Leading Edge
Review

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Synaptic Neurexin Complexes: A Molecular Code for the Logic of Neural Circuits

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SUMMARY

Synapses are specialized junctions between neurons in brain that transmit and compute information, thereby connecting neurons into millions of overlapping and interdigitated neural circuits. Here, we posit that the establishment, properties, and dynamics of synapses are governed by a molecular logic that is controlled by diverse *trans*-synaptic signaling molecules. Neurexins, expressed in thousands of alternatively spliced isoforms, are central components of this dynamic code. Presynaptic neurexins regulate synapse properties via differential binding to multifarious postsynaptic ligands, such as neuroligins, cerebellin/GluD complexes, and latrophilins, thereby shaping the input/output relations of their resident neural circuits. Mutations in genes encoding neurexins and their ligands are associated with diverse neuropsychiatric disorders, especially schizophrenia, autism, and Tourette syndrome. Thus, neurexins nucleate an overall *trans*-synaptic signaling network that controls synapse properties, which thereby determines the precise responses of synapses to spike patterns in a neuron and circuit and which is vulnerable to impairments in neuropsychiatric disorders.

Synapses and the Molecular Logic Of Neural Circuits

Synapses Construct Neural Circuits

Neural circuits are thought to underlie all brain function and are formed by synapses that act as communication nodes and connect neurons into vast networks (Figure 1A). Neural circuits are useful theoretical constructs to probe brain function but, as theoretical constructs, suffer from inherent limitations that need to be considered when evaluating the role of synapses in neural information processing. Two features of neural circuit organization in particular are notable.

First, a map of the synaptic connections of a particular circuit (its "wiring diagram") alone does not allow predicting its input/output relations because synapses exhibit diverse and plastic properties. Synapses are not linear information transfer units. Synapses differentially process information encoded by a neuron's spike pattern. The transformation of presynaptic neuronal spikes into postsynaptic signals at the thousands of input and output synapses of a neuron varies dramatically between synapses depending on their properties and plasticity and is further modulated by diffusible signals (e.g., endocannabinoids) and modulatory inputs (e.g., dopamine or serotonin). As a result, a neuron integrates multifarious input signals into a spike pattern, which in turn is then transformed into output signals that differ greatly not only between neurons, but also between the various output synapses of a given neuron.

Second, most neurons in the brain simultaneously participate in many neural circuits, not just one. Separation of the different neural circuits in which a neuron participates is often difficult. The standard approach to circuit analysis is to monitor the activity of a particular class of neurons during a behavior, to determine the consequences of silencing and/or activating these neurons for this behavior and to map the synaptic connections of the monitored neurons. However, the resulting data may not allow conclusions about a neural circuit's function without the same information for upstream and downstream neurons in the circuit and for other behaviors to which these neurons contribute, as well as information about the task-specific properties of their synapses. Without such comprehensive information, conclusions about the "functions" of a neural circuit (such as "A neural circuit for [your favorite behavior here]") are likely premature. Thus, although neural circuits are useful theoretical constructs, their interpretive power is constrained by limitations that need to be considered when studying the role of synapses in specific computational tasks of the brain.

The present Review focuses on one particular question: How are synapses specified? We approach this question with the overall premise that the magical complexity of the brain—its construction from millions of overlapping neural circuits containing trillions of synapses—can be accounted for by molecular rules. These rules are thought to govern the dynamic assembly and restructuring of neural circuits throughout life. In discussing how synapses are constructed, I will focus on one particular class of synaptic proteins, the neurexins, as central effectors of molecular rules governing synapse properties (Südhof, 2008). Neurexins are presynaptic cell-adhesion molecules that are currently the best understood regulators of

