Synapse Assembly and Neurodevelopmental Disorders

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In this review we examine the current understanding of how genetic deficits associated with neurodevelopmental disorders may impact synapse assembly. We then go on to discuss how the critical periods for these genetic deficits will shape the nature of future clinical interventions.

Neuropsychopharmacology Reviews (2015) **40**, 4–15; doi:10.1038/npp.2014.163; published online 30 July 2014

INTRODUCTION

During development, the human central nervous system (CNS) carries out an extraordinary undertaking: it forms on the order of 10^{15} connections between $\sim 10^{12}$ neurons. Considering this huge bioengineering challenge, it may be surprising that for the majority of humans the end result is immensely successful at performing a wide range of tasks. These tasks range from monitoring of sensory inputs to motor control output, and to higher cognitive tasks such as memory storage and retrieval, and social interaction. Unfortunately, though, things do go wrong. This is underlined by the appearance of symptoms of neurodevelopmental disorders (NDDs) such as autism spectrum disorders (ASD) and intellectual disability that coincides with the period of massive synaptogenesis in the developing cortex during the first 2 years of an infant's development (Huttenlocher and Dabholkar, 1997).

Here, we will use NDDs to collectively designate all disorders that impair the development of the CNS. These commonly include autism and ASDs, attention deficit-hyperactivity disorder (ADHD), intellectual disability (ID), Down's syndrome, and Tourette's syndrome (TS). In addition, a large body of evidence suggests that schizophrenia (SCZ) and some forms of bipolar disorder may have developmental roots. Some studies indicate a correlation between individuals destined to manifest SCZ as adults and delays in motor and cognitive milestones during childhood, reaching back to as early as 6 months of life (reviewed by Weinberger and Levitt, 2011). It is possible that some forms of bipolar disorder manifest themselves in children as pediatric mania; however, this is difficult to diagnose and

is often misdiagnosed with ADHD and conduct disorder (Biederman *et al*, 2000). Thus, we consider any possible CNS disorder that may have roots during childhood as a NDD.

The temporal correlation between diagnosis of a subset of NDDs and the period of synapse assembly led to the hypothesis that the deficit underlying many cases of these disorders may reside at the synapse (Bourgeron, 2009; Zoghbi, 2003). Since formulation of the idea of a synaptic pathology (or 'synaptopathy') lying at the heart and being a common denominator for NDDs (Brose *et al*, 2010), many of the genes that have been implicated in these disorders by genetic studies have been examined either in the test tube, in tissue cultures, or in genetically modified organisms (primarily mice) with a possible synaptic etiology in mind. Astonishingly, a large proportion of the genes that have been identified in cases of NDDs have identified roles at the synapse either during development or in the adult (Aldinger *et al*, 2011; Peca *et al*, 2011b). Although not all of the studies argue for strictly developmental deficits, a few recent observations argue for shortfalls during synapse assembly and maturation in at least some forms of ID, ASD, and SCZ. Here we will examine the current understanding of the assembly of synapses and how mutations in some genes identified in NDDs affect this process.

THREE STEPS OF SYNAPSE ASSEMBLY

Synapse formation, as we currently understand it at the molecular and cellular level, can be dissected into three major steps: (1) contact formation, (2) transport of synaptic components within those neurites, and (3) immobilization of the synaptic components at the nascent synaptic contact sites (McAllister, 2007) (Figure 1). Genes implicated in NDDs have been found to be involved with all three aspects of synaptogenesis. In general, deficits in NDD susceptibility genes are uncommon, up to 2% of NDD cases for the more common variants (Toro *et al*, 2010), and collectively only

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Received 5 March 2014; revised 23 June 2014; accepted 26 June 2014; accepted article preview online 3 July 2014

Figure 1. The three basic steps of synapse assembly. (1) First neuronal contact triggers a cascade of events through cell adhesion molecules such as Nlgns, Nrns, and SynCAMs. Pre- and postsynaptic precursors need to be efficiently transported in axons and dendrites (2), by associating with motor proteins, such as Kinesin17, that 'walk' along microtubules. Neuronal contact must result in stopping, perhaps by means of kinases such as Cdk5, and maintenance of the synaptic material at the nascent synaptic site because of scaffolding molecules, such as SHANKs and CASK (3). This process eventually leads to the formation of a functional synaptic contact (4).

account for between 10 and 20% of NDDs. However, identifying mutations that genuinely cause dysfunction of a gene, and thus implicating it in the etiology of NDDs, tells us much about the underlying biology of CNS and synapse development. Below, we present examples of genes with mutations and copy number variations (CNVs) that are both highly likely to result in protein function deficits or protein expression level differences, and for which studies clearly indicate an involvement in the three steps of synapse assembly (Table 1). This should not be understood as a comprehensive list, but serves as a demonstration of how genetic studies of the NDD population and basic research, primarily in mouse models, have helped further our understanding of synapse assembly and some aspects of NDDs. We then examine genes identified in syndromic forms of ASDs, and how the encoded proteins might function during synaptic development.

