Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models

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Proper brain function requires stringent balance of excitatory and inhibitory synapse formation during neural circuit assembly. Mutation of genes that normally sculpt and maintain this balance results in severe dysfunction, causing neurodevelopmental disorders including autism, epilepsy and Rett syndrome. Such mutations may result in defective architectural structuring of synaptic connections, molecular assembly of synapses and/or functional synaptogenesis. The affected genes often encode synaptic components directly, but also include regulators that secondarily mediate the synthesis or assembly of synaptic proteins. The prime example is Fragile X syndrome (FXS), the leading heritable cause of both intellectual disability and autism spectrum disorders. FXS results from loss of mRNA-binding FMRP, which regulates synaptic transcript trafficking, stability and translation in activity-dependent synaptogenesis and plasticity mechanisms. Genetic models of FXS exhibit striking excitatory and inhibitory synapse imbalance, associated with impaired cognitive and social interaction behaviors. Downstream of translation control, a number of specific synaptic proteins regulate excitatory versus inhibitory synaptogenesis, independently or combinatorially, and loss of these proteins is also linked to disrupted neurodevelopment. The current effort is to define the cascade of events linking transcription, translation and the role of specific synaptic proteins in the maintenance of excitatory versus inhibitory synapses during neural circuit formation. This focus includes mechanisms that fine-tune excitation and inhibition during the refinement of functional synaptic circuits, and later modulate this balance throughout life. The use of powerful new genetic models has begun to shed light on the mechanistic bases of excitation/inhibition imbalance for a range of neurodevelopmental disease states.

Keywords: neurodevelopment, synapse, GABA, glutamate, excitation, inhibition, *E/I* ratio, Fragile X syndrome

INTRODUCTION

The integration of excitatory and inhibitory inputs at the level of the individual neuron, and in the organization of functional units constituting neural circuits, is fundamental to the information processing that mediates brain function. Establishing and maintaining the appropriate ratio of excitatory versus inhibitory synapses (*E/I* ratio) is a critical factor that enables circuit threshold definition and balances measured output responsiveness. Disruption of the *E/I* set point beyond acceptable tolerances leads to aberrant hyper/hypo-transmissive states, which when chronically unresolved cause severe dysfunction. A growing body of evidence suggests that disrupted *E/I* ratios within the central nervous system may be implicated in a range of neurodevelopmental disorders. Contributing factors may include the selective loss of either excitatory or inhibitory synapses, without compensatory changes, or genetic conditions that actively favor the formation or maintenance of one class of synapse relative to the other. Such imbalances may arise during initial neural circuit formation, as a failure of 'refinement' mechanisms that address balance during preliminary circuit use periods, or as a selective inability to maintain the *E/I* ratio into maturity, perhaps owing to defective plasticity properties. This review will discuss these alternate possibilities in the context of genetic models of neurodevelopmental disorders.

We examine *E/I* balance based on the two primary, opposing central neurotransmitters, glutamate and γ-amino-butyric acid (GABA). Their respective excitatory and inhibitory synapses are characteristically divergent in a range of features (Boeckers, 2006; Craig et al., 2006; Okabe, 2007; Tretter and Moss, 2008; Tyagarajan and Fritschy, 2010). First, the cellular position of the synapses differs dramatically. Glutamatergic synapses form almost exclusively on dendritic spines after preferential association with, and stabilization of, an incoming, exploratory filopodial scout (Lohmann and Bonhoeffer, 2008). In contrast, GABAergic synapses do not form on spines, but rather reside on dendritic shafts, nerve cell somata and axon initial segments. These inhibitory synapses arise from predecessor axon-dendrite contacts without any apparent protrusive activity from either axon or dendrite (Wierenga et al., 2008). Second, ultrastructural properties of pre- versus post-synaptic specializations are readily distinguishable between synaptic classes (Gray, 1959; Colonnier, 1968; Peters and Palay, 1996). Excitatory contacts maintain discrete asymmetry with an electron-dense postsynaptic density opposing a presynaptic active zone. Inhibitory contacts, however, appear relatively symmetric with presynaptic vesicles clustered opposite a synaptic cleft without a robust postsynaptic density. Third, the molecular constituents of the synapses differ consid-

erably. Beyond the ionotropic and metabotropic glutamate and GABA receptors that confer transmitter specificity, presynaptic transporters responsible for vesicle loading and organizing postsynaptic scaffolding molecules often serve as reporters for immunohistochemical synapse discrimination (Boeckers, 2006; Craig et al., 2006; Okabe, 2007; Tretter and Moss, 2008; Tyagarajan and Fritschy, 2010). Excitatory synapses maintain vesicular glutamate transporters (VGLUTs) and the postsynaptic density protein-95 kDa (PSD-95), whereas inhibitory synapses are marked by the presence of the vesicular GABA (VGAT) and vesicular inhibitory amino acid (VIAAT) transporters and the postsynaptic adaptor protein gephyrin.

In considering the overall ratio of excitatory to inhibitory neurons, in the mammalian cortex roughly 80% of neurons are excitatory and 20% inhibitory (Rubenstein and Merzenich, 2003). However, this compositional ratio may vary dramatically with brain region, development or aging, and it does not necessarily reflect the *E/I* synaptic balance per individual neuron. The *E/I* ratio influencing a given neuron is often functionally studied at an electrophysiological level by examining the contribution of glutamatergic and GABAergic synaptic inputs. The ratio can be determined by variably clamping the membrane potential to differentially record excitatory and inhibitory postsynaptic currents (EPSCs or IPSCs) (Liu, 2004). In addition, anatomical methods can be employed to structurally characterize the *E/I* ratio. Postembedding immunostaining or assays of symmetrical versus asymmetrical synapses via electron microscopy can assess the prevalence of excitatory and inhibitory synapses amongst convergent contacts (Megias et al., 2001). Serial electron microscope reconstructions have shown that *in vivo* rat hippocampal CA1 pyramidal cell dendrites receive approximately 30,000 excitatory inputs and 1,700 inhibitory inputs, yielding an *E/I* ratio of ~18:1 (Megias et al., 2001). Notably, *in vitro* hippocampal cultures display an immature dendritic compartment ratio of ~3:2 at 16-days shifting to ~4:1 by 19 days (Liu, 2004), which nevertheless remains strikingly different from the *in vivo* condition. Examination of the local inhibitory interneurons in the hippocampus reveals salient differences: Parvalbumin-containing neurons receive 16,000 inputs of which 6% are inhibitory (*E/I* = ~14:1); calbindin D (28 k) neurons receive 4,000 inputs of which 30% are inhibitory (*E/I* = ~2:1); and calretinin-positive neurons maintain 2,000 inputs of which 20% are inhibitory (*E/I* = ~3:1) (Gulyas et al., 1999). Clearly then, considerable differences in *E/I* ratios are discernable between, and even amongst, neurons of particular classes, and ratios may be highly dynamic as a function of developmental stage.

As studies have shown the relative excitatory drive upon each neuronal subtype is likely quite distinct, the *E/I* ratio therefore must be carefully regulated. Establishing this balance is further complicated by the developmental and activity-dependent shifts influencing ratio modulation. For example, *Xenopus* tectal neurons can bidirectionally modify GABAergic inputs as a function of the prevalent *E/I* ratio, with the level of convergent glutamatergic input appearing to be the determining factor (Liu et al., 2007). Indeed, GABA can serve as a depolarizing, excitatory transmitter in immature neurons (Ben-Ari, 2002; Akerman and Cline, 2007), and, when coupled with the relatively limited glutameteric inputs

at this stage, repetitive stimulation can lead to attenuating long-term depression (LTD) in GABAergic inputs (Liu et al., 2007). However, with the developmental elevation of glutamatergic strength and transition of GABAergic inputs to their canonical inhibitory role, similar stimulation at maturity yields GABAergic long-term potentiation (LTP) (Liu et al., 2007). An excitatory role for GABA is also prevalent in the developing murine neocortex. A combination of the resting membrane potential (E_m) and reversal potential for GABA (E_{GABA}) in immature animals (postnatal days 2–10) leads to pyramidal neuron and interneuron depolarization that mediates action potential generation within cortical layers 5/6 (Rheims et al., 2008). Moreover, excitatory and inhibitory neurotransmitters can be employed coincidentally with their co-release critical to proper circuit refinement (Noh et al., 2010). In the mammalian auditory system, VGLUT3 and glutamate are found at developing inhibitory GABA/glycinergic synapses in the sound localization pathway of the lateral superior olive, prior to the onset of hearing (Gillespie et al., 2005). Genetic disruption of the glutamatergic component in a *Vglut3*−/−mouse impairs the coordinate, requisite synaptic strengthening and silencing necessary for normal tonotopic map organization (Noh et al., 2010). Thus, successfully implementing a strategy to influence the *E/I* ratio depends upon not only synapse enumeration but also how specific neurotransmitters function as a consequence of developmental progression.

How then do neural circuits establish and maintain balanced excitation and inhibition? For the sake of stable, long-term effectiveness and integrity, network activity is proposed to be maintained within a given dynamic range by compensatory alterations preventing runaway signaling, a concept termed 'synaptic homeostasis' (Turrigiano and Nelson, 2004; Davis, 2006; Maffei and Fontanini, 2009). The initial characterization of this phenomenon was done in cultured cortical neurons (Turrigiano et al., 1998). Pharmacological activity blockade with tetrodotoxin resulted in an upregulation of miniature excitatory postsynaptic current (mEPSC) amplitudes to 192% of control; whereas, disruption of GABAergic inhibition with bicuculline resulted in a 2.5-fold increase in neuronal firing and decreased mEPSC amplitudes to 70% of control (Turrigiano et al., 1998). Thus, in response to influences forcibly altering activity, compensatory mechanisms are enlisted in an apparent attempt to restore the initial circuit set point. In addition to the modulation of synaptic strength, balance may be achieved through adaptation of synaptic efficacy, membrane excitability and/or synapse number. For example, in independent studies, activity deprivation also reduced miniature inhibitory postsynaptic current (mIPSC) amplitudes with a concomitant 50% reduction in synapses reactive for the ionotropic GABA$_A$ receptor (GABA$_A$R) (Kilman et al., 2002), and diminished activity in hippocampal cultures has been shown to increase the prevalence of synaptic pairing in efforts to enhance connectivity (Nakayama et al., 2005). Nevertheless, many conditions exist that permanently alter the *E/I* balance, indicating regular failure of the apparent homeostatic mechanism. This fact suggests either that homeostasis is far from a universal property of circuits, or that many, disparate defects can prevent appropriate compensation mechanisms from being engaged or maintained, compromising neurological function.

