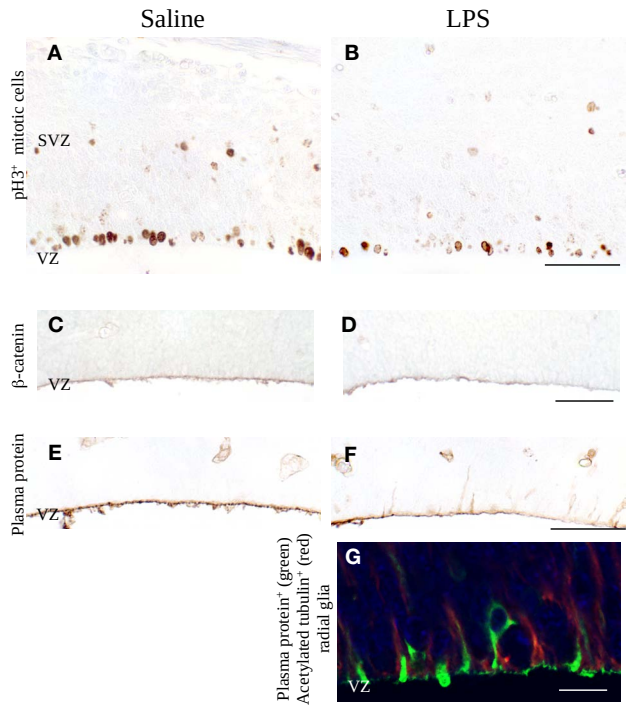

FIGURE 5 | Effect of maternal inflammation on the ventricular zone at E13. Maternal inflammation at E13 has profound effects on cortical development. There is a decrease in proliferation in the ventricular zone **(A,B)**. As well as this, there is a disruption of the ventricular surface, indicated by decreased β-catenin immunoreactivity **(C,D)** and uptake of proteins from the CSF **(E,F)**, by acetylated tubulin positive radial glia **(G)**. Scale bars = 50 μm for **(A–F)**, 10 μm for **(G)**. Modified from Stolp et al. (2011).

and Patterson, 2009), but also astrogliogenesis in later development. However, this is mainly related to local expression within the developing CNS, rather than the inflammation-associated endocrine functions of cytokines, which have been associated with a wide variety of neurodevelopmental disorders including schizophrenia (Ellman and Susser, 2009), autism and periventricular leukomalacia (Deverman and Patterson, 2009). Due to the prevalence of IL-6/gp130 family cytokines acting in the developing brain, there has been substantial investigation of the contribution of IL-6 of brain injury induced by maternal immune activation. IL-6 has been shown to increase in the fetal brain following many forms of early life systemic inflammation (Golan et al., 2005; Stolp et al., 2009), and there is some evidence that IL-6 in the maternal circulation can cross the placenta from mid-gestation (Dahlgren et al., 2006). The most compelling evidence for the important contribution of IL-6 to early life neurodevelopmental disorders is the work of Patterson and colleagues. In their recent paper (Smith et al., 2007), substantial evidence was present that IL-6 alone could stimulate long-term changes in neurological behavior and that inhibiting IL-6 when inflammation is induced by a general immune mediator prevents these behavioral phenotypes. A further recent study also implicated maternal IL-6 in influencing placental expression of immune- and endocrine-related genes, including factors known to influence fetal development such as growth hormone and insulin-like growth factor I (Hsiao and Patterson, 2011), which poignantly illustrates the interconnectedness of immune, endocrine, and CNS development.

HYPOXIA-ISCHEMIA

While the brain grows in a hypoxic environment, it is protected from extremes of hypoxia, as with many other organs, by vascular auto-regulation (Pearce, 2006). As part of normal brain function, neurovascular coupling allows distribution of nutrients in a spatial and temporal fashion to brain areas with the highest neuronal activity and, therefore, metabolic demand. This form of auto-regulation is typically due to the function of smooth muscle cells around the pial, penetrating and pre-capillary arterioles (Lok et al., 2007). However, at the level of the capillaries, pericytes and astrocytes have been found to have some contractile capacity and are able to regulate vessel diameter (Haydon and Carmignoto, 2006; Peppiatt et al., 2006). It is currently unclear how much this contributes to the vascular auto-regulation (discussed by Quaegebeur et al., 2011), and there is also currently limited data indicating when these auto-regulatory mechanisms are established. Clinical and experimental research demonstrated evidence of regulation during late stages of human pregnancy and pericyte- and astrocyte-induced contraction of capillary beds in postnatal rat brains (Peppiatt et al., 2006; Degani, 2009), suggesting that auto-regulatory mechanisms occur in the fourth phase of telencephalic development.