Neuronal Contact

Genes involved in neuronal cell adhesion and synaptic scaffolding are collectively the most prevalent genes found to date in genome-wide association studies (GWASs) of

NDDs (Corvin, 2010; Kumar *et al*, 2011; O'Dushlaine *et al*, 2011). This suggests that cell adhesion events are critical for the correct assembly of synapses (Figure 1, top left). There are hundreds of genes encoding potential cell adhesion molecules (CAMs) expressed in the CNS; however, to date only a handful of them have been found to be able to induce synapse assembly (Bukalo and Dityatev, 2012; Washbourne *et al*, 2004). This has largely been defined by the expression of candidate CAMs in nonneuronal cells and their ability to induce either pre- or postsynaptic assembly in cocultured neurons (Biederer *et al*, 2002; Biederer and Scheiffele, 2007; Fu *et al*, 2003; Scheiffele *et al*, 2000; Siddiqui *et al*, 2010).

Neuroligins. The first family of CAMs that were shown to be sufficient to drive synapse assembly *in vitro* was the neuroligins (Scheiffele *et al*, 2000). These had been identified as ligands of the presynaptically localized α -latrotoxin receptors, the neuroligins (Song *et al*, 1999). Indeed, subsequent studies demonstrated that binding of neuroligins (Nlgns) to the neuroligins (Nrns) resulted in assembly of presynaptic terminals in axons (Dean *et al*, 2003), and that binding of Nrns to Nlgns drove postsynaptic differentiation in dendrites (Barrow *et al*, 2009; Nam and Chen, 2005). The *in vitro*

TABLE 1 Summary of Genes Associated with NDDs That May Be Involved in Steps of Synapse Assembly

Step in synapse assembly	Gene	Chromosome	Protein function	Alleles	NDD diagnosed	Prevalence	Reference
Neuronal contact	<i>NLGN3</i>	Xq13.1	Synaptic CAM	Mutations	ASD	0.26% (1774) [2]	Jamain <i>et al</i> (2003); Glessner <i>et al</i> (2009)
	<i>NLGN4X</i>	Xp22	Synaptic CAM	Mutations	ASD, ID, TS	2.65% (498) [2]	Jamain <i>et al</i> (2003)
	<i>NLGN1</i>	3q26.31	Synaptic CAM	CNV	ASD	*5.01% (2195) [1]	Glessner <i>et al</i> (2009)
	<i>NRXN1</i>	2p16.3	Synaptic CAM	CNV, mutations, microdel.	ASD, SCZ	0.42% (2380) [3]	Awadalla <i>et al</i> (2010); Reichelt <i>et al</i> (2012)
	<i>CADMI</i>	11q23.2	Synaptic CAM	Mutations	ASD	1.00% (194) [1]	Zhiling <i>et al</i> (2008)
Transport	<i>MAPT</i>	17q21.31	Microtubule associated	CNV	ASD, SCZ, ID	0.14% (14270) [2]	Rovelet-Lecrux <i>et al</i> (2012)
	<i>KIF17</i>	1p36.12	Motor protein	Nonsense mutation	SCZ	0.29% (710) [2]	Awadalla <i>et al</i> (2010); Tarabeux <i>et al</i> (2010)
	<i>GSK3B</i>	3q13.33	Kinase	SSM	SCZ	NA (459) [1]	Blasi <i>et al</i> (2013)
Recruitment and maintenance	<i>SHANK1</i>	19q13.3	Scaffolding protein	Microdeletions	ASD	0.31% (1614) [2]	Sato <i>et al</i> (2012)
	<i>SHANK2</i>	11q13.2	Scaffolding protein	Deletions, SSM	ASD, ID	3.41% (851) [2]	Berkel <i>et al</i> (2010); Leblond <i>et al</i> (2012)
	<i>SHANK3</i>	22q13.3	Scaffolding protein	Mutations	ASD, SCZ, mild ID	0.74% (1466) [2]	Awadalla <i>et al</i> (2010)
	<i>CDK5R1</i>	17q11.2	Kinase regulator	Mutations	ID	1.00% (100) [1]	Venturin <i>et al</i> (2006)
	<i>CASK</i>	Xp11.4	Scaffolding protein	Mutations	XLMR, MIC-PCH	***4.43% (557) [3]	Hackett <i>et al</i> (2009); Najm <i>et al</i> (2008)

Abbreviations: ASD, autism spectrum disorder; CAM, cell adhesion molecule; CNV, copy number variation; ID, intellectual disability; MIC-PCH, microcephaly and disproportionate pontine cerebellar hypoplasia; NA, not available; SSM, splice site mutation; TS, Tourette's syndrome; XLMR, X-linked mental retardation.

Prevalence is quantified as the average percentage of the sample's affected population with the identified alleles. The total sample size and the number of studies are in round and square parentheses, respectively. *NLGN1 CNVs were detected in control populations, resulting in an odds ratio of only 1.47; **CASK mutations were determined using a very specific disease population, XLMR and MIC-PCH, and thus does not represent the prevalence in the NDD population.

studies suggest that *Nlgn1* is key in triggering the recruitment of NMDA-type glutamate receptors (Barrow *et al*, 2009; Hoy *et al*, 2013), but this may be a late maturational step of synapse formation.