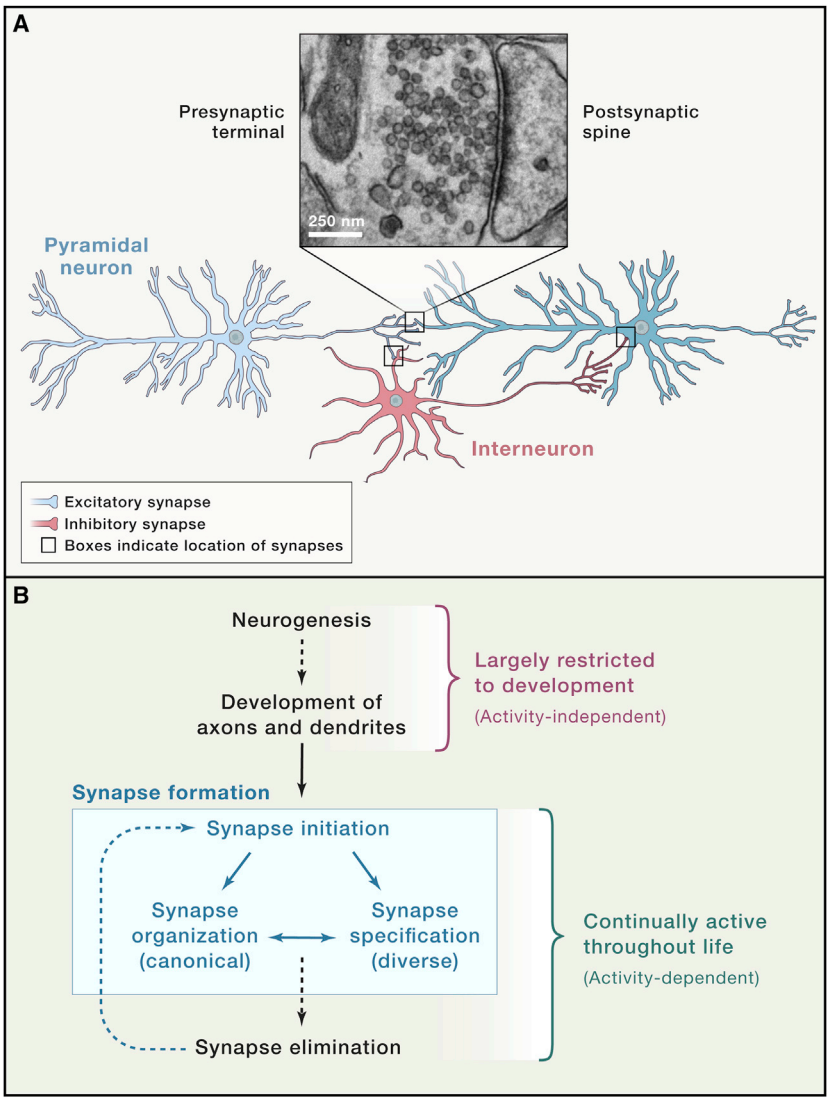


Figure 1. Synapses Construct Neural Circuits

(A) Schematic of a neural microcircuit mediating feed-forward inhibition. A presynaptic pyramidal neuron (blue) forms synapses on both a postsynaptic excitatory pyramidal neuron and a postsynaptic inhibitory neuron (red) that in turn also forms a synapse the second pyramidal neuron (top, electron micrograph of an excitatory synapse). Boxes indicate positions of synapses.

(B) Schematic of neural circuit development. Neurogenesis is followed by neural migration (not shown) and elaboration of axons and dendrites, including extension of axons over long distances (axon pathfinding). Guided axon-dendrite contacts then form synapses, with three proposed components of synapse formation: target recognition that causes synapse initiation, organization of the canonical components of synapses such as synaptic vesicles and active zones, and specification of synapse properties such as transmitter identity, release probability, or competence for long-term plasticity. Synapse formation is often followed by synapse elimination, resulting in continuous turnover of some synapses.

synapse properties and that perform a key role in neural circuit assembly and restructuring via interacting with multifarious pre- and postsynaptic ligands. These neurexin ligands in turn interact with other extra- and intracellular signaling proteins in a regulated manner, creating a dynamic molecular network. We propose that the properties and functions of neurexins may serve as a paradigm for how the molecular logic of neural circuits is constructed, with the notion that neurexins are just one component of a comprehensive mosaic of signaling molecules that determines synapse formation and function. Although focused on neurexins, this Review is not meant to be comprehensive but is intended as a conceptual discussion of how we can understand the molecular logic of neural circuits using neurexins as an example.

Synapse Formation and Elimination

During development, neurons are born, migrate, and extend axons, often over long distances to specific target areas (axonal pathfinding; Figure 1B). The axons then form synapses on target neurons (or other cells) to construct neural circuits. Postnatally, an excess of synapses is produced; 40%–50% of synapses are subsequently pruned during adolescence (Bourgeois and Rakic, 1993; Markus and Petit, 1987). Neurogenesis and formation of long-distance axon tracts are largely restricted to development, whereas synapses are continuously formed and eliminated throughout life (Figure 1B). Neurons exhibit rates of 5%–10% synapse loss per month depending on the age of the animal and the type of neuron (Qiao et al., 2016); some neurons may replace as much as 40% of synapses per month (e.g., synapses formed by layer 6 neurons in layer 1 and layer 2 of the sensory mouse neocortex; De Paola et al., 2006). Newly formed synapses are less stable than pre-existing synapses, and synapse loss primarily affects recently formed synapses. Synapse turnover is partly activity dependent, although the overall activity dependence of synapse formation and elimination appears to be surprisingly modest (Qiao et al., 2016).