A host of devastating neurological diseases have been proposed to result from underlying *E/I* imbalance, ranging from neuropsychiatric conditions such as schizophrenia (Kehrer et al., 2008) and Tourette's syndrome (Wassef et al., 2003), to neurodegenerative conditions like Parkinson's and Huntington's diseases (Choonara et al., 2009; Cummings et al., 2009). In particular, a large number of neurodevelopmental diseases are proposed to result from an *E/I* shift. This review will specifically highlight a subset of such disorders that harbor identifiable genetic components and have been successfully modeled to enable rigorous preclinical investigations. Neurodevelopmental disorders include a myriad of conditions, which share the common theme that symptomatic presentation and disease onset is identifiable in early postnatal years, prior to full brain maturation, when critical periods of activity-dependent refinement and circuit remodeling are vulnerable. Such disorders include the autism spectrum, epilepsy, Rett syndrome (RTT), and Fragile X syndrome (FXS) (**Figure 1**). For each case, we will survey indicative *E/I* imbalance conditions and then provide a more thorough discussion of FXS as our prime example. We will then discuss a range of causative genetic alterations that skew *E/I* balance by interfering with various aspects of normal gene expression programs and synaptic formation, organization and maintenance.
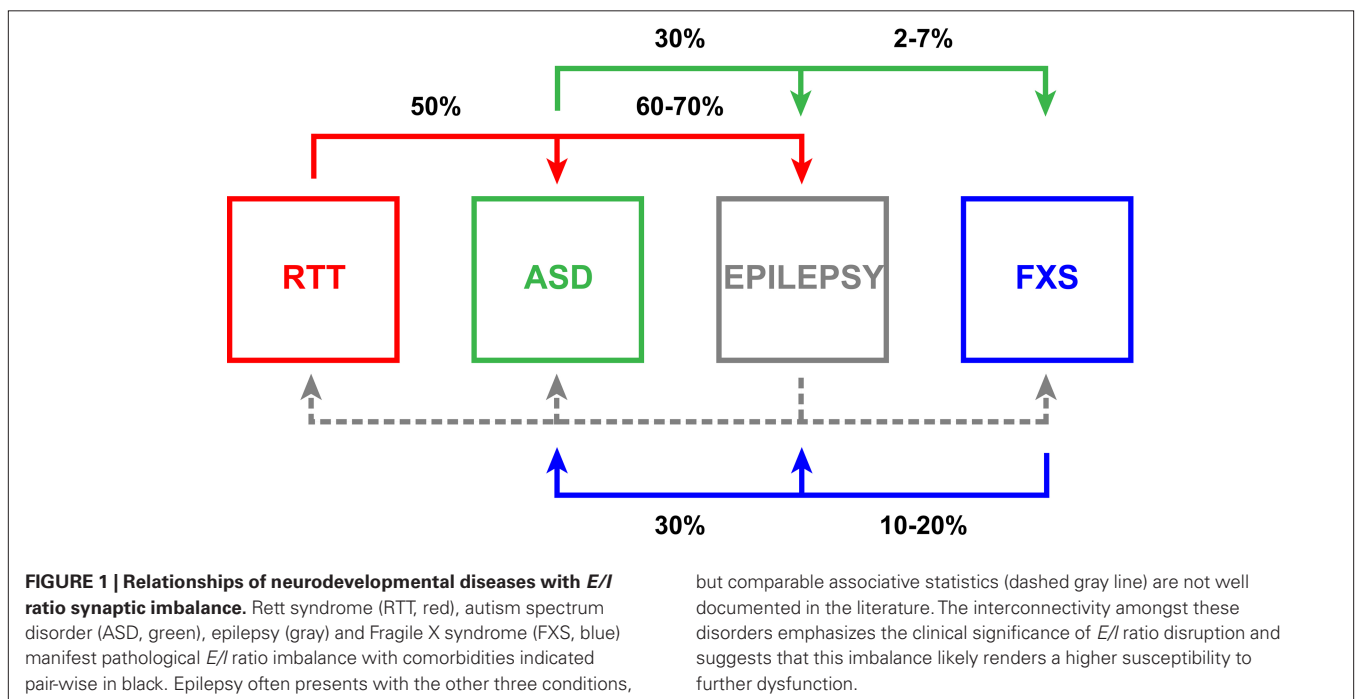
NEURODEVELOPMENTAL DISEASES WITH *E/I* IMBALANCE
AUTISM
With an incidence that some recent estimates place as high as 1:110 (Mulvihill et al., 2009), autism spectrum disorders (ASDs) are diseases of neurodevelopment with pervasive impact. The spectrum is characterized by compromised communication, social skills and cognition, often coupled with stereotypic repetitive behaviors and perception anomalies including hypersensitivity

to tactile and auditory stimulation (APA, 2000; Levy et al., 2009; O'Hare, 2009). Autism is clinically obvious typically by 3 years of age; however, abnormalities in socialization and play can be ascertained as early as 12 months (Landa et al., 2007; Barbaro and Dissanayake, 2009; Zwaigenbaum, 2010). The early onset of ASD implicates defects in either fetal brain development or the earliest phases of postnatal maturation. Thus for effective intervention, it is critical that we push downward the age of effective diagnosis as early as possible. In addition, autism often presents with other neurological disorders (**Figure 1**). Approximately 5% (2–7%) of ASD children have the common mutation causing FXS (Rogers et al., 2001; Hagerman, 2008b; Harris et al., 2008). Thirty percent of ASD patients also struggle with epilepsy (Tuchman and Rapin, 2002; Tuchman et al., 2009), and though current classification guidelines prevent comorbid diagnoses of autism and RTT, a recent study indicated that approximately 50% of RTT patients have been shown to meet ASD criterion employing the Developmental Behavior Checklist and the Diagnostic Interview for Social and Communication Disorders (Wulffaert et al., 2009).

Defects in synaptogenesis, synaptic refinement and connectivity have long been postulated to be a leading cause of the autism spectrum (Rubenstein and Merzenich, 2003; Rippon et al., 2007). Specifically, autism has been suggested to result from 'an increased ratio of excitation/inhibition in sensory, mnemonic, social and emotional systems' (Rubenstein and Merzenich, 2003). This putative imbalance is thought to contribute to the prevalence of poor signal-to-noise ratios, whereby hyperexcitable, non-tunable cortical circuits lead to critical developmental periods passing without appropriate differentiation being achieved, resulting in systemic instability (Rubenstein and Merzenich, 2003; Rippon et al., 2007). In support of this hypothesis, cytological abnormalities in the

FIGURE 1 | Relationships of neurodevelopmental diseases with *E/I* ratio synaptic imbalance. Rett syndrome (RTT, red), autism spectrum disorder (ASD, green), epilepsy (gray) and Fragile X syndrome (FXS, blue) manifest pathological *E/I* ratio imbalance with comorbidities indicated pair-wise in black. Epilepsy often presents with the other three conditions, but comparable associative statistics (dashed gray line) are not well documented in the literature. The interconnectivity amongst these disorders emphasizes the clinical significance of *E/I* ratio disruption and suggests that this imbalance likely renders a higher susceptibility to further dysfunction.

organization of mini-columns have been observed in neocortical post-mortem tissue. These vertical arrays of functionally related glutamatergic and GABAergic neurons process thalamic input and, in the autistic cortex, are smaller, more numerous and display disordered peripheral neuropil space (Casanova et al., 2002, 2003; Casanova, 2006). Also potentially contributing to unchecked hyperexcitability, post-mortem studies have revealed that neurons in autistic patients likely display depressed GABA production as the level of glutamic acid decarboxylase (GAD), the rate limiting enzyme in GABA synthesis, is reduced by half (Fatemi et al., 2002). Additionally, GABA responsiveness may itself be compromised as GABA$_A$R subunit depression is also observed with mRNA and protein studies showing altered α1–5 and β1 subunits (Fatemi et al., 2009, 2010). These studies suggest a shift in the E/I ratio favoring an elevated preponderance of glutamatergic connections with inhibitory insufficiency.