The neuroligins are encoded by five genes in humans (1, 2, 3, 4X, and 4Y), and mutations in *NLGN1*, 3, and 4X genes have been associated with autism (Sudhof, 2008) (Table 1). Whereas loss-of-function mutations have been found in patients with ASDs for *NLGN3* and *NLGN4X* (Jamain *et al*, 2003; Laumonnier *et al*, 2004), an increase in copy number of the *NLGN1* gene has been associated with ASD (Glessner *et al*, 2009; Szatmari *et al*, 2007) (Table 1). Analyses in mice have confirmed that loss-of-function mutations in *Nlgn3* and *Nlgn4* result in abnormal synaptic function and behavioral deficits (Ellegood *et al*, 2011; Jamain *et al*, 2008; Radyushkin *et al*, 2009; Tabuchi *et al*, 2007). It is important to note that studies performed with the same *Nlgn3* and *Nlgn4* mutations as characterized previously (Jamain *et al*, 2008; Tabuchi *et al*, 2007), but carried out in different labs, showed no significant effects on social behaviors or ultrasonic vocalizations (Chadman *et al*, 2008; Ey *et al*, 2012). These results open the possibility that genetic background and environmental effects may contribute to the subtle behavioral phenotypes. Since then, social deficits have been confirmed for the *Nlgn3* knock-in mice, expressing a mutant form of *Nlgn3* (R451C) identified in patients with autism, using multiple background strains of mice (Jaramillo *et al*, 2014). This result adds weight to the

argument that mutations in these genes may be the cause of some cases of ASD in humans. Similarly, mice in which *Nlgn1* was overexpressed, to mimic the copy number increase, also displayed anomalous synaptic characteristics (Dahlhaus *et al*, 2010; Hoy *et al*, 2013). Although overexpression of *Nlgn1* resulted in deficits in learning and memory behavior, social behaviors tested were not affected (Dahlhaus *et al*, 2010; Hoy *et al*, 2013). In conclusion, the *Nlgn* family of genes has been clearly implicated in ASD etiology through genetic association and mouse studies.

Neurexins. The *Nrxns* are encoded by three genes in mammals; and all of them generate two forms of the protein from different start codons: the long α -*Nrxns* and the short β -*Nrxns* (Tabuchi and Sudhof, 2002). Approximately 0.5% of ASD cases harbor mutations in *Nrxn1* (*NRXN1*), underlining the importance of this gene in the etiology of NDDs (Reichelt *et al*, 2012). Analyses with loss-of-function mutations of the *Nrxn* genes have confirmed the role of α -*Nrxns* in shaping synapse function (Missler *et al*, 2003; Sons *et al*, 2006) and complex behaviors (Etherton *et al*, 2009; Laarakker *et al*, 2012), including social behaviors (Grayton *et al*, 2013). Deletion of all three α -*Nrxn* genes results in death of newly born mouse pups because of reduced synaptic transmission (Missler *et al*, 2003).

Further study of the extracellular domains of *Nrxns* demonstrated that they are relatively promiscuous, participating in molecular interactions with a suite of other

synaptic proteins in addition to the Nlgns. These include LRRTMs, cerebellin, and the GABA A receptor. LRRTMs appear to act in a manner similar to Nlgns in postsynaptic assembly (de Wit *et al*, 2009; Ko *et al*, 2009; Linhoff *et al*, 2009; Wright and Washbourne, 2011). One important difference is that Nlgns primarily affect NMDA-type glutamate receptor recruitment and maintenance at synapses (Barrow *et al*, 2009; Hoy *et al*, 2013), whereas LRRTMs influence AMPA-type glutamate receptor presence at synapses (Soler-Llavina *et al*, 2011). Binding of Nrnxns to cerebellin (Uemura *et al*, 2010), which in turn binds to glutamate receptor subunit $\delta 2$ (Matsuda *et al*, 2010), may be a specialization in the cerebellum to control synapse number between parallel fibers and Purkinje cells (Hirai *et al*, 2005). It is unclear how the *cis* interaction between Nrnxns and the GABA A receptor contributes to synapse formation (Zhang *et al*, 2010). Presumably this interaction occurs in dendrites at an early stage during development, before Nrnxns redistribute to axons (Barker *et al*, 2008). Thus, these studies suggest that Nrnxns are key organizers of the pre- and postsynaptic specializations.

SynCAMs. Another family of molecules, identified by the ability to induce the formation of presynaptic terminals in coculture experiments, is the SynCAMs (Biederer *et al*, 2002). These proteins resemble nectins, in that they contain three extracellular immunoglobulin domains for homo- and heterophilic interaction. There are four SynCAM genes in humans (*CADMI–4*) (Biederer, 2006), one of which (*CADMI*) has been linked to ASD by virtue of two missense mutations that render SynCAM1 unable to be transported out of the endoplasmic reticulum to the plasma membrane (Zhiling *et al*, 2008). Knockout mice for SynCAM1 present a subtle reduction in synapse number (<10%), perhaps because of compensation by the other family members (Robbins *et al*, 2010). Examination of synapses by electron microscopy suggests an impact of SynCAM1 ablation on the size of both the presynaptic active zone and the postsynaptic density (Robbins *et al*, 2010). SynCAM1 suppresses long-term depression (LTD), as knockout mice show enhanced LTD, with no effect on long-term potentiation (Robbins *et al*, 2010). As a result, SynCAM1 KO mice exhibit enhanced spatial reference memory. The *in vitro* studies of SynCAM1 have suggested that the majority of this CAM function resides in its ability to modulate cytoskeletal dynamics during assembly of the presynaptic terminal (Stagi *et al*, 2010) and during formation of the postsynaptic spine (Cheadle and Biederer, 2012). Thus, SynCAM1 is clearly implicated in the development of fully functional synapses.