Here, we divide synapse formation conceptually into three components: initial synapse establishment upon contact of an axon with a target cell, organization of synapse components to construct the canonical synaptic machinery shared by all synapses, and specification of synapse properties to confer characteristic features on a given synapse (Figure 1B). The range of synapse properties is large. It includes parameters such as neurotransmitter type, release probability, short- and long-term plasticity, postsynaptic receptor composition, and neuromodulatory signaling. Synapse specification is dynamic and activity dependent. Not only does the strength of a synapse change during activity-dependent synaptic plasticity, even its neurotransmitter type can switch in an activity-dependent

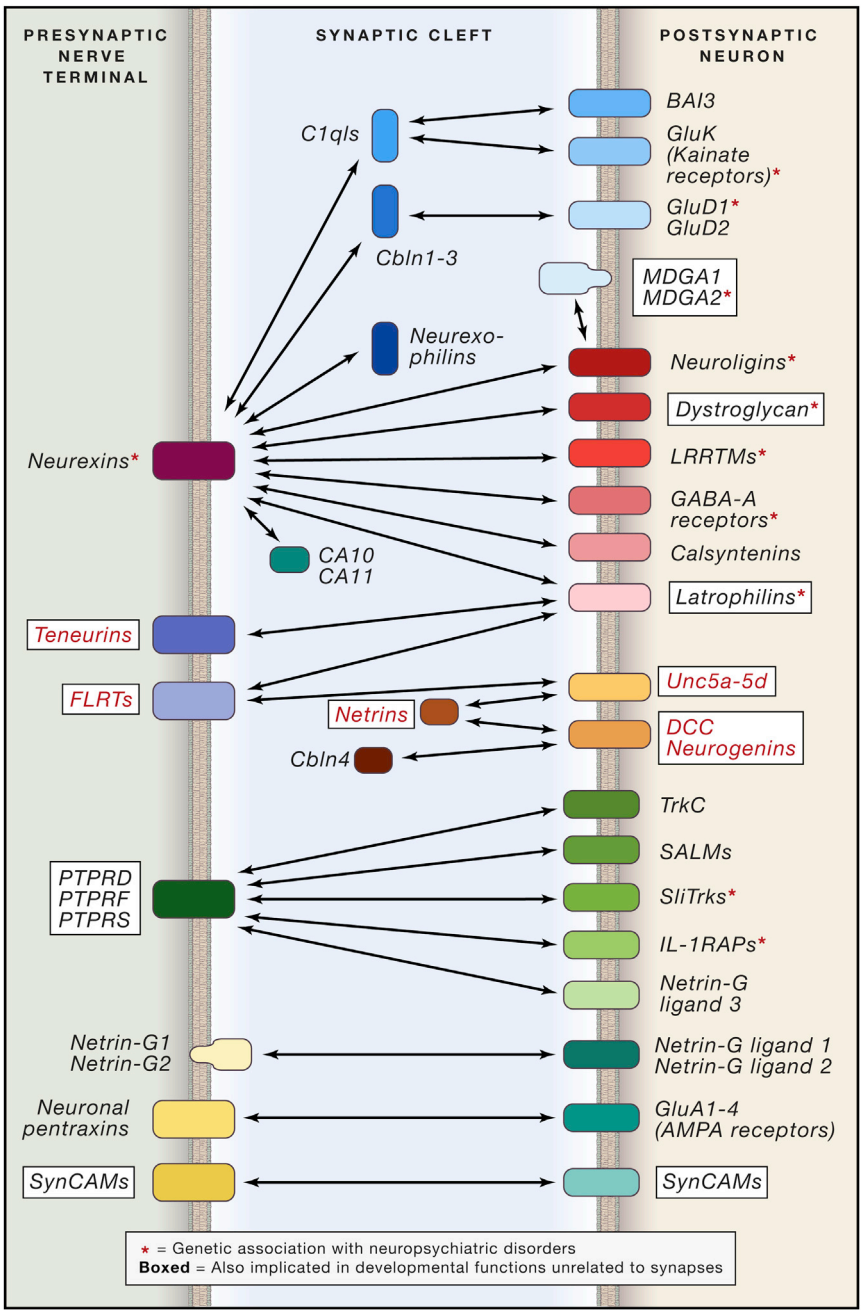


Figure 2. Overview of *trans*-Synaptic Interaction Complexes

Proteins are shown schematically; arrows signify physical binding. Interactions that are specific to particular isoforms, such as neurexin splice forms or LAR-type PTPR variants, are not shown, and interactions shown may not apply to all members of a protein family. Although only selected interactions are shown that were chosen based on the level of evidence, significant uncertainty still exists about the validity of some of the interactions shown, and even the pre- versus postsynaptic localizations of some of the proteins (shown in red typeface) have not be definitively established.

manner (Spitzer, 2017)! As a result, the number of synapse types is enormous, and defining a synapse functionally is no trivial task.