In addition to FXS and RTT, a number of genetic susceptibilities have been linked to elevated autism risk including maternal 15q11–13 chromosomal duplications, anomalies in the tumor suppressor genes *NF1*, *TSC1/TSC2*, and *PTEN* that activate mammalian target of rapamycin (mTOR)/phosphatidylinositol 3-kinase (PI3K) signaling pathways, and mutations in a range of synaptic genes such as the *neurexins*, *neuroligins*, *SHANK3* and *CNTNAP2* (Betancur et al., 2009; Bourgeron, 2009; Kumar and Christian, 2009). For example, mutations in the postsynaptic cell adhesion molecule neuroligin-3 (NLGN3), particularly a single amino acid substitution at the Arg451 to Cys451 (R451C), have been well-characterized (Jamain et al., 2003). This genetic variant has been introduced in mice and behaviorally produces impaired social interaction, albeit with a surprisingly enhanced capacity for spatial learning (Tabuchi et al., 2007). At the E/I level, the R451C–NLGN3 knock-in shows increased VGAT and gephyrin levels. Although no change in inhibitory synapse number was observed, a 50% increased mIPSC frequency and increased amplitude of evoked IPSCs in whole-cell recordings of layer 2/3 somatosensory barrel cortex slices was observed in the absence of any accompanying changes in excitatory transmission (Tabuchi et al., 2007). Interestingly, these changes did not occur in the complete NLGN3 knockout (KO), indicating R451C–NLGN3 presumably represents a gain-of-function variation. *In vitro* connectivity studies employing dissociated rat hippocampal cultures indicated that NLGN3 introduction enhances optically observable spontaneous synchrony, whereas R471C-NLGN3 reduces this capacity (Gutierrez et al., 2009). Further, studies of axonal topography revealed R471C-NLGN3 expression results in shorter, less interconnected neurons and the preferential degeneration of inhibitory neurons. Thus, the overall network influence trends toward a reduction of inhibitory activity (Gutierrez et al., 2009). Collectively, these ASD model studies indicate that E/I imbalance can present differentially in various circuits; and, importantly, structural and functional changes do not always coincide.

EPILEPSY

Epilepsy refers to a family of chronic neurological disorders with a frequency of approximately 7:1,000 individuals (Hirtz et al., 2007), characterized by a tendency toward recurrent non-provoked, spontaneous seizures (Scharfman, 2007; Jacobs et al., 2009; Reid

et al., 2009). Epilepsy is often discussed as resulting from disruption in the balance of neuronal excitation and inhibition coupled with asynchrony generating episodic periods of abnormal activity (Fritschy, 2008) and can be caused by neurological channelopathies with mutations in voltage-gated (N$^+$, K$^+$, Ca^{2+} and Cl$^-$) and ligand-gated (nicotinic acetylcholine and GABA$_A$R) channels (Kang and Macdonald, 2009; Reid et al., 2009; Kullmann, 2010). Furthermore, epilepsy is often strikingly associated with other neurodevelopmental disorders (**Figure 1**). Epilepsy develops in 60–70% of RTT patients (Huppke et al., 2007; Moser et al., 2007), and abnormal electroencephalographs (EEGs) are observed in 60% of autistic children with 30% having recurrent seizures (Tuchman and Rapin, 2002; Tuchman et al., 2009; Coury, 2010). Likewise, altered EEGs are observed commonly in FXS children, and 10–20% display benign focal epilepsy with childhood seizures, typically remitting in late adolescence (Musumeci et al., 1999; Sabaratnam et al., 2001; Berry-Kravis, 2002; Qiu et al., 2008; Hagerman and Stafstrom, 2009). This remission prevalence and the incidence of other childhood epilepsies that wane with age are indicative of higher epileptogenic susceptibility to E/I imbalance in the immature brain (Rakhade and Jensen, 2009). During development, homeostatic mechanisms may not be effectively engaged, or may require a substantial temporal window to effect the functional compensation necessary to offset alterations in activity. However, resolution is not a consistent feature of epileptic disease progressions, indicating an insufficiency and/or failure of long-range homeostatic influence under certain circumstances.

The clear propensity of E/I imbalance involvement in epilepsy obviates the need to explore and define this in effective epilepsy model systems (Fritschy, 2008). In rodents, experimental cortical dysplasia induced via *in utero* irradiation mirrors altered cortical abnormalities seen in intractable epilepsy (Roper, 1998; Porter et al., 2003). Initial findings showed reduced inhibitory synaptic currents in pyramidal cells (Zhu and Roper, 2000) and reduced density of inhibitory interneurons (Roper et al., 1999) with depressed excitatory drive (Xiang et al., 2006). A more recent study specifically assessed the inhibitory synaptic activity in fast-spiking (FS) interneurons in these animals, showing a reduction in frequency, though not amplitude, of spontaneous IPSCs (sIPSCs) and aberrant short-term facilitation upon evocation (Zhou et al., 2009). Moreover, simultaneously monitoring excitatory and inhibitory postsynaptic currents revealed an E/I shift favoring inhibition in these interneurons, which typically serve to temper activity, thus leading to a net circuit effect of unchecked excitation. Altered E/I parameters are also seen upon modeling temporal lobe epilepsy, which is characterized by neuron loss in the hippocampal dentate gyrus (Dudek and Sutula, 2007). A recent study of the pilocarpine-elicited status epilepticus model revealed a preliminary loss of GABAergic neurons and decrement of inhibitory synapses to minimally 70% of control levels with concomitant excitatory synaptic loss onto the granule cells (Thind et al., 2010). Excitatory synapses were reported to later reestablish normally, whereas the surviving interneurons initiate robust synaptogenesis and supersede control levels by 120–150%. However, it was previously shown that disrupted chloride regulation, via loss of Cl$^-$ extrusive function in the K$^+$/Cl$^-$ KCC2 co-transporter, leads to a reduction in inhibition efficacy and enhanced granule

cell excitability (Pathak et al., 2007). These results suggest that the *E/I* quantitative ratio is not always solely indicative of the exerted circuit influence, and coordinate functional determinations remain imperative.

RETT SYNDROME

Rett syndrome (RTT) is a relatively rare (1:10,000) but insidious, progressive X-linked neurodevelopmental disease that is a leading cause of intellectual disability in females (Glaze, 2002; Percy and Lane, 2005; Segawa and Nomura, 2005; Chahrour and Zoghbi, 2007; Monteggia and Kavalali, 2009). Development appears to progress normally in RTT children until 6–18 months of age, when symptomatic presentation begins. The disorder is then associated with developmental reversion involving loss of acquired proficiencies including expressivity and motor skills, continuing cognitive impairment, autistic behaviors and seizures (**Figure 1**). The most common etiology is *de novo* mutation in the transcriptional regulator, methyl-CpG-binding protein 2 (MECP2) (Amir et al., 1999). *MECP2* disruptions account for up to 90% of RTT cases, although *MECP2* duplication events (Shi et al., 2005; Van Esch et al., 2005; del Gaudio et al., 2006; Bunyan and Robinson, 2008; Smyk et al., 2008) and mutations in cyclin-dependent kinase-like 5 (*CDKL5*) (Weaving et al., 2004; Mari et al., 2005; Sprovieri et al., 2009) and the Forkhead family transcriptional repressor *FOXG1* (Ariani et al., 2008; Jacob et al., 2009a; Mencarelli et al., 2010) have also been implicated.

Rett syndrome has been successfully modeled in mice via deletion of all or the third exon of *MECP2* (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). Mutant animals display age-progressive motor impairment, tremors, increased anxiety and seizures. In agreement with analyses of post-mortem tissue samples (Belichenko et al., 1994; Chapleau et al., 2009), *MECP2* mutants display a significant reduction in dendritic spine number (Belichenko et al., 2009; Chapleau et al., 2009), suggesting decreased excitatory synapse capacity. Electrophysiological examinations of cortical slices from the *MECP2* KO mouse indicate a reduction in spontaneous pyramidal neuron activity resulting from a reduction in mEPSC amplitude as compared to maintained mIPSCs, thus favoring inhibition (Dani et al., 2005). Deficits in LTP upon MECP2 truncation have also been reported (Moretti et al., 2006); however, cortical LTP induction mechanisms appear to be retained in early-symptomatic *MECP2* KO animals though with fewer and weaker connections, as revealed by a 50% reduction in connection probability and a 45% reduction in excitatory postsynaptic potential (EPSP) amplitude, respectively (Dani and Nelson, 2009). Recent *in utero* transfection of short hairpin RNA (shRNA) constructs directed against *MECP2* support these findings with intracortical networks showing at least 30% reduction in excitatory synaptic input from cortical layers L3/5A (Wood et al., 2009). Glutamate uncaging coupled with laser scanning photostimulation likewise revealed a 40% reduced excitatory input to layer 2/3 neurons (Wood et al., 2009). In addition, impaired hippocampus-dependent spatial memory, contextual fear memory, social memory, and LTP arise with MECP2 truncation (Moretti et al., 2006). In contrast to cortical studies, hippocampal network examination of the *MECP2* KO animals revealed a tendency toward hyperexcitability, as spontaneous inhibitory rhythmic activity was selectively reduced in frequency (Zhang et al., 2008).

However, the *MECP2* KO displays a 20% reduction in excitatory synapses in the stratum radiatum of hippocampal CA1 neurons *in vivo*, although this appears to resolve by 5 weeks of age (Chao et al., 2007). Thus, as in ASD, *E/I* imbalance in RTT may be circuit specific and temporally modulated.

FRAGILE X SYNDROME

Fragile X syndrome (FXS) is the leading monogenic heritable cause of intellectual disability and ASD, conservatively affecting 1:4,000 males and 1:8,000 females (O'Donnell and Warren, 2002; Penagarikano et al., 2007; Cornish et al., 2008; Garber et al., 2008), although recent estimates place the full mutation frequency as high as 1:2,500 in the general population (Hagerman, 2008a). Clinical FXS symptoms include delayed and depressed developmental trajectories (Bailey et al., 2001a,b; Hall et al., 2008), short-term working memory deficits (Munir et al., 2000; Cornish et al., 2001; Kwon et al., 2001; Lanfranchi et al., 2009), disordered sleep (Gould et al., 2000; Miano et al., 2008), seizures (Kluger et al., 1996; Musumeci et al., 1999; Singh et al., 1999; Sabaratnam et al., 2001; Berry-Kravis, 2002; Di Bonaventura et al., 2006; Qiu et al., 2008), elevated anxiety (Tsiouris and Brown, 2004), and attention-deficit hyperactivity disorder (Hatton et al., 2002; Sullivan et al., 2006). Significantly, 30% of FXS patients are also diagnosed with autism (**Figure 1**) (Kau et al., 2004; Hagerman, 2008b; Harris et al., 2008).