Mechanisms of CAM-triggered synapse assembly. To date, there appears to be a discrepancy between the sufficiency for CAMs to induce the formation of synaptic terminals in coculture systems and their necessity in the formation of synapses, especially *in vivo*. Expression of SynCAMs and Nlgns in nonneuronal cells and culturing of these cells with neurons suggests a potent ability of presynaptic SynCAMs

and Nrnxns in coordinating the recruitment of presynaptic components (Biederer *et al*, 2002; Scheiffele *et al*, 2000). But *in vitro* and *in vivo* studies of loss-of-function mutations of these genes have not shed light on how these molecules might perform this function (Robbins *et al*, 2010; Varoqueaux *et al*, 2006). This discrepancy may be because of compensation by other genes within the family, or by other CAM families. Alternatively, the effects of the loss of synaptic CAM function may only be discerned when there is competition between individual neurons, such that complete knockout does not show a difference, whereas if some neurons have reduced CAM expression they will exhibit reduced synapse numbers (Kwon *et al*, 2012).

Despite the lack of a strong effect on synapse formation by loss-of-function mutations, it was hypothesized that protein–protein interactions of the short intracellular tails of both Nrnxns and SynCAMs might be the trigger for the accumulation of presynaptic components. Specifically, it was assumed that the presynaptic MAGUK protein CASK, which can bind to the terminal PDZ domain of both Nrnxns and SynCAMs, might be the presynaptic organizer. This view was recently challenged by experiments in which the PDZ domain was switched for one that cannot bind to CASK, yet still resulted in functional presynaptic assembly (Gokce and Sudhof, 2013). Indeed, expression of a mutant form of Nrnxn in which the entire intracellular tail was eliminated was still sufficient for presynaptic assembly to occur, including CASK recruitment (Gokce and Sudhof, 2013). These experiments suggest that Nrnxns, and based on the similarity of the intracellular regions of perhaps also SynCAMs, function through an as yet unidentified transmembrane receptor to induce presynaptic assembly.

In contrast, the intracellular regions of Nlgns appear to play a central role in coordinating postsynaptic assembly and maturation. Protein–protein interactions of the intracellular tails of Nlgn1 help traffic NMDA receptors to synapses (Barrow *et al*, 2009) and mediate the switch in NMDA receptor type (from NR2B to NR2A containing) that occurs at synapses during development (Hoy *et al*, 2013). In addition, novel interaction domains within the C-terminus of Nlgns have been identified that are critical for both NMDA and AMPA receptor recruitment to synapses (Etherton *et al*, 2011; Shipman *et al*, 2012). Thus, whereas recruitment and maintenance of presynaptic components is not mediated by intracellular interactions of Nrnxns and SynCAMs, it appears that protein–protein interactions within the C-terminal tail of Nlgns has a profound effect on postsynaptic assembly.

Axonal and Dendritic Transport of Synaptic Components

The vast majority of synaptic components are synthesized within the cell body. Thus, for the assembly of synapses to occur, synaptic precursor material must be transported within dendrites and axons to nascent synaptic sites (see Figure 1, top right). Transport of synaptic precursors to

synapses involves motor proteins, dynamins, and kinesins that walk along microtubule tracks in both dendrites and axons (Goldstein *et al*, 2008; Hirokawa *et al*, 2010). Synaptic vesicle precursors and active zone precursors are transported in a microtubule-dependent manner in both antero- and retrograde directions (Ahmari *et al*, 2000; Bury and Sabo, 2011; Sabo and McAllister, 2003). Similarly, NMDA and AMPA receptors are transported along microtubules in both directions in dendrites (Washbourne *et al*, 2002). Although it is possible that some transmembrane synaptic components diffuse within the plasma membrane to synaptic sites (Groc *et al*, 2009), it is unlikely that this mechanism would deliver enough receptors, especially during peak synaptogenesis. Supporting a role for microtubule-based transport in synapse assembly, genes that encode axonal or dendritic motor proteins, microtubule-organizing proteins or regulators of microtubule transport have been uncovered as candidate genes in NDDs.

MAPT. As microtubules are the rails along which long-distance axonal and dendritic transport is achieved, one would expect mutations that affect microtubule stability or organization to result in NDDs. Indeed, rearrangements of human chromosome 17 that affect the microtubule-associated protein Tau (*MAPT*) have been identified in patients with learning disability and developmental delay (Koolen *et al*, 2006; Rovelet-Lecrux *et al*, 2012; Shaw-Smith *et al*, 2006). Although *MAPT* has been more commonly associated with neurodegenerative disorders, because of its presence in neurofibrillary tangles (Goedert *et al*, 2006), this gene plays an active role during development. *MAPT* promotes microtubule assembly and stability and may act as a linker protein between the microtubules and the plasma membrane. Thus, a reduction in *MAPT* can affect neuronal migration and deficits in dendritic arbor elaboration and synapse number (Sapir *et al*, 2012).