All components of synapse formation are cell-biological processes that are likely mediated by specific signal-transduction pathways. The potent induction of synapses by contact of neurons with synaptic cell-adhesion molecules exposed on non-neuronal cells, a process referred to as artificial synapse formation (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004; Nam and Chen, 2005), suggests that activation of a general signal transduction cascade by different receptors is sufficient to trigger assembly of canonical synaptic structures. Although many synaptic cell-adhesion molecules induce artificial synapses, little is known at present about the biological significance of this assay or the signaling cascades involved.

Synapse elimination ("synaptic pruning") may be as important as synapse formation for understanding how circuits are constructed (Changeux et al., 1973). After all, the majority of synapses are eliminated at one time or another during the life of an organism (Bourgeois and Rakic, 1993). Little, however, is known about the mechanisms that mediate synapse elimination. Synapse elimination is so widespread that it probably represents an intrinsic neuronal process. At the neuromuscular junction and at cerebellar climbing-fiber synapses, synapse elimination is an activity-dependent developmental process (Goda and Davis, 2003), and at least some synapse elimination involves an activity-dependent CaMKII-signaling cascade (Ko et al., 2011). However, synaptic inactivity does not necessarily lead to synapse elimination, and different types of synapse elimination may exist. The complement cascade appears to make a contribution to developmental synapse elimination (Stevens et al., 2007; Chu et al., 2010), but the effects are too small to explain the majority of synapse elimination. Synapse elimination clearly is a fertile ground for future studies!

Synapse Formation Involves Many Synaptic Cell-Adhesion Molecules

Many *trans*-synaptic interactions likely shape synapse formation (Figure 2). This multitude of molecules is not surprising considering the molecular requirements that need to be met in the development and continuous reconstruction of synaptic circuits during the lifetime of an animal. Presynaptic cell-adhesion molecules comprise at least five groups, of which neurexins and the LAR-type receptor tyrosine phosphatases (PTPRD, PTPRF, and

PTPRS) constitute "hubs" that each interact with multiple classes of postsynaptic cell-adhesion molecules. Postsynaptic cell-adhesion molecules, as far known, are more numerous and diverse than presynaptic cell-adhesion molecules and likely feature more than 50 genes. In Figure 2, only well-supported molecules are depicted, and many more plausible synaptic cell-adhesion candidates were reported that are not shown because of limited information (e.g., diverse cadherins and Ig-domain proteins).

Synaptic cell-adhesion molecules can be broadly classified into proteins whose only known functions are synaptic (such as neurexins and neuroligins) and proteins with prominent non-synaptic, primarily developmental roles (e.g., dystroglycan, teneurins, or LAR-type PTPRs). Several synaptic cell-adhesion molecules also function in brain development, in particular in neuronal migration (e.g., MDGAs and FLRTs) or axonal pathfinding (e.g., teneurins and DCC), but probably use different ligands for the various functions as illustrated by LAR-type PTPRs (Takahashi and Craig, 2013). In the case of netrin-G's, interestingly, proteins that are evolutionarily derived from a conserved axonal pathfinding molecule (netrin) have assumed a synapse-restricted function (Woo et al., 2009).

Although other key biological processes, such as gene transcription or neurotransmitter release, are as complex as synapse formation, synapse formation differs fundamentally from these processes. In gene transcription and neurotransmitter release, a complex set of molecular interactions leads to a final common pathway consisting of DNA synthesis and Ca^{2+}-triggered vesicle exocytosis, respectively. Synapse formation, on the other hand, displays no final common pathway because there is no single "endproduct." Instead, there is a continuum of synaptic states, from an initial contact to diverse types of mature synapses. These states are likely created by the convergence of multiple collaborating trans-cellular interactions processed in different combinations. As a consequence, no "master synapse formation molecule" exists. To resolve the resulting complexity of synapse formation, we need to reduce it to its constituent signaling components one by one. The collaboration between these components can then predict the properties of synapses, i.e., the collaboration determines the molecular logic of synaptic connections.