FXS is caused by the loss of *fragile X mental retardation 1* (*FMR1*) gene function (Verkerk et al., 1991), most commonly due to unstable CGG-trinucleotide repeat expansion in the 5′ regulatory region that leads to hypermethylation and transcriptional silencing (Sutcliffe et al., 1992). The *FMR1* product (FMRP) is an mRNA-binding, polysome-associated protein that typically acts as a negative regulator of protein translation (Laggerbauer et al., 2001; Li et al., 2001; Sung et al., 2003; Zalfa et al., 2003; Lu et al., 2004; Qin et al., 2005; Tessier and Broadie, 2008), facilitates mRNA trafficking (Dictenberg et al., 2008; Estes et al., 2008), and influences mRNA stability (Zalfa et al., 2005; Zhang et al., 2007). Murine and *Drosophila* FXS models allow mechanistic examinations into the disease (Bakker et al., 1994; Zhang et al., 2001; Mientjes et al., 2006), and both models recapitulate numerous clinical FXS features, displaying neuronal overgrowth, altered synaptogenesis and synaptic plasticity coupled with defective circadian rhythms, learning and memory (Bassell and Warren, 2008; Gatto and Broadie, 2009b; Mercaldo et al., 2009; Pfeiffer and Huber, 2009).

In light of *E/I* balance, it is important to note that FMRP is activity-regulated. Downstream of glutamatergic neurotransmission, metabotropic glutamate receptor (mGluR)-mediated translational induction elevates FMRP levels (Weiler and Greenough, 1993; Weiler et al., 1997). Moreover, peak endogenous FMRP levels *in vivo* correlate with postnatal periods of significant use-dependent synaptic refinement and later plasticity (Singh et al., 2007; Tessier and Broadie, 2008). As we have discussed previously (Gatto and Broadie, 2009b; Tessier and Broadie, 2009), considerable work has shown that sensory stimulation also serves as an effective means to elicit activity-responsive FMRP enhancement and can be achieved via exposure to complex, stimulating environments and motor skill training (Irwin et al., 2000, 2005). Levels of sensory stimulation directly impact FMRP levels and function. In rodents, whisker stimulation increases FMRP expression in synapse- and polysome-containing fractions (Todd and

Mack, 2000) as does visual experience in dark-reared/light-exposed animals (Gabel et al., 2004). Consistently, in *Drosophila*, sensory deprivation yields depressed dFMRP levels, as does genetic disruption of visual or olfactory sensory modalities (Tessier and Broadie, 2008). Thus, activity positively regulates FMRP, and FMRP functions in activity-dependent synaptic mechanisms.

E/I BALANCE IN FXS

The above studies suggest that conditions favoring an excitation-dominant *E/I* ratio elevate FMRP levels and function. Conversely, FMRP itself may impinge upon the balance of excitatory and inhibitory synapse formation during the genesis and maintenance of neural circuits (**Figure 2**). Post-mortem examinations in FXS patients reveal aberrant, supernumerary cortical dendritic spines, suggesting an architectural framework for elevated glutamatergic synaptogenesis (Rudelli et al., 1985; Hinton et al., 1991). However, as with the potential *E/I* ratio variability, FMRP function may be

brain region specific. GluR1-containing α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPAR) and LTP are depressed in the mouse FXS model cortex (Li et al., 2002; Zhao et al., 2005; Desai et al., 2006; Wilson and Cox, 2007), whereas mGluR-mediated LTD is enhanced in the hippocampus (Huber et al., 2002; Nosyreva and Huber, 2006). Moreover, in conditioned hippocampal slices from *FMR1* KO mice, synaptically released glutamate induced prolonged group I mGluR-mediated epileptic discharges, whereas induction of this type of activity in wild-type animals normally requires mGluR activation with (RS)-3,5-dihydroxyphenylglycine (DHPG). The induced epileptiform activity in the KO mouse could be blocked with translational inhibitors and suppressed with mGluR antagonists (Chuang et al., 2005). In *Drosophila fmr1* (*dfmr1*) mutants, electrophysiological studies have indicated increased glutamatergic vesicle fusion event frequency and enhanced exocytic vesicle cycling (Zhang et al., 2001; Gatto and Broadie, 2008). High frequency stimulation paradigms
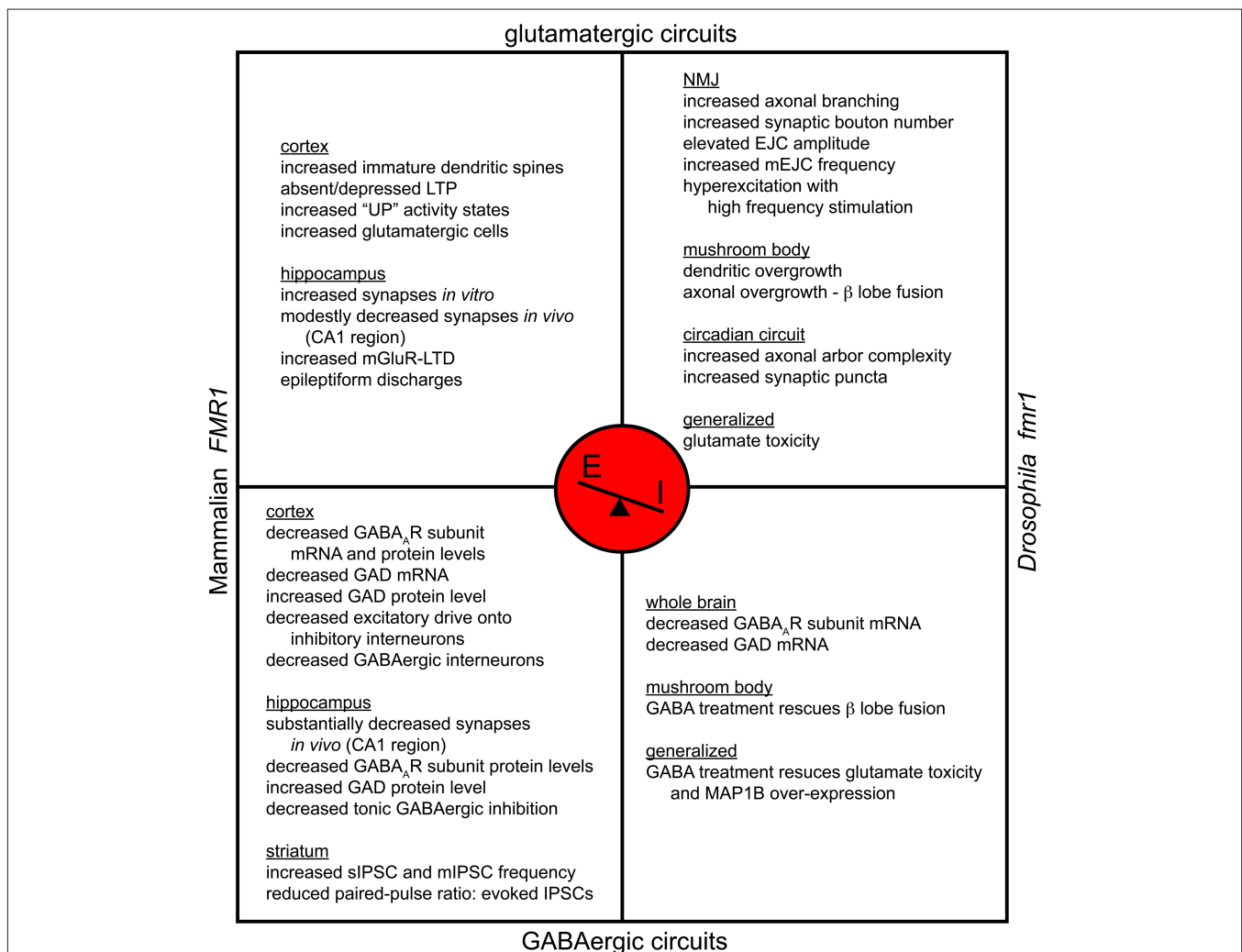

FIGURE 2 | Fragile X syndrome is a disease of excitation-dominance. FXS models have been established via mutation of mouse and *Drosophila FMR1*. Both systems manifest a shift in synaptic *E/I* ratio with hyperexcitation favored. Model-specific alterations are diagrammed for glutamatergic and GABAergic circuits. Both models share the prevalence of increased numbers of excitatory synaptic boutons, elevated glutamatergic transmission, susceptibility to hyperexcitability and decreased GABA$_A$R expression. FXS is thus characterized by glutamatergic elevation as well as GABAergic depression.

revealed multiple synaptic transmission events in response to discrete stimuli and periodic amplitude cycling during the high frequency challenge. These defects are partially rescued in *dfmr1; DmGluRA* double null mutants, which lack all mGluR-mediated signaling (Repicky and Broadie, 2008). Taken together, these studies indicate exaggerated mGluR signaling function in FXS models, establishing a hyperexcitable state (**Figure 2**).

The FXS hyperexcitable state resulting from an *E/I* imbalance was recently demonstrated in acute neocortical slices isolated from the *FMR1* KO mouse (Gibson et al., 2008). In layer 4 recordings, mutants showed ~50% reduction in excitatory drive onto FS inhibitory neurons, with EPSC frequency and amplitude diminished due to compromised connectivity. In addition, there was a ~20% decrease in excitatory drive onto excitatory neurons (Gibson et al., 2008). Together, these conditions foster an inhibition of inhibitory neurons within the circuit promoting an *E/I* balance in favor of elevated excitation. Moreover, the intrinsic excitability of the excitatory neurons was itself enhanced yielding a 2-fold increase in epochs of persistent activity, or 'UP' states, albeit with altered synchrony ascribed to the disrupted FS circuitry (Gibson et al., 2008). Consistently, the *FMR1* KO may yield more cells that are responsive to mGluR activation (Castren et al., 2005) and increased neocortical glutamatergic cell differentiation (Tervonen et al., 2009), further predisposing the FXS state for hyperexcitation.