Hypoxic injury is generally considered to be associated with birth complications and neurodevelopmental injury such as cerebral palsy (Gunn and Bennet, 2009; Volpe, 2009). There has been a recognized difficulty in diagnosing consequences of antenatal oxygen deprivation as there is not a clear correlation with markers of oxygen deficit (e.g., acidosis, lactate levels) and later neuropathology (Gunn and Bennet, 2009). This is partly due to the protective mechanism of the brain and cerebrovascular system and the regenerative capacity of the still developing CNS (see below). However, despite the presence of auto-regulatory mechanisms, the clearest link between hypoxia-ischemia and brain injury is during this stage of brain development (Lou et al., 2004). White matter damage has been found to occur following inflammation or hypoxia-ischemia (Hagberg et al., 2002). The precursor oligodendrocytes are susceptible to damage at this stage of development (Back et al., 2001; Back and Rivkees, 2004) and a number of studies have shown that high density of microglia within the white matter contributes to this injury, facilitating the immune activation of the susceptible oligodendrocyte precursors (Verney et al., 2010). It is likely that the mechanisms of injury in these two experimental conditions are linked, as hypoxia-ischemia can cause the production of inflammatory mediators and inflammation can cause an alteration in auto-regulation and potentially reduce oxygenation.

Astrocytes have been shown to undergo functional and structural long term changes following neonatal hypoxia/ischemia in the pig brain (Sullivan et al., 2010a,b). There are significant decreases in the average astrocyte size, number of processes, and length of processes following hypoxia-ischemia treatment. Furthermore, D-aspartate uptake studies revealed that

the hypoxia-ischemia insult resulted in impaired astrocytes function, with significantly reduced clearance of the glutamate analog, D-aspartate. Sullivan et al. (2010b) argue that the initial reduction in astrocytes size and impaired function in the study are detrimental to the survival of neurons. Changes in the morphology of astrocytes have the potential to alter functions reliant on the location of astrocytic membrane proteins, for example the glutamate transporters located in close proximity to synapses play a role in clearing the excitatory amino acids preventing excitotoxicity (Sullivan et al., 2010b).

Subplate neurons have been suggested to be selectively sensitive to hypoxic-ischemic injury in postnatal rodents (McQuillen et al., 2003) and perinatal humans (Volpe, 2009) that might account for some behavioral deficits observed following hypoxic-ischemic injury. However, the majority of thalamic connections have been made by these stages of brain development, and no damage to these connections was observed following injury (McQuillen et al., 2003). It has recently been questioned whether the subplate is actually at increased sensitivity that other neurons in the cerebral cortex (Nguyen and McQuillen, 2010), with deficits in layer V also observed in autopsy studies of human periventricular leukomalacia (Andiman et al., 2010).

DRUGS/TOXINS

Unlike the relatively general actions of inflammation and hypoxia, a number of drugs and toxins appear to have very specific actions on the central nervous system, based on binding to channels or receptors within the brain. Lead is one example of this, where its presence within the brain appears to interfere with Ca^{2+} entry into synaptic terminals, which normally facilitates neurotransmitter release (Suszkiw, 2004). Interestingly, this appears to have an immediate effect, as well as a long-term, developmental effect. This may be due to the inhibition of NMDA receptor-dependent BDNF signaling, that is important for the stabilization of synaptic regions during presynaptic development (Neal et al., 2010). The CB_1-receptor is differentially regulated during neurogenic commitment, and there is evidence suggesting that cannabis exposure can affect fate regulation of cells in the developing brain by switching the balance of normal endocannabinoid signaling (Keimpema et al., 2011). Cocaine exposure during the prenatal period appears to have long term consequences on dopamine transporter function (Harvey, 2004) as well as impairing tangential and radial migration within the developing telencephalon (Lee et al., 2011; McCarthy et al., 2011), providing a possible double-hit that results in long-term behavioral deficits.

Ethanol is another environmental toxin with known effects on the central nervous system, and is currently considered to be the major preventable cause of mental retardation worldwide (Centre of Disease Control). In the cortex, observed changes include ectopias and microgyri-like structures and a general reduction in cortical volume (see Fukui and Sakata-Haga, 2009 for review). These changes, and the critical period identified for ethanol-induced microcephaly (Guerri, 2002), suggest early stages of cortical development are disrupted, with the radial progenitors the main target for the effects of ethanol. In support of this hypothesis, a decrease proliferation of these progenitors has been observed in a number of models of prenatal ethanol exposure (Rubert et al., 2006), as well as increased death of this population (Rubert et al., 2006; Aronne et al., 2008). There is also a reduction in the ability of the radial progenitors to differentiate into neurons and glia in an *ex vivo* assessment of progenitor function following prenatal ethanol exposure (Rubert et al., 2006). Interestingly, a recent study suggests that alcohol induces inflammation via activation of TLR4 (Alfonso-Loeches et al., 2010), which could mean that inflammation and ethanol exposure have similar underlying mechanisms of damage in the early developing brain.