Kinesin17. A nonsense mutation in Kinesin17 gene (*KIF17*) was found in a case of schizophrenia (Awadalla *et al*, 2010; Tarabeux *et al*, 2010). The mutation resulted in truncation of the protein before the tail domain, the region important for interaction with the transport cargo (Goldstein *et al*, 2008). Similar truncations result in dominant negative motor proteins (Setou *et al*, 2002), explaining how such a mutation could result in deficits even when present in the heterozygous state (Tarabeux *et al*, 2010). Kinesin17 is an interesting candidate with regard to NDDs, as it was originally identified as a motor protein for NMDA receptor transport (Kayadjanian *et al*, 2007; Setou *et al*, 2000). Thus, overexpression of Kinesin17 results in enhanced spatial learning and memory in transgenic rodents (Wong *et al*, 2002). The ability to release the NMDA receptor-containing cargo is critical, as mutations that block phosphorylation of serines in the tail domain result in reduced synaptic NMDA receptor localization and deficient spatial learning and memory (Yin *et al*, 2012).

Gsk-3 β . Mutations affecting splicing and expression levels of the *glycogen synthase kinase 3 β* gene (*GSK3B*) are associated with schizophrenia (Blasi *et al*, 2013). Many of the roles of this signaling enzyme in neural function remain unclear. However, with regard to synapse formation, Gsk-3 β has been implicated in the transport of amyloid precursor protein (APP) in axons, and in the wnt signaling pathway. Although the role of APP outside of the pathway to Alzheimer's disease remains elusive, interactome studies suggest that APP can bind synaptotagmin-1, the calcium sensor for synaptic vesicle release (Kohli *et al*, 2012) and the axonal motor protein Kinesin-1 (Kamal *et al*, 2001). However, there has been some controversy as to whether APP is important for the transport of these components in axons (Lazarov *et al*, 2005). Gsk-3 β phosphorylates APP, reducing its axonal transport (Takashima *et al*, 1995), suggesting that Gsk-3 β may play a generalized role in regulating transport of synaptic components to synapses. In another potential synaptic function, Gsk-3 β regulates wnt signaling at vertebrate synapses and the invertebrate neuromuscular junction to modulate presynaptic microtubules during synapse formation (Salinas, 2005). Gsk-3 β plays a role in this pathway by phosphorylating microtubule-associated protein 1B (*MAP1B*), resulting in more dynamic microtubules (Goold *et al*, 1999). In addition, Gsk-3 β plays a role in the mobilization of postsynaptic density protein-95 (*PSD-95*) (Nelson *et al*, 2013), suggesting a wide variety of roles for this kinase in shaping the synapse.

Recruitment and Maintenance of Synaptic Components at Nascent Synaptic Sites

Once synaptic precursors reach the nascent synaptic site, they need to stop, reorganize, and be maintained at the developing synapse (see Figure 1, bottom left). Kinases may regulate synaptic precursor stopping at the synapses, whereas scaffolding molecules will lock synaptic components into a network of interactions to hold them at the mature synapse (Figure 1, bottom right).

SHANKs. Mutations and microdeletions in the *SHANK* genes have been found among individuals with ASDs (Awadalla *et al*, 2010; Berkel *et al*, 2010; Guilmatre *et al*, 2014; Leblond *et al*, 2012; Lennertz *et al*, 2012; Sato *et al*, 2012). These genes encode scaffolding molecules, molecules whose sole job is to bind to other proteins inside the cell exclusively at synapses (Grabrucker *et al*, 2011). Studies of knockout mice for SHANKs 1, 2, and 3 suggest that reduced levels of these SHANK proteins result in synaptic deficits (Bozdagi *et al*, 2010; Sala *et al*, 2001; Wang *et al*, 2011) with consequences for cognitive and social behavior (Bozdagi *et al*, 2010; Peca *et al*, 2011a; Silverman *et al*, 2011; Wang *et al*, 2011; Wöhr *et al*, 2011; Won *et al*, 2012). SHANKs are interacting partners of Nlgns and form a link between the transsynaptic CAMs and important synaptic signaling molecules inside the postsynaptic terminal including NMDA receptors (Petralia *et al*, 2005). Indeed, SHANK2

deficits can be reverted in mice by restoring NMDA receptor function (Won *et al*, 2012). The wealth of mutational information from the ASD population and the strong indications from mouse experiments point toward a significant contribution of SHANK gene deficits underlying ASD etiology.