At present, however, few candidate synaptic cell-adhesion molecules are well understood, although many are described. No common theme emerges, no simple way of categorizing the processes involved can currently be envisioned. In view of this situation, here, we focus on one subset of synaptic cell-adhesion molecules, neurexins, and their ligands for three reasons: (1) because the most in-depth analyses are available for these molecules, (2) because they are centrally involved in neuropsychiatric diseases, and (3) because they may be paradigmatic of the field as a whole. However, as noted above, neurexins are just one component of a multifaceted *trans*-synaptic molecular machine. Owing to space restrictions, our discussion will be limited to vertebrates, and I apologize to our colleagues using flies or worms to study these interesting proteins for not including their work.

Experimental Considerations in Studying Synapse Formation

In order to understand how neural circuits are built, it is not sufficient to simply map them, nor does characterizing the properties of the synapses of a neural circuit alone unravel the logic of its construction. Instead, we need to identify the molecules that guide the formation and activity-dependent, continuous restructuring of synapses. Identifying such molecules requires a multidisciplinary approach that is quite different from the rather straightforward methods of tracing synaptic connections. To understand the molecular logic of synapse formation, we need to establish a catalog of the molecules involved and analyze the functions of these molecules in circuits at all levels—from atomic structures and biochemical properties to their specific roles in a particular synaptic context. Progress towards this goal involves not only structural biology and studies of synapses in reduced culture systems, but also sophisticated genetics combined with slice physiology and behavioral experiments. Owing to the diversity of synapses, rigid quality control is essential. Because molecular or pharmacological manipulations that decrease synapse numbers could act by either impairing synapse formation or increasing synapse elimination (for example by destabilizing synapses), changes in synapse numbers do not reveal whether synapse formation or elimination is impaired. Many cell-adhesion molecules with a large array of functions—more than are plausible—have been reported to be not only synaptic, but to be essential for synapses as such. Indeed, the field of synaptic biology occasionally gives the impression of a rich animal habitat featuring a bewildering array of species, some of which have been sighted only once and may be mirages. Thus, for the purpose of this Review and in order to focus the discussion, I will discuss only selected studies that were chosen based on the experimental criteria described below.

First, manipulations involving RNA interference (RNAi, using shRNAs, microRNAS, or oligonucleotides) often produce dramatic effects (especially in cultured neurons or cultured slices) that are sometimes undetectable with genetic manipulations. Because of the many inherent problems of RNAi experiments—which inevitably interfere with the endogenous microRNA processing machinery of a cell, can have incalculable off-target effects, and never completely ablate expression of a gene—I will discuss only RNAi experiments that are validated genetically.

Second, overexpression experiments are subject to a myriad of interpretational difficulties, e.g., gain-of-function effects, inappropriate outcompeting of specific ligands, misfolding-induced unfolded protein responses, and engagement in low-affinity interactions that do not normally occur. Thus, although overexpression experiments are valuable, they will be considered with caution. Similarly, rescue approaches to perform structure-function analyses in knockout neurons or to validate the RNAi invariably involve overexpression and also warrant reservations.

Third, many studies report protein-protein interactions that plausibly explain the results of experimental manipulations. Frequently, however, major conclusions are based on non-quantitative procedures such as co-immunoprecipitations or GST-pull-downs of overexpressed proteins. Co-immunoprecipitations (coIPs), if not validated by more direct measurements and/or quantifications of endogenous protein complexes, are prone to artifacts. For example, a large number of cell-adhesion molecules were reported to bind to AMPA-receptors based

purely on coIPs, even though systematic analyses of the AMPA-receptor interactome failed to identify these complexes (Schwenk et al., 2012). Candidate protein-protein interactions should be assessed by multiple assays and validated by affinity measurements or by characterizations of stable protein complexes reconstituted from purified proteins. To save space, I will thus not discuss any studies in which conclusions play a major role that are based solely on co-immunoprecipitations or GST-pull-downs.

Fourth, artificial synapse formation assays (in which an adhesion protein is expressed in a non-neuronal cell and induces pre- or postsynaptic specializations in co-cultured neurons; see Scheiffele et al., 2000; Biederer et al., 2002) are central components of most studies on synapse formation. These assays provide a tremendous opportunity to dissect signaling pathways involved in synapse formation. The very fact, however, that many diverse molecules (> 30) are active in these assays demonstrates that these assays alone do not constitute evidence for a function in synapse initiation. For the purpose of this review, we will consider these assays as valuable tools that do not in themselves provide functional evidence.