In addition to enhanced excitation, dysfunction in FXS neuronal circuits can also be attributed to more direct GABAergic involvement (**Figure 2**). In *FMR1* KO animals, initial examination of subicular neuron function, located in the most inferior aspect of the hippocampus, revealed electrophysiological responses indicating decreased GABAergic inhibition (D'Antuono et al., 2003). Further functional characterization with patch-clamp recordings in *FMR1* null organotypic slice cultures revealed a 91% decrease in current density for tonic, but not phasic, inhibition in subicular pyramidal cells, suggesting a defect in peri- and extra-synaptic GABA receptors (Curia et al., 2009). This tonic depression was associated with corollary α5 and δ GABA$_A$R subunit under-expression, with mRNA levels depressed 26 and 35% by real-time RT-PCR and protein levels depressed 13 and 28% by Western blot, respectively (Curia et al., 2009). In the somatosensory neocortex, there was a 20% reduction in parvalbumin-reactive GABAergic interneurons, and GABAergic neurons were also decreased in Layers II/III/IV (Selby et al., 2007), suggesting aberrant local inhibitory contributions. These changes are clearly brain region specific and may be absent, or even opposite, in other brain regions. In fact, in light of frontostriatal circuit disruption reported in clinical FXS evaluations (Menon et al., 2004b), striatal slices from the *FMR1* KO reveal abnormal GABA-mediated transmission in spiny neurons, although within this region inhibitory transmission was enhanced with an increased frequency of sIPSCs and mIPSCs (Centonze et al., 2008). Interestingly, this change was not due to increased GABAergic synapse number, as VGAT-reactive puncta were actually decreased, but rather an enhanced release probability as evidenced by the reduced paired-pulse ratio of evoked IPSCs (Centonze et al., 2008).

MECHANISTIC THEORIES BEHIND *E/I* IMBALANCE IN FXS

The 'mGluR Theory of FXS' places FMRP function downstream of mGluR activation at the synapse (Bear et al., 2004; Bear, 2005), based on observations that Group I mGluR1/5 signaling via Gq-mediated

transduction stimulates synaptic translation via FMRP (Weiler and Greenough, 1993; Weiler et al., 1997, 2004; Greenough et al., 2001; Job and Eberwine, 2001; Muddashetty et al., 2007; Westmark and Malter, 2007). In wild-type mice, mGluR-dependent LTD requires stimulus-responsive protein synthesis (Massey and Bashir, 2007), but *FMR1* KO mice do not display mGluR-triggered polysome assembly or activity induced protein synthesis (Todd et al., 2003; Weiler et al., 2004; Muddashetty et al., 2007; Westmark and Malter, 2007; Park et al., 2008). The result is elevated mGluR-triggered LTD (Huber et al., 2002; Nosyreva and Huber, 2006), suggesting that FMRP usually contributes feedback by repressing LTD-enhancing elements. mGluR5 antagonists rescue many *FMR1* KO defects, including audiogenic seizure susceptibility, open-field exploratory hyperactivity and prepulse startle inhibition (Yan et al., 2005; de Vrij et al., 2008). Similarly, genetic reduction of mGluR5 signaling (*mGluR5/+* heterozygotes) in the *FMR1* null background alleviates most defined neurological dysfunctions, including altered ocular dominance plasticity, cortical neuron dendritic spine density, increased basal protein synthesis, inhibitory avoidance extinction and audiogenic seizure sensitivity (Dolen et al., 2007).

Despite the clear importance of the mGluR signaling mechanism, it alone does not account for the full range of FXS abnormalities. More recent work has established links to fast cholinergic transmission and slow modulatory transmission mediated by biogenic amines (e.g. dopamine) and neuropeptides (Zhang et al., 2005; Wang et al., 2008). Examination of M$_1$ muscarinic acetylcholine receptor (mAChR) activated-LTD revealed a protein synthesis-dependent mechanism and AMPAR internalization associated with elevated FMRP levels (Volk et al., 2007). mAChR-mediated LTD was enhanced in *FMR1* KO mice, associated with misregulation of FMRP targets, i.e. *elongation factor 1α* (*EF1α*) and *calcium/calmodulin-dependent kinase II* (*CaMKII*), uncoupling LTD induction from requisite protein synthesis. Thus, Gq-coupled ACh and glutamate receptors both appear to mediate protein synthesis-dependent synaptic changes mechanistically involving FMRP. Moreover, female *FMR1* KO mice display increased dopamine turnover in cortical regions, striatum and hippocampus (Gruss and Braun, 2004), although this is reported not to occur in KO males (Gruss and Braun, 2001). A proteomic approach employing 2D electrophoresis in *dfmr1* mutants indicated elevations in phenylalanine hydroxylase and GTP cyclohydrolase, driving the upregulated biosynthesis of dopamine and serotonin (Zhang et al., 2005). Critically, as dopamine D1 receptors contribute to ionotropic glutamate AMPAR trafficking, D1 stimulation in *FMR1* KO cultured prefrontal cortex neurons caused a 37% decrease in GluR1-containing AMPAR surface expression and compromised phosphorylation (Wang et al., 2008). Moreover, D1 receptor signaling was itself impaired, and the redistribution of its G protein-coupled receptor kinase 2 (GRK2) to the membrane was also observed, likely yielding D1 receptor hyperphosphorylation and disrupted function. The *in vivo* relevance of these studies was highlighted by finding that amphetamine introduction in the *FMR1* KO mouse elevated dopamine release in the prefrontal cortex and improved object recognition (Ventura et al., 2004), and D1 agonist treatment reduced open-field hyperactivity (Wang et al., 2008). These results indicate that FMRP's mode of action is more complex than being solely mGluR-reactionary and, in fact, suggest that FMRP serves more generally as part of a

feedback loop linking neurotransmitter signaling via Gq-coupled receptors for a range of neurotransmitters and neuromodulators. This model is described as the 'Gq Theory of FXS'.

Disruption of inhibitory GABAergic signaling is also a vitally important component of the disease state (**Figure 2**), as expressed by the 'GABA$_A$R Theory of FXS' (D'Hulst and Kooy, 2007, 2009). In addition to the functional defects in inhibitory signaling discussed above, GABA$_A$R subunit mRNA and protein levels are depressed in both murine and *Drosophila* FXS models, suggesting that FMRP must contribute to the stability and/or translation of GABA$_A$R transcripts (El Idrissi et al., 2005; D'Hulst et al., 2006, 2009b; Gantois et al., 2006). The *Drosophila* model further manifests reduced mRNA levels for the GABA synthesizing enzyme GAD (D'Hulst et al., 2009b), and likewise, the *FMR1* KO mouse has been reported to show decreased *GAD* mRNA in the cortex (D'Hulst et al., 2009b). However, in sharp contrast, the *FMR1* KO displays increased GAD protein expression where decreased GABA$_A$R β subunits are detected (i.e. in cortex, hippocampus, diencephalon and brainstem), perhaps indicating a partial compensatory mechanism (El Idrissi et al., 2005). Most notably, GABA administration in the *Drosophila* FXS model reportedly blocks glutamate toxicity and rescues microtubule-associated protein 1B (MAP1B)/Futsch over-expression, neuronal overgrowth defects and courtship memory impairment (Chang et al., 2008). Thus, FXS is likely a disease of neuronal hyperexcitation, not only due to overactive Gq-signaling downstream of glutamate and other transmitters, but also due to hypoinhibition, due to reduced expression and function of GABA$_A$Rs.

GENETIC MECHANISMS REGULATING *E/I* BALANCE
Although homeostatic mechanisms and corrective programs may be in play to address synaptic stability, *E/I* imbalance remains as a prevalent underpinning in many types of neurological dysfunction. It is therefore critical to understand at what level the genetic and molecular regulation goes awry to compromise circuit integrity. In recent years, a growing number of genes have been identified as participating in sculpting and maintaining the delicate balance of excitatory and inhibitory synaptogenesis, in different animal models and in different classes of circuit. These factors range from transcriptional and translational regulators, to specific resident synaptic proteins; moreover, mutations in these same genes are often clinically linked to the manifestation of the neurodevelopmental diseases discussed above (**Figure 3**).