CDK5. Although scaffolding molecules such as SHANKs can explain the maintenance of a variety of components at synapses, it is less clear how these components might initially be recruited to a nascent synaptic site. An inkling into mechanisms of recruitment can be gained by examining cyclin-dependent kinase 5 (CDK5). The gene for the regulatory subunit of CDK5 (*CDK5R1*) has been linked to nonsyndromic intellectual disability (Venturin *et al*, 2006) and dysregulation of CDK5 expression has been found in the brains of patients with schizophrenia (Engmann *et al*, 2011; Ramos-Miguel *et al*, 2012). CDK5 has been ascribed many neuronal functions ranging from neuronal migration to the regulation of synaptic strength (Cheung and Ip, 2007; Dhariwala and Rajadhyaksha, 2008; Smith and Tsai, 2002). However, a number of studies have specifically examined the role of CDK5 in transport of synaptic components to synapses. In the assembly of presynaptic terminals in *C. elegans*, CDK5 regulates the deposition of all presynaptic components (Park *et al*, 2011). In contrast, in vertebrates CDK5 specifically regulates the appearance of CASK (Samuels *et al*, 2007) and synapsin at synapses (Easley-Neal *et al*, 2013). In fact, CDK5 activity must be local to allow synapsin transport packets to stop at sites of nascent synapse formation (Easley-Neal *et al*, 2013). These studies suggest that kinases may play critical roles in regulating the recruitment of presynaptic components by generating a local STOP signal at sites of synapse formation. What exactly constitutes the STOP signal is unclear, but could include inhibition of the motor proteins (Yin *et al*, 2012) or uncoupling of the cargo from the motor proteins by phosphorylation (Samuels *et al*, 2007).

CASK. Mutations in the *calcium/calmodulin-dependent serine kinase* (CASK) gene have been identified in cases of X-linked optic atrophy, X-linked mental retardation, and microcephaly with disproportionate pontine cerebellar hypoplasia with associated intellectual disability (Dimitratos *et al*, 1998; Froyen *et al*, 2007; Hackett *et al*, 2009; Hayashi *et al*, 2008; Najm *et al*, 2008). CASK protein appears to have two very distinct functions (Hsueh, 2006). During embryonic development, ~20% of the protein is present in the nucleus where it can interact with the transcription factor TBR1 (Hsueh *et al*, 2000). However, the protein also contains PDZ, SH3, and a guanylate kinase domain that are characteristic of membrane-associated guanylate kinase (MAGUK) proteins. MAGUK proteins, the most famous of which is perhaps PSD-95, are classic synaptic scaffolding proteins (Craven and Bredt, 1998). Indeed, CASK localizes at synapses and can interact with the intracellular tail of SynCAMs and Nrnxns (Biederer *et al*, 2002; Hata *et al*, 1996). CASK associates with the α_{1B} subunit of the voltage-gated calcium channel (VGCC)

and this interaction determines VGCC localization at synapses (Maximov and Bezprozvanny, 2002; Maximov *et al*, 1999). As mentioned above, the synaptic localization is regulated by phosphorylation by Cdk5 (Samuels *et al*, 2007). Interestingly, from the perspective of synaptogenesis, CASK assembles with Mint1 and Veli to form a motor protein/cargo adaptor (Setou *et al*, 2000). This suggests that CASK may not only help maintain synaptic proteins, such as calcium channels at synapses, but also be involved in the transport of synaptic cargo to developing synapses (Figure 1, top right). Thus, mutations in CASK may contribute to neuronal and synaptic development at multiple levels; as a transcription factor during neuronal differentiation and migration, and during synaptic assembly and maturation.

SYNAPTIC FUNCTIONS OF SYNDROMIC NDD GENES

There are several syndromes that manifest with social deficits, bringing them into the autism spectrum. Three of these strictly mendelian syndromes, tuberous sclerosis, fragile X mental retardation, and Rett syndrome, are because of mutations in genes that have now been shown to have roles in synaptic function and potentially also synapse formation. Here we discuss how the gene products of the TSC1 and 2, FMR1, and MECP2 genes may be involved in synapse formation.

Tsc1 and Tsc2

Tuberous sclerosis (TSC) is an inherited disease manifested with nonmalignant growths in the brain, skin, and other tissues. These growths, often called tubers in the brain, arise because of imbalances in cell growth signals. Importantly, ~60% of individuals with TSC also manifest deficits in intellectual ability and social behavior, placing TSC within the autism spectrum (Napolioni and Curatolo, 2008). The genes affected are the tumor suppressor genes *Tsc1* and *Tsc2*. The encoded proteins, hamartin and tuberin, interact with Rheb GTPase thereby inactivating the mammalian target of rapamycin (mTOR) complex (Napolioni and Curatolo, 2008). The mTOR is a kinase that regulates not only cell growth, but also cell motility, transcription, and protein synthesis. Importantly, recent studies have uncovered the importance for mTOR signaling at the synapse, potentially explaining the association with neurodevelopmental disorders: mTOR acts as a regulator of activity-dependent transcription of synaptic plasticity genes (Gipson and Johnston, 2012; Troca-Marin *et al*, 2012). More recently, *Tsc1* and *Tsc2* have been shown to function antagonistically with another mendelian NDD gene, *Fmr1* (Auerbach *et al*, 2011). Thus, *Tsc1* and *Tsc2* may be involved in the activity-dependent consolidation of synapses during development.