Finally, genetic manipulations also have potential problems but are considered here as a baseline for functional evaluations. In constitutive knockouts (KOs), developmental compensation is often cited as a confounding issue but is rarely documented; in fact, we are only aware of a single instance in synapse formation (Zhang and Südhof, 2016). Two other potential problems with constitutive KOs may be more significant. Constitutive KOs of developmentally active genes preclude an analysis of the role of these genes in synapse formation, and constitutive KOs can produce inadvertent effects on nearby genes. Therefore, conditional KOs are superior because they allow spatially and temporally restricted manipulations even in mature animals and enable precise control of genetic background, leading us to consider them the method of choice. It is occasionally argued that conditional KOs may be less "acute" than RNAi experiments, but both manipulations require enzymatic reactions to suppress expression of an mRNA, and the time course of both methods depends more than anything on the lifetime of the target protein—thus, conditional KOs are probably no slower than RNAi. Another manipulation that needs to be considered here is CRISPR, which is likely to become a major tool in postmitotic neurons but has not yet been fully optimized.

Neurexins: Form and Function

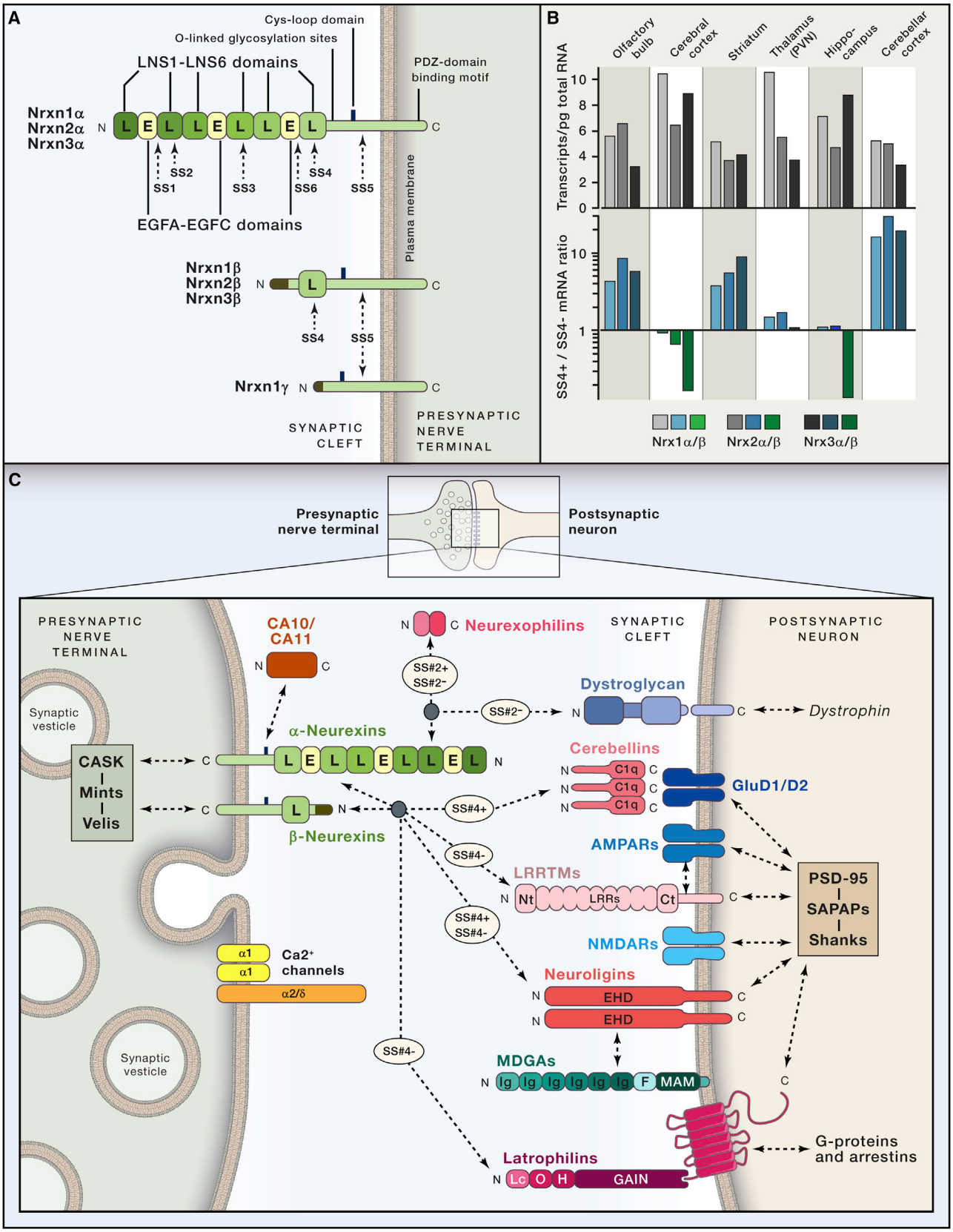
Mammalian neurexins are type-1 membrane proteins expressed from three genes as α-, β-, and γ-neurexins (Figure 3; *Nrxn1-3* in mice and *NRXN1-3* in humans; Ushkaryov et al., 1992, 1993, 1994; Sterky et al., 2017). α-neurexins contain 6 LNS-domains (for laminin/neurexin/sex-hormone-binding globulin domains) with three interspersed EGF-like repeats, followed by an O-linked sugar modification sequence, a short cysteine-loop domain, a transmembrane region (TMR), and a cytoplasmic sequence of 55-56 residues. β-neurexins are transcribed from an internal promoter in all three α-neurexin genes and are composed of an N-terminal β-neurexin-specific sequence that then splices into the α-neurexin sequence N-terminal of its LNS6 domain; thus, β-neurexins are effectively N-terminally truncated α-neurexins with a short specific N-terminal motif (Figure 3A). A γ-neurexin is only transcribed from an internal promoter in the *Nrxn1* gene and splices into the Nrxn1α and Nrxn1β sequence C-terminal of the LNS6 domain, therefore only containing the cysteine-loop as an extracellular domain followed by the TMR and cytoplasmic sequence (Sterky et al., 2017). In invertebrates, a single neurexin gene encodes only an α-isoform (Tabuchi and Südhof, 2002). Comparison of neurexin genes shows that *Nrxn1* and *Nrxn3* are more closely related to each other than to *Nrxn2*, suggesting that evolutionarily *Nrxn2* diverged from a common progenitor of *Nrxn1* and *Nrxn3* (Treutlein et al., 2014). Neurexins are homologous to other cell-surface proteins containing LNS-domains, in particular CASPRs (Peles et al., 1997), and are thus part of a larger family of cell-adhesion molecules. Crystal structures of a fragment from Nrxn1α spanning the LNS2 to LNS6 domains revealed an L-shaped form (length = ~14 nm; width = ~6 nm), with a long arm composed of the LNS2-LNS3-EGFB-LNS4-LNS5 domains and a short arm composed of the EGFC-LNS6 domains (Chen et al., 2011; Miller et al., 2011). In the crystal structure, the "arms" of Nrxn1α were stabilized by extensive interdomain contacts that stably connected some domains (e.g., at the hinge between LNS5 with EGFC or at the contact between EGFC with LNS6 that is predicted to be loosened by the alternatively spliced SS6 insert). The three neurexin genes are transcribed in brain at similar levels, with α-neurexins being much more abundant than β-neurexins (Aoto et al., 2013; Schreiner et al., 2014; Anderson et al., 2015).