GENE-ENVIRONMENT INTERACTIONS

The original evidence supporting gene-environmental interactions in neurodevelopmental disorders were based largely on observations of disease incidence. For example, siblings of autistic patients have approximately a 3–8% risk of disease incidence. This risk increases to 10% in non-identical twins and to 60–90% in identical twins (Parker-Athill and Tan, 2010). The incidence in siblings suggests a genetic component of the disease, while the increased incidence in non-identical twins highlights the important contribution of a shared maternal environment. The high concordance in identical twins, where gene and maternal environment are shared, has now been recognized not just to indicate genetic susceptibility, but also to highlight the important of the gene-environment interaction (van Os et al., 2010).

There are two main mechanisms by which gene-environment interactions can contribute to disease etiology. The first is a direct interaction, where a genetic polymorphism or copy number variant increases damage (or confers protection) after an environmental insult. The second is the ability of an environmental insult to produce an epigenetic change, potentially causing a long-term modulation of gene function (Van Winkel et al., 2010). There is evidence for both of these interaction paradigms in a number of neurological and mental disorders.

There are, as yet, few experimental studies testing the link between genes and environment, although those that do, using a number of outcome measures, have all indicated a synergistic effect of genetic modification is combined with environmental challenge, above and beyond what was observed with a single challenge (Oliver, 2011). One example, relevant for the etiology of schizophrenia is the combination of early life inflammation and a DISC1 mutation, which has been recognized in a number of schizophrenic patients. PolyI:C induced inflammation in both prenatal and postnatal mice carrying a DISC1 mutation is associated with a number of behavioral changes that are not observed in the DISC1 animals or polyI:C animals alone (Abazyan et al., 2010; Ibi et al., 2010).

The neurodevelopmental injury models of schizophrenia have also lead to the identification of new candidate genes for increased disease risk. Considering the increased risk of schizophrenia associated with perinatal hypoxia, Schmidt-Kastner et al. (2012) re-evaluated a number of GWAS and CNV studies to determine if genes associated with the hypoxic-ischemic response are altered in the schizophrenic patients. Their findings substantially supported this hypothesis, identifying a number of additional gene modifications that may increase the risk of schizophrenia following birth complications, and supporting the earlier work of

Nicodemus et al. (2008), who showed a significant interaction between hypoxia-ischemia genes and obstetric complications in a cohort of schizophrenia patients.

A later life example of gene-environment interaction is that of the association between cannabis use and psychosis. Caspi et al. (2005) conducted a longitudinal study on an epidemiological birth cohort and found that presence of a polymorphism in the COMT gene in combination with adolescent cannabis use increased the risk of adult-hood psychosis. The presence of the polymorphism explains the strong association between cannabis use and psychosis while also accounting for the small risk of psychosis in cannabis users in general.

Epigenetic changes in neurological disease are less well defined in neuropathology, and the potential heritability of the epigenetic change confounds the gene-environment calculation. However, altered histone methylations (one of the key forms of epigenetic regulation) have been described in brains from patients that had been diagnosed with schizophrenia or depression (Peter and Akbarian, 2011). Drug exposure (e.g., to methamphetamine) and stress are examples of environmental challenges that my cause epigenetic changes, and have been linked to changes in DNA methylation in the brains of schizophrenic patients and relevant animal models (Oh and Petronis, 2008). Additionally, transcriptional repressor MeCP2 has been identified in autism, with gene silencing produced by this mechanism linked to changes in neuronal structure (Currenti, 2010). It is likely that epigenetic modification of gene function will be increasingly recognized as a key contributor to neurological disorders as our scientific investigations continue in this area.

CONCLUSIONS

It is clear that there are a number of factors that contribute to neurodevelopmental disorders. When considering which are the most important in predicting neuropathology following insult it is necessary to consider the likelihood that multiple "windows of vulnerability" exist within the developing brain. These are likely to be dependent on the type and severity of the environmental insult and the genetic background of the individual. The task to decipher the links between genetic susceptibility and environmental insults is hindered by the fact that barrier functions, inflammatory, and endocrine factors have to be taken into account along with brain development. These will have to be considered in different combinations, rather than in isolation. Our current approaches are not broad enough. We are determined to link diseases to a single genetic or environmental insult. Instead, a wide variety of broadly linked functional systems have been identified as possibly effected, and the genes involved in various stages of neuronal development might be just a small part of the puzzle.

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