Fmr1

Fragile X mental retardation is the most common form of inherited intellectual disability and the most common single

gene cause of autism. The disease arises because of chromosomal instabilities of the X chromosome (Xq27.3). The affected gene, *FMR1*, encodes for an mRNA binding protein that has been shown to regulate the transcription of synaptic mRNAs in an activity-dependent manner (McLennan *et al*, 2011). Examples of the genes that are regulated include those encoding glutamate receptor subunits and calcium-calmodulin kinase II (CaMKII). The chromosomal instabilities result in increased numbers of CGG repeats in the promoter region that in turn cause decreased expression levels of Mecp2 protein. This decrease in expression level results in synaptic deficits, including an increase in synaptic spine density and an increase in immature spine shapes in humans (Irwin *et al*, 2001) and mice (Greenough *et al*, 2001). In addition, knockout mice exhibit an increase in metabotropic glutamate receptor-dependent LTD (Huber *et al*, 2002; Martin and Huntsman, 2012). Interestingly, as the LTD effect was opposite to the LTD deficit observed in Tsc2 mice, crossing of the two mutations into the same mouse resulted in the effects canceling each other out (Auerbach *et al*, 2011). Thus, it has been hypothesized that Tsc2 and Fmr1 lie in parallel, antagonistic pathways that both control the synthesis of LTD-specific proteins at synapses (Auerbach *et al*, 2011). In another comparative study of knockout mice, it was shown that both Nlgn3 knockout mice and Fmr1-null mice share a similar phenotype, including perturbed metabotropic glutamate receptor-dependent LTD (Baudouin *et al*, 2012). These studies suggest that both syndromic and nonsyndromic forms of autism may share synaptic mechanisms. In addition, one can conclude that, in the case of FMR1 and Tsc2, mutations affect common pathways, although in different directions. In conclusion, with regard to synapse development, FMR1 appears to be critical in the pruning back of inappropriate synapses.

Mecp2

Rett syndrome is caused by mutations in the *methyl-CpG binding protein 2 (MECP2)* gene. This gene is located on the X chromosome in humans, leading to more severe defects in affected males than females. In fact, the majority of males die within the first 2 years after birth. The encoded protein has a DNA-binding domain that preferentially interacts with methylated DNA in chromatin. Based on this observation, it was assumed that the protein is a transcriptional repressor (Boyes and Bird, 1991); however, it has been also seen to drive the expression of some genes (Della Ragione *et al*, 2012). Importantly, it has been shown to affect the development of dendrites and synapses, regardless of when the gene is inactivated using a conditional mouse knockout model (Nguyen *et al*, 2012). Furthermore, it appears that the deficit may lie within glial cells. Coculturing of neurons with Mecp2-deficient astrocytes is sufficient to elicit the neuronal phenotype (Ballas *et al*, 2009). This suggests that Mecp2 may regulate the expression of secreted glial factors that contribute to neuronal development (see below).

GLIAL FACTORS CONTRIBUTING TO SYNAPSE ASSEMBLY

During early studies of the development of synapse formation in primary neuronal cultures, it was apparent that 'feeder layers' of glial cells, in particular astrocytes, were critical to drive synapse assembly between various populations of neurons (Banker, 1980; Kaech and Banker, 2006; Pfrieger and Barres, 1997). Since then, a number of factors released by astrocytes have been identified, including thrombospondins, glypicans, even cholesterol, and their mechanisms for driving synapse assembly and elimination have been examined (comprehensively reviewed by Clarke and Barres, 2013). As mentioned previously, mutations in Mecp2 (Ballas *et al*, 2009), and also FMR1 (Jacobs and Doering, 2010), result in astrocytes that do not maintain correct synaptic development, clearly implicating some of these factors in the etiology of ASD-linked disorders.

For this review, we will consider the *glypican 6 (GPC6)* gene in more detail, as it has been linked to ADHD and neuroticism (Calboli *et al*, 2010; Lesch *et al*, 2008), a character trait characterized by anxiety and depressed mood. Biochemical fractionation studies of astrocyte-conditioned media led to the identification of Gpc4 and Gpc6 as factors that could significantly increase the formation of synapses (Allen *et al*, 2012). Whereas astrocyte-secreted thrombospondins induce the formation of structural, but nonfunctional, synapses (Christopherson *et al*, 2005), Gpc4 and Gpc6 administration to neurons in culture results in the addition of functional synapses. Conversely, knockout mice for Gpc4 exhibit deficits in synapse formation (Allen *et al*, 2012). Together, these results argue for a significant role for this astrocyte-secreted factor in synaptic development. It will be interesting to discover the neuronal ligand that mediates the effects of Gpcs, thereby uncovering the molecular mechanism for astrocyte-induced synaptogenesis.

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

In conclusion, genetic studies combined with studies primarily in mouse models of the candidate gene defects have contributed to our understanding of the underlying mechanisms of NDDs. Although much work is still needed, it is apparent that, at least for a subset of NDDs, deficits during development have a significant impact on synapse assembly and function, and consequently on behavior. Much work still needs to be carried out. The number of mouse models of candidate genes needs to be expanded to gain a more complete picture of the underlying molecular mechanisms of NDDs. Existing mouse models, or perhaps even zebrafish with high-throughput capacity (Tropepe and Sive, 2003), can be systematically tested for pharmacological rescue of behavioral deficits, thereby generating lead drug candidates for treatment studies. In addition, it will be

important to study whether candidate gene function is absolutely necessary during development of the synapse or whether some aspects of synaptic function can be regained in the adult.