Neurexins are primarily expressed by neurons in and outside of brain and are localized to synapses (Ushkaryov et al., 1992). In addition, *Nrxn1* mRNA is abundantly produced in astrocytes (Zhang et al., 2014; Gokce et al., 2016). Several reports suggested neurexin expression outside of neurons and glia using sensitive RT-PCR assays or immunological approaches, but given the absence of non-brain phenotypes in neurexin mutant mice and the lack of reliable antibodies, these observations need to be validated. Similar to other synaptic genes, neurexins are expressed early in development long before synapse formation begins, presumably to synthesize synaptic proteins in preparation to synapse assembly (Daly and Ziff, 1997).

Neurexin Diversification by Alternative Splicing into Thousands of Isoforms

When neurexins were identified, their extensive alternative splicing was proposed to suggest a role as surface recognition molecules that specify synapses (Ushkaryov et al., 1992). This hypothesis is increasingly being validated.

Neurexins are alternatively spliced at six canonical sites (referred to as SS1 to SS6) into thousands of isoforms that are differentially expressed throughout brain (Figures 3A and 3B; Ullrich et al., 1995; Treutlein et al., 2014; Schreiner et al., 2014). In a given neuron and for a given splice site, neurexin alternative splicing is almost never all or none but generally covers a continuum. Individual neurons express different fractions of mRNAs that include or exclude the alternatively spliced sequences and that are typical for a neuron type (Fuccillo et al., 2015). Both at the level of brain regions (Figure 3B) and at the single-cell level (Fuccillo et al., 2015), alternative splicing



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of *Nrxn1*, *Nrxn2*, and *Nrxn3* is often uncorrelated, resulting in dramatic differences in alternative splicing at a given site between neurexins. Thus, alternative splicing is differentially regulated between neurexin genes despite their homology.