TRANSCRIPTIONAL REGULATORS
To keep the synaptic *E/I* ratio balanced, transcriptional regulators likely provide a first line of defense (**Figure 3**). For example, MECP2 binds methylated DNA to mediate recruitment of a repressor complex including Sin3A and histone deacetylases; its mutation is the leading cause of RTT (Amir et al., 1999). Exemplifying the interconnectedness of neurodevelopmental dysfunction, *MECP2* mRNA levels are also dramatically misregulated in *FMR1* KO mice; decreased in hippocampus, cortex, diencephalon and brain stem, and conversely elevated in the cerebellum (Zhang et al., 2009). Reduced MECP2 expression is also noted in the frontal cortex in non-FXS autism (Nagarajan et al., 2006). To control *E/I* balance, MECP2 serves to promote excitatory synapse formation. *MECP2* null neurons maintain a 40% reduction in VGLUT/PSD-95 puncta
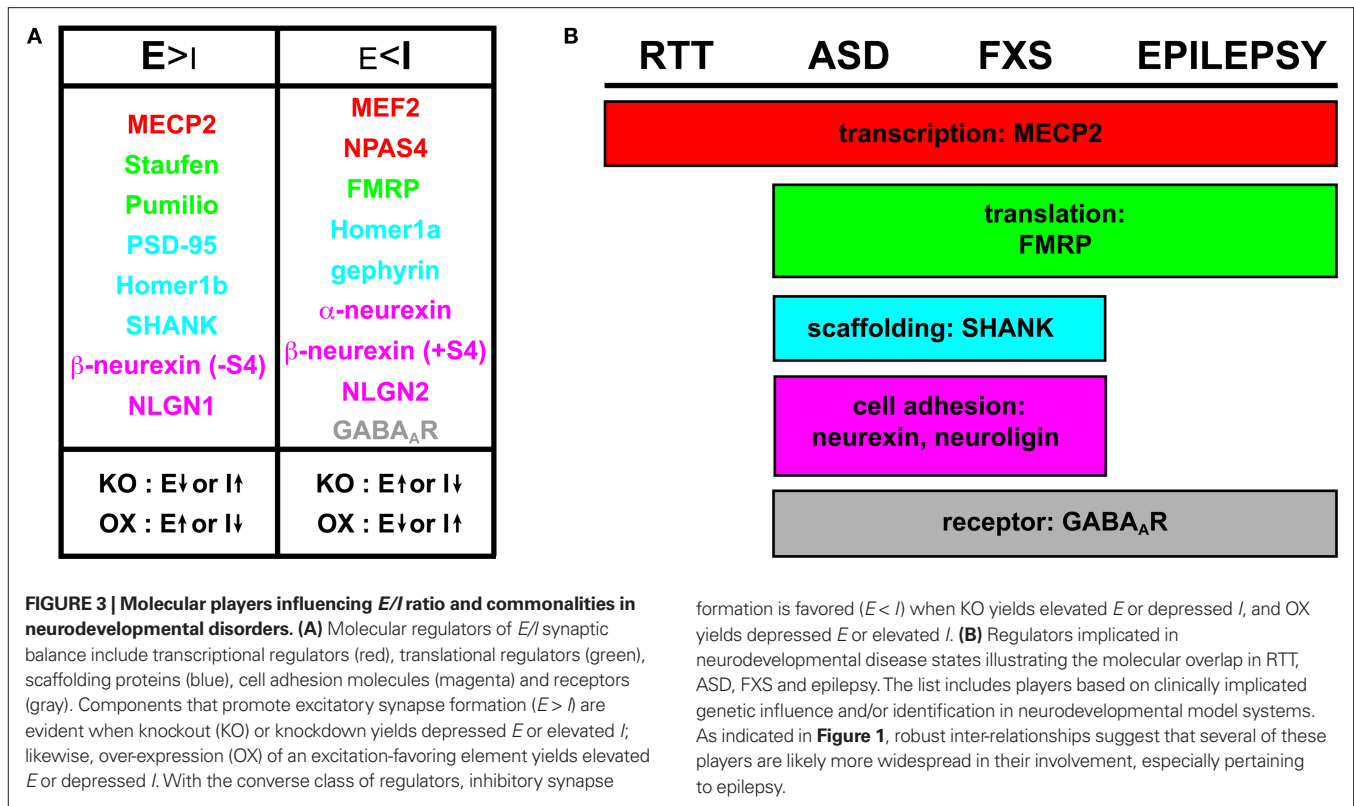
and a 46% reduction in EPSC amplitude, whereas transgenic 2-fold over-expression of MECP2 yields a 60% increase in synapse number with a 116% increase in EPSC amplitude (Chao et al., 2007). As a range of neurodevelopmental disorders display compromised MECP2 levels, and thus potentially favor depressed excitatory synapse formation and function, how these findings align with the network-specific hyperexcitability characteristic of RTT, FXS and autism remains a confounding issue.

The myocyte enhancer factor 2 (MEF2) family of transcription factors, initially implicated in muscle development (Buskin and Hauschka, 1989; Gossett et al., 1989), also regulates excitatory synapse number (Flavell et al., 2006; Barbosa et al., 2008). In contrast to MECP2, however, the MEF2 family serves a suppressive role. In cultured hippocampal neurons, RNAi-mediated knockdown of *MEF2A* and *D* caused a significant increase in the number of synapsin/PSD-95 reactive synapses and increased mEPSC frequency (Flavell et al., 2006). These effects are attributed to MEF2 promoting the transcription of targets that negatively regulate excitatory synapse development, including activity-regulated cytoskeletal-associated protein (Arc) and synaptic Ras guanosine triphosphate activating protein (synGAP), as indicated by real-time quantitative PCR and Western blotting (Flavell et al., 2006). MEF2-dependent negative regulation of excitatory synapse number and function was confirmed *in vivo* using a brain-specific deletion of *MEF2C* (Barbosa et al., 2008), although surprisingly, neurons formed normal synapses in culture. Behaviorally, *MEF2C* deletion significantly impaired hippocampal-dependent learning and memory (Barbosa et al., 2008). Thus, there is a correlation in this case between increased excitatory synapse number, potentiated synaptic transmission, and impaired behavioral output.

Inhibitory synapse development appears to be independently driven by a different dedicated set of transcription factors. The basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family member *Npas4* was identified in a screen for neuronal genes induced upon membrane depolarization via calcium influx (Lin et al., 2008). Target genes were selected based on excitatory activity being temporally coordinated with inhibitory synapse development. RNAi against *Npas4 in vitro* significantly reduced inhibitory synapse number, to less than 50% of control, whereas *Npas*-minigene over-expression doubled inhibitory synapse density (Lin et al., 2008). These studies showed no impact on excitatory synapse number, suggesting *Npas4* activity is specifically driving inhibitory synapse formation. Functionally, deletion of *Npas4* in organotypic slice cultures decreased inter-event intervals (increased frequency) of spontaneous mEPSCs, while over-expression increased their inter-event interval (decreased frequency) and decreased their amplitude (Lin et al., 2008). Thus, Npas4 selectively and positively regulates inhibitory synaptogenesis. Taken together, the above studies show that different transcription factors control excitatory and inhibitory synapse formation independently and, apparently, without a detectable level of *E/I* balance compensation.

TRANSLATIONAL REGULATORS
Downstream of transcription, activity-dependent regulators of protein expression are prime candidates to modulate the *E/I* ratio. The importance of spatially restricted, local translation modulating functional synapse changes is well documented, particularly in mediating

FIGURE 3 | Molecular players influencing *E/I* ratio and commonalities in neurodevelopmental disorders. (A) Molecular regulators of *E/I* synaptic balance include transcriptional regulators (red), translational regulators (green), scaffolding proteins (blue), cell adhesion molecules (magenta) and receptors (gray). Components that promote excitatory synapse formation (*E > I*) are evident when knockout (KO) or knockdown yields depressed *E* or elevated *I*; likewise, over-expression (OX) of an excitation-favoring element yields elevated *E* or depressed *I*. With the converse class of regulators, inhibitory synapse formation is favored (*E < I*) when KO yields elevated *E* or depressed *I*, and OX yields depressed *E* or elevated *I*. **(B)** Regulators implicated in neurodevelopmental disease states illustrating the molecular overlap in RTT, ASD, FXS and epilepsy. The list includes players based on clinically implicated genetic influence and/or identification in neurodevelopmental model systems. As indicated in **Figure 1**, robust inter-relationships suggest that several of these players are likely more widespread in their involvement, especially pertaining to epilepsy.

plasticity (Steward and Schuman, 2001; Sutton and Schuman, 2006; Bassell and Warren, 2008; Waung and Huber, 2009). The FMRP translational regulator perhaps represents the archetypal example. FMRP was initially proposed to bind ~4% of human fetal brain mRNAs *in vitro* (Ashley et al., 1993), but thus far only a handful of *in vivo* targets have been validated. The best-supported FMRP targets include *Arc*, *PSD-95*, *AMPAR subunits GluR1/2*, *GABA$_A$R subunit δ*, actin-binding *profilin*, *MAP1B* and *CaMKII* (Bassell and Warren, 2008; Gatto and Broadie, 2009b). While much attention had been paid to the morphological phenotypes associated with FXS model neurons, including the excessive formation of dendritic filopodia/immature spines and supernumerary bouton formation (Comery et al., 1997; Nimchinsky et al., 2001; Zhang et al., 2001; Morales et al., 2002; Galvez et al., 2003; Michel et al., 2004; Pan et al., 2004; Gatto and Broadie, 2008, 2009a; Tessier and Broadie, 2008), relatively little information is available regarding any corollary alteration in *E/I* ratio at the level of synapse number. One recent study indicated *FMR1*-deficient neurons manifest 10% fewer excitatory synapses and 50% more inhibitory synapses in the hippocampus CA1 region (Dahlhaus et al., 2009). Importantly, FMRP re-introduction in a range of FXS model systems, from mammalian dissociated *FMR1* null hippocampal cultures to the *Drosophila dfmr1* mutant NMJ and circadian circuits (Pfeiffer and Huber, 2007; Gatto and Broadie, 2008, 2009a), has been shown to rescue synapse over elaboration by inducing synapse loss. These findings highlight the normal function of FMRP in restricting synapse number.