Although determining whether a genetic defect primarily affects development of synapses rather than mature synapse function may seem, at first glance, an esoteric endeavor, this difference has important implications for therapy. With the advent of viral-based gene therapy applications almost a realistic possibility in humans (Witt and Marks, 2011), it will be important to know where and also when an intervention is critical for a successful outcome. For example, if a synaptic deficit can be overcome in the adult, then this provides hope for many adults with this form of ASD. In contrast, when it is known that a developmental synaptic deficit is hardwired into the mature CNS, it advocates for an intervention as early as possible. Although the genetic critical periods are not the only deciding factors in the ability to treat a deficit, especially by pharmacological means, information on when and where a gene product's function is necessary will certainly give insight into windows of opportunity.

To date, only a few of the NDD candidate genes have been studied with temporal requirements in mind. These kinds of experiments are difficult, requiring either the 'rescue' of deficits with viruses in a known location during development and in the adult, or the generation of conditional mutations, allowing for genetic rescue at given times. Such studies have been performed for *Mecp2* and *Nlgn3*, and for *Nlgn1* overexpression, but are necessary for more models of NDDs.

The most striking temporal rescue was demonstrated using a floxed STOP allele of the *Mecp2* gene in mice. Re-expression of *Mecp2* protein starting at 10 weeks after birth ameliorated neurological symptoms and promoted survival of male null mice many weeks beyond their null littermates that did not re-express *Mecp2* (Guy *et al.*, 2007). These experiments conclusively demonstrated that Rett-like defects in mice can be rectified in adult mice. Similarly, late expression (P30) of *Nlgn3* in a null background rescued an ectopic synapse formation phenotype in the cerebellum (Baudouin *et al.*, 2012). *Nlgn3* knockout mice demonstrate a motor coordination phenotype that is rescued by re-expression during development. Amelioration of the motor coordination phenotype was not tested in the juvenile rescue experiment (Baudouin *et al.*, 2012). It is also important to note that pharmacological intervention has been successful in improving synaptic and social deficits in adult mice. *Shank2*-deficient mice, which have the same microdeletion as seen in cases of ASD, manifest decreased NMDA receptor function, reduced social interaction, and reduced ultrasonic vocalization (Won *et al.*, 2012). Treatment of adult mice with D-cycloserine, a partial NMDA receptor agonist, or CDPPB, a positive allosteric modulator of the metabotropic glutamate receptor 5 (mGluR5, which modulates NMDA receptor function), reversed the synaptic deficits and enhanced social interaction (Won *et al.*, 2012).

To contrast these examples of juvenile/adult rescue of neuronal and neurological deficits, adult reduction of Neuroligin1 overexpression did not rescue synaptic or behavioral symptoms (Hoy *et al.*, 2013). Neuroligin1 overexpression in the hippocampus results in increased spine head size and reduced learning and memory behavior. When overexpression was turned off at 2 months of age, spine heads remained larger in size and repeated testing with the novel object recognition test showed no improvement of memory for up to 4 weeks (Hoy *et al.*, 2013). In contrast, 4 weeks of overexpression that began in the adult did lead to decreased novel object recognition. This study suggests that overexpression of Neuroligin1 is sufficient to hardwire synapses into a pathological state during development, and that this state cannot be reversed by reducing expression levels in the adult. It is, however, possible that a pharmacological intervention might be successful in improving the behavioral deficits.

Given the heterogeneity of results from these models, that is, that Neuroligin1 overexpression deficits cannot be rescued after development (Hoy *et al.*, 2013) whereas *MeCP2*, *Neuroligin3*, and *SHANK2* deletions can (Baudouin *et al.*, 2012; Guy *et al.*, 2007; Won *et al.*, 2012), it is imperative that we widen these types of studies to other genetic models. It is tempting to hypothesize that overexpression can generally not be rescued, whereas lack of expression can. However, this general rule does not seem to be the case as the increased number of synapses driven by *SynCAM1* overexpression during development can only be maintained by continuous *SynCAM1* overexpression in the adult (Robbins *et al.*, 2010). As rescue is not equally possible in developing and adult mice, it seems that each individual mutation may have a defined critical period and will have to be experimentally determined in each case.

With the advent of personalized medicine, we can expect to see prenatal genomic profiling to become increasingly routine. Genomic data will need to be supplemented with a basic understanding of (1) the genes involved in NDDs, (2) the parts of these genes that are essential for synaptic function, and (3) the critical periods for the function of each gene. It is difficult to extrapolate genetic critical periods from mouse studies to humans, and much work is necessary to confirm these trends in human patients. However, an integration of these studies with an individual's genome sequence may eventually allow personalized interventions, such as gene therapy and/or pharmaceutical treatments for the most severe cases of NDDs.

FUNDING AND DISCLOSURE

The author declares no conflict of interest.

ACKNOWLEDGEMENTS

The research in the author's laboratory is supported by a grant from the National Institute of Neurological Disorders and Stroke (R01 NS065795).

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