The control of alternative splicing of neurexins is only beginning to be explored. Pioneering studies by Zisapel and colleagues showed that activation of cultured cortical neurons causes a dramatic, Ca^{2+}-dependent exclusion of the *Nrxn2* SS3 insert that depends on specific sequence motifs associated with the alternatively spliced exon (Rozic-Kotliroff and Zisapel, 2007; Rozic et al., 2013), consistent with the large regional differences in SS3 alternative splicing (Ullrich et al., 1995). Subsequent, more extensive studies showed that SS4 is also regulated by neuronal activity (Ding et al., 2017). The regulation of neurexin alternative splicing at SS3 and SS4 by activity appears to be physiologically important because it can be observed *in vivo*, both as a consequence of fear learning (Rozic et al., 2011; Ding et al., 2017) and as a diurnally regulated process (Shapiro-Reznik et al., 2012).

The protein factors mediating SS4 alternative splicing are now being examined. Compelling evidence implicates members of the STAR family of RNA-binding proteins, although uncertainty remains about the precise isoforms and mechanisms involved. STAR family RNA-binding proteins comprise Sam68, SLM1, and SLM2 (gene symbols *KHDRBS1-3*). They are characterized by a central KH-type RNA-binding domain and a C-terminal Sam68 domain, and they function broadly in many events of alternative splicing in neuronal and non-neuronal cells (Chawla et al., 2009). Puzzlingly, all STAR family proteins have been shown to be separately essential for neurexin alternative splicing at SS4 (Iijima et al., 2011 and 2014; Ehrmann et al., 2013; Traunmüller et al., 2016; Danilenko et al., 2017). Of these studies, the best evidence exists for SLM2, whose deletion causes a dramatic loss of SS4− variants for all three neurexins (Ehrmann et al., 2013; Traunmüller et al., 2016).

How Sam68, SLM1, and SLM2 collaborate and are regulated in controlling the differential SS4 alternative splicing of *Nrxn1*, *Nrxn2*, and *Nrxn3* remains unclear. *In vitro* experiments suggested that both SLM2 and Sam68 can enhance excision of SS4 in both *Nrxn1* and *Nrxn3*, but that only SLM2 and not Sam68 can do so in *Nrxn2* because the *Nrxn2* gene contains fewer binding sites for these factors (Danilenko et al., 2017). Additional mechanisms may contribute. Ding et al. (2017) showed that neuronal activity induces increases in the repressive histone marker H3K9me3 at the SS4 exon of *Nrxn1*, which is mediated at least in part by the histone methyl-transferase Suv39h1. Strikingly, suppression of Suv39h1 blocked the activity-dependent exclusion of SS4 in Nrxn1 (Ding et al., 2017). Thus, both STAR domain RNA-binding proteins and histone modifications may control neurexin SS4 alternative splicing. The challenge now arises to understand their respective mechanisms and to learn if and how these mechanisms also apply to other sites of alternative splicing in neurexins.

Neurexin Ligands

A panoply of neurexin ligands have been described over the last decades, starting with the discovery of neuroligins (Ichtchenko et al., 1995, 1996), and most recently identifying neurexin-binding to cerebellins and C1qls as a link that connects neurexins to GluDs and GluK2, respectively (Uemura et al., 2010; Matsuda et al., 2016). As a result, neurexins are now known to anchor a ligand interaction network that includes interactions with members of at least seven postsynaptic protein families as well as soluble adaptor proteins (Figures 2 and 3).

At present, three ligand-binding sites on neurexins have been described: (1) the LNS2 domain that is specific for α-neurexins binds to neurexophillins and dystroglycan (Missler et al., 1998; Sugita et al., 2001); (2) the LNS6 domain that is shared by α- and β-neurexins binds to neuroligins, LRRTMs, GABA-A-receptors, cerebellins, and latrophilins (Ichtchenko et al., 1995,1996; Ko et al., 2009a; de Wit et al., 2009; Siddiqui et al., 2010; Uemura et al., 2010; Zhang et al., 2010; Boucard et al., 2012); and (3) the juxtamembranous sequences that are also shared by α- and β-neurexins bind to CA10 and CA11 and to C1qls (Sterky et al., 2017; Matsuda et al., 2016). Many of these ligands, as discussed in greater detail below, are major postsynaptic cell-adhesion molecules within their own rights. For each of the three binding sites on neurexins, the corresponding ligands cannot bind simultaneously, whereas ligands for different sites can bind at the same time. Because, for most domains of α-neurexins, no ligand has yet been identified, more neurexin ligands likely remain to be discovered, suggesting that the neurexin-anchored interaction network is even more extensive than currently envisioned.

Importantly, many ligand interactions depend on neurexin alternative splicing (Figure 3C). Neuroligins were initially thought only to bind to β-neurexins lacking an insert in SS4 (Ichtchenko et al., 1995, 1996). Subsequent studies, however, revealed a more complex interaction pattern that is not only regulated by SS4 of neurexins, but also by alternative splicing of neuroligin-1 (Nlgn1