Numerous other translational regulators, many with direct or indirect interactions with FMRP, similarly regulate *E/I* balance in developing neuronal circuits, including Staufen and Pumilio

(**Figure 3**). The double-strand RNA-binding protein Staufen has been implicated in mRNA localization, translational control and decay in dendrites important for long-term memory formation (Tang et al., 2001; Dubnau et al., 2003; Dugre-Brisson et al., 2005; Kim et al., 2005). Staufen co-purifies with FMRP in ribonucleoprotein complexes (Villace et al., 2004), which are dendritically recruited in an activity-dependent mechanism (Kim and Kim, 2006). More significantly, in *Drosophila*, *dfmr1* and *staufen* (*stau*) interact to mediate long-term memory consolidation (Bolduc et al., 2008). Coincident reduction in double heterozygotes (*stau^{D3}/+*; *Fmr1^3/+*) showed defective 1-day memory after spaced training in an olfactory conditioning paradigm. With respect to *E/I* balance, examination in cultured hippocampal neurons demonstrated that RNAi-mediated *Staufen2* knockdown decreased the number of PSD-95-reactive synapses by ~70% (Goetze et al., 2006). This postsynaptic effect was associated with a less marked ~25% decrease in presynaptic synapsin puncta, indicating compromised excitatory connectivity. In addition to synapse loss, there was a significant reduction in the amplitude of recorded mEPSCs (Goetze et al., 2006). Similarly, downregulation of *Staufen1* in hippocampal slice cultures decreased mEPSC frequency and amplitude (Lebeau et al., 2008). Interestingly, *Staufen1* disruption caused structural changes resulting in elongated dendritic spines without altered density (Lebeau et al., 2008). Notably, as previously indicated, similar immature-type dendritic spines are a clinical hallmark in the cytological presentation of FXS (Rudelli et al., 1985; Hinton et al., 1991).

The mRNA-binding protein Pumilio has been shown to serve as a sequence-specific translational repressor and, like Staufen and FMRP, facilitates long-term memory formation (Dubnau et al., 2003;

Bolduc et al., 2008). In fact, murine microarray screens have identified *pumilio* as an FMRP target (Brown et al., 2001). In *Drosophila*, Pumilio positively regulates synapse number while negatively regulating the GluRIIA glutamate receptor subunit (Menon et al., 2004a, 2009), and Pumilio regulates neuronal excitability by repressing the voltage-gated sodium channel, paralytic (Mee et al., 2004). Efforts to elucidate the synaptic components regulated by *Drosophila* Pumilio revealed a host of key partners including Discs-Large (DLG; PSD-95 homolog), Cysteine String Protein (CSP) and GAD (Chen et al., 2008). Most recently, RNAi-mediated knockdown of mammalian *pumilio2* in cultured hippocampal neurons caused a reduction in mature dendritic spine number coupled with an elevation in elongated dendritic filopodia (Vessey et al., 2010). Despite this alteration in spine morphology, and contrary to results in the *Drosophila* studies, increased numbers of excitatory synapses presented on the dendritic shafts and were functionally associated with an increased mEJC frequency (Vessey et al., 2010). Despite some differences, it is clear that FMRP, Staufen, and Pumilio coordinately regulate synaptic mechanisms in both *Drosophila* and mouse systems. These three translational regulators possess the capacity to modulate relative expression of a range of synaptic constituents and provide an effective means of altering *E/I* ratios within neural circuits underlying memory formation and likely other behavioral outputs.

SCAFFOLDING PROTEINS

Beneath the synaptic membrane, numerous scaffold/adaptor proteins provide positional anchoring, coupling membrane proteins to underlying cytoskeletal elements and signaling machinery. The composition of this scaffold network, and its preferential retention of specific membrane proteins, directly promotes the differential formation of excitatory versus inhibitory synapses (**Figure 3**). A prime example of such a key scaffolding element is the membrane-associated guanylate kinase (MAGUK) family member PSD-95 found exclusively at glutamatergic excitatory synapses (Kim and Sheng, 2004). Despite this apparent specificity, however, PSD-95 seems to exert influence upon both excitatory and inhibitory synapse classes (Levinson and El-Husseini, 2005b; Han and Kim, 2008; Keith and El-Husseini, 2008). PSD-95 over-expression in primary hippocampal neuron cultures was shown to enhance excitatory synapse size and spontaneous firing frequency, while reducing the number of VGAT-reactive inhibitory contacts by 50% (Prange et al., 2004). In agreement, *PSD-95* siRNA induced a 30% decrease in excitatory synaptic contacts and 1.5-fold increase in inhibitory synapses (Prange et al., 2004). With such dual capacity, it is important to reiterate that PSD-95 has been linked to FXS. FMRP binds the 3′-UTR of *PSD-95* conferring enhanced message stability (Zalfa et al., 2007), which could perhaps contribute to elevated glutamatergic synapse formation. *PSD-95* is also rapidly translated upon mGluR stimulation with DHPG, and this transient response is lost in FMRP deficient neurons (Todd et al., 2003; Muddashetty et al., 2007).

Other important scaffolding components in excitatory synapses include Homer and SH3 and ankyrin repeat containing-protein (SHANK) (Boeckers, 2006; Okabe, 2007). The neuronal immediate early gene Homer, with its Ena/VASP homology 1 (EVH1) and coiled-coil domains, dimerizes and serves to link mGluR, inositol 1,4,5-triphosphate receptor (IP3R) and *N*-methyl-*D*-aspartate (NMDA) receptor complexes (Brakeman et al., 1997; Kato et al.,

1998; Xiao et al., 2000; Duncan et al., 2005). As a putative *E/I* modulator, Homer functions bidirectionally depending on its prevailing isoform. Homer1b has been shown to induce synaptic growth and maturation cooperatively with SHANK (Sala et al., 2001), whereas Homer1a, lacking the coiled-coil domain and potentially serving as an endogenous dominant negative incapable of dimerization, induces a 50% reduction in excitatory spine density (Sala et al., 2003). Homer1a over-expression also results in the redistribution of SHANK away from its synaptic clusters and a reduction in the size of PSD-95 and GKAP (a PSD-95/SHANK linker) clusters by 30% (Sala et al., 2003).

SHANK proteins are multi-modal adaptors that harbor ankyrin repeats, SH3, PDZ and proline rich motifs (Sheng and Kim, 2000). These scaffolds multimerize and couple to actin-binding proteins and mGluR through interaction with Homer (Naisbitt et al., 1999; Tu et al., 1999). Mutations in SHANK3 are linked to autism (Durand et al., 2007; Moessner et al., 2007). Importantly, SHANK1 is elevated in *FMR1* KO mice at 2 weeks of age in the neocortex and in the hippocampus by 2 months, and SHANK3 is elevated in the *FMR1*-deficient neocortex at 2 months (Schutt et al., 2009). *In vitro*, SHANK is required for spine maintenance in hippocampal neurons and transgenic over-expression induces *de novo* functional spine formation in aspiny cerebellar granule cell cultures (Roussignol et al., 2005). In addition, the *SHANK1* KO displays altered postsynaptic density composition with reduced GKAP and Homer1b/c levels, smaller dendritic spines and weaker basal synaptic transmission (Hung et al., 2008). Thus, in concert, Homer and SHANK appear to mediate both structural and functional synaptic alterations in establishing *E/I* balance.

Different scaffolds reside at inhibitory synapses. Gephyrin is a key tubulin-binding scaffold restricted to inhibitory synapses that establishes a hexagonal lattice serving to anchor both glycine and GABA$_A$ receptors (Kirsch et al., 1991; Schrader et al., 2004; Sola et al., 2004; Fritschy et al., 2008; Tretter et al., 2008); although, some controversy remains as to whether gephyrin is obligatory in mediating GABA$_A$R clustering (Kneussel et al., 1999; Levi et al., 2004). Knockdown experiments with shRNAs and titration of endogenous gephyrin with an aggregating gephyrin-EGFP fusion in cultured hippocampal neurons yield depression of GABA$_A$R clusters, without affecting the number of glutamatergic synapses (Yu et al., 2007; Yu and De Blas, 2008). This impairment was associated with the loss of ~50% of GAD-positive GABAergic innervating boutons and corollary reduction in the sIPSC amplitude and frequency (Yu et al., 2007). Importantly, there was no disruption of whole-cell GABA currents, indicating the specificity of the effect is restricted to the synapse. These studies indicate a highly specific role of gephyrin in inhibitory synapses. However, perhaps due to opportunistic compensation, gephyrin reduction has been shown to increase the size of PSD-95 clusters and their apposing glutamatergic VGLUT1-reactive presynaptic termini (Yu and De Blas, 2008). This interaction again reveals the likely dynamic interplay between inhibitory and excitatory synapses in establishing the *E/I* ratio.

CELL ADHESION MOLECULES

Cell adhesion molecules mediating synaptic partner recognition and persistent trans-synaptic signaling are ideally situated to modulate excitatory and inhibitory synaptic formation and stabilization.

In particular, presynaptic neurexin and postsynaptic neuroligin form a heterophilic adhesion complex possessing the capacity to drive *E/I* balance (**Figure 3**). These proteins present isoforms differentially expressed between excitatory and inhibitory synapse classes and have therefore been proposed to specify synapse identities (Levinson and El-Husseini, 2005a,b; Craig and Kang, 2007). The influence they exert may be substantial, although their roles remain somewhat elusive *in vivo*.

In mammals, the 3-gene neurexin family provides considerable diversity based on the formation of α and β isoforms, splice variants and post-translational glycosylation (Rowen et al., 2002; Tabuchi and Sudhof, 2002; Comoletti et al., 2003). *Neurexin1* (α and β) disruptions are associated with autism (Feng et al., 2006; Kim et al., 2008; Yan et al., 2008), and deletion and copy number variants are also linked to schizophrenia (Kirov et al., 2008; Vrijenhoek et al., 2008; Rujescu et al., 2009). Importantly, a recent proteomic analysis revealed decreased neurexin1α in the *FMR1* KO mouse (Liao et al., 2008). To modulate *E/I* balance, α isoforms typically associate with inhibitory synapses, while the more promiscuous β isoforms are present at both excitatory and inhibitory synapses (Craig and Kang, 2007). In fibroblast-neuronal co-culture studies, α-neurexins were able to cluster gephyrin, but not PSD-95, whereas β-neurexins could cluster gephyrin or PSD-95 (Graf et al., 2004; Kang et al., 2008). However, in β-neurexins, a particular splice variant (S4) shifts its capacity, such that its inclusion (+S4) favors GABAergic synapse development and its omission (−S4) favors glutamatergic synapses. Significantly, the developmental acquisition of an elevated −S4:+S4 utilization in β-neurexins was observed both *in vivo* (embryonic day 18 vs. postnatal day 18) and *in vitro* (embryonic day 18 cultures maintained for 7–22 days *in vitro*) (Kang et al., 2008). Despite these compelling findings, the *in vivo* significance of α-neurexins has been questioned as double- and triple-KO animals, viability issues aside, display only marginal alterations in synapse presentation (Missler et al., 2003; Dudanova et al., 2007). However, in *Drosophila* the sole neurexin gene, *dnrx*, does influence synapse formation in the central nervous system and at the glutamatergic NMJ (Li et al., 2007; Zeng et al., 2007). In the brain, *dnrx* disruption depressed levels of the active zone component Bruchpilot (Brp) and resulted in 50% fewer synapses identifiable by electron microscopy (Zeng et al., 2007). At the NMJ, *dnrx* nulls display shortened axonal branches and 40–60% fewer synaptic boutons with reduced Brp (Li et al., 2007; Sun et al., 2009; Zweier et al., 2009). Moreover, the pre- and post-synaptic engagement appeared compromised in *dnrx* mutants with evident membrane detachments (Li et al., 2007), and neurotransmission is defective as evidenced by decreased evoked excitatory junctional potential (EJP) amplitude and increased miniature EJP amplitude and frequency (Li et al., 2007; Sun et al., 2009). Thus, the *Drosophila* studies provide *in vivo* evidence for a neurexin role, at least in excitatory synapse development.

Mammalian neuroligins (NLGN) are a 4-member family, with mutation in NLGN3 (single amino acid substitution – R451C) and NLGN4 (frameshift resulting in premature termination) presenting in autism (Jamain et al., 2003; Laumonnier et al., 2004). Importantly, *NLGN1/2* mRNAs interact with FMRP, and protein analysis revealed NLGN1 levels depressed in the hippocampus and cerebellum of *FMR1* KO mice (Dahlhaus and El-Husseini, 2009). NLGN1 primarily localizes to excitatory synapses (Song et al., 1999), whereas NLGN2

resides at inhibitory synapses (Varoqueaux et al., 2004). However, in terms of influencing *E/I* ratio, neuronal transfection of hemagglutinin-tagged NLGN1 (HA-NLGN1) caused a 3-fold increase in VGAT-positive and a 1.5-fold increase in VGLUT-positive presynaptic contacts (Prange et al., 2004); this trend was also observed with NLGN2 and NLGN3 over-expression (Chih et al., 2005; Levinson et al., 2005). These results suggest that neuroligins possess more generalized synaptogenic properties, although notably, NLGN1 over-expression increased the density of PSD-95 and Homer containing puncta, and NLGN2 was more effective at inhibitory synaptic induction than either NLGN1 or NLGN3 (Chih et al., 2005). The influences that neuroligins exert on *E/I* balance are also complicated by their interactions with PSD-95. PSD-95 over-expression altered NLGN2 distribution, delivering it to excitatory synapses (Levinson et al., 2005). Functionally, NLGN1 over-expression enhanced excitatory synaptic transmission but not inhibitory, and the converse occurred with NLGN2 (Chubykin et al., 2007). *In vivo* NLGN1 over-expression increased the number of asymmetric synapses assayed by electron microscopy without influencing symmetric synapses, thereby apparently increasing the *E/I* ratio, despite elevations in gephyrin/VGAT as well as PSD-95/VGLUT (Dahlhaus et al., 2009). Conversely, *in vivo* NLGN2 over-expression decreased the *E/I* ratio by increasing symmetric synapse number (Hines et al., 2008). RNAi directed against *NLGN1*, *2* or *3*, expressed singly or in combination, reduced excitatory synapse formation, with the triple knockdown yielding a 70% reduction (Chih et al., 2005). However, confoundingly, no corresponding alteration in mEPSCs was detected. Even more surprising, although excitatory and inhibitory transmission largely fail in the brainstem of the *NLGN1-3* triple-KO mouse yielding perinatal respiratory arrest, the density of synaptic contacts was largely unaltered (Varoqueaux et al., 2006). These complications aside, it is of particular interest that transgenic over-expression of HA-NLGN1 in the *FMR1*-deficient background alters the *E/I* ratio. This transgenic intervention overcorrects the modestly depressed excitatory synapse prevalence in *FMR1* nulls, shifting it from 10% less to 50% more than is seen in wild type. Conversely, introduction of HA-NLGN1 in the *FMR1* KO mediates the reciprocal effect on inhibitory synapses, taking them from 50% more to 10% less as compared to control (Dahlhaus et al., 2009). Behaviorally, this transgenic intervention improves socialization and reduces hyperactivity in the mouse FXS model, but provides no remediation of memory deficits.

CHANNELS/RECEPTORS

By directly mediating membrane ion flux, synaptic channels and receptors are clearly positioned to shift the functional *E/I* balance (**Figure 3**). For example, GABA$_A$R is a heteropentameric ion channel permeant to Cl$^-$/HCO$_3^-$ ions, which is most prevalently comprised of 2α, 2β, and either δ or γ subunits (Tretter and Moss, 2008; D'Hulst et al., 2009a). GABA$_A$Rs have been linked to autism via a two-locus gene-gene effect involving α4 through interaction with β1 (Ma et al., 2005). Moreover, discrete abnormalities on chromosome 15 region q11–13, containing three GABA$_A$R subunits (α5, β3, and γ3), are also linked to autism (Cook et al., 1998; Shao et al., 2003). In these disease conditions, significant GABA$_A$R subunit reductions were detected in parietal cortex, cerebellum and superior frontal cortex (Fatemi et al., 2009, 2010). Moreover, the maternal

GABA$_A$R β3 signal peptide variant P11S, associated with 15q11–q13 duplication, has been implicated in both childhood absence epilepsy and autism owing to reduced functionality of receptors containing the β3 subunit (Delahanty et al., 2009). Importantly, GABA$_A$R abundance was also reportedly decreased in FXS, at both the mRNA and protein level (El Idrissi et al., 2005; D'Hulst et al., 2006, 2009b; Gantois et al., 2006), and α5 or δ GABA$_A$R KO mice display induced epileptiform hyperexcitability due to reduced tonic inhibition (Spigelman et al., 2002; Glykys and Mody, 2006).

Several lines of evidence implicate a role for the GABA$_A$R itself in directly influencing synaptogenesis. Studies of the *GABA$_A$R* γ2 subunit KO mouse indicated that the subunit was necessary for the clustering and retention of GABA$_A$Rs (Essrich et al., 1998; Schweizer et al., 2003). γ2 shRNA *in vitro* and *in vivo* demonstrate the subunit was also required to establish normal postsynaptic gephyrin accumulation, and its disruption lead to the depression of incoming presynaptic GABAergic innervation (Li et al., 2005). In addition, these receptors typically show significant and constitutive turnover; as such, impairing receptor subunit binding to the clathrin adaptor protein-2 elevated GABA$_A$R retention at the membrane. By over-expressing an altered β3 subunit (β3$^{S408/9A}$) in cultured hippocampal neurons, this mechanism increased the number of inhibitory synapses formed to 144% of control values and enhanced inhibitory synaptic transmission by elevating mIPSC amplitudes (Jacob et al., 2009b). Moreover, the elevated GABA$_A$R presentation impaired dendritic spine maturity and shifted the immature filopodia versus mature mushroom-shaped spine ratio from 1:1 in controls to 2:1 in β3$^{S408/9A}$-expressing neurons and decreased the density of PSD-95 clusters by 40% (Jacob et al., 2009b). These recent findings again highlight the interdependence of excitatory and inhibitory synaptogenesis and bolster the conclusion that synapse class-specific alterations are not mutually exclusive in their synapse class effects.

CONCLUDING REMARKS

Proper brain function requires a delicate balance of excitatory and inhibitory synaptogenesis during neural circuit establishment and employment. Examination of ASD, epilepsy, RTT, and FXS demonstrate a high incidence of non-compensated disruption of *E/I* synapse balance. This suggests that disease-related homeostatic

insufficiency and/or failure of compensatory mechanisms impair the development, connectivity and transmissive properties of neural circuits. *E/I* ratio imbalance may often be attributed to mutations in genes that facilitate synaptogenesis and/or synaptic maintenance through transcriptional control, translational control, structural scaffolding, cell adhesion and signaling. Defining the cross-talk between these levels will be vital to further understanding their contribution to the processes that differentially modulate each class of synapse. Crucial to the advancement of this work is identification of genetic targets regulated at the transcriptional level by factors such as MECP2, MEF2 and Npas4. Parallel examination of elevated and/or depressed targets should allow identification of key *E/I* switches. Likewise, the continuing definition of mRNA-binding proteins such as FMRP, Staufen and Pumilio, and their interactors, will be vital in determining how differential protein translation preferentially subserves different synaptic classes. Finally, much progress has been made in elucidating the functional *E/I* shift toward hyperexcitation in FXS, with robust examination of both the Gq and GABA$_A$R theories. While each of these views of FXS provides insight into the *E/I* imbalance that contributes to the disease state, a number of key questions remain. Are hyperexcitation and/or hypoinhibition apparent in all circuits, or are these features circuit specific? How do the alterations in excitation and inhibition intersect; are they fully independent, or is there evidence of interacting compensation? Do transmissive changes act in concert, or is there mutual exclusivity? Can developmentally specific timing be assigned to these circuit alterations? Are the *E/I* shifts maintained? Perhaps most importantly from a clinical perspective, could combinatorial therapies targeting both glutamatergic and GABAergic systems in FXS prove most beneficial? Answers to these questions will help in understanding the mechanistic bases of disorders that affect brain development and should provide direct avenues to the most effective disease interventions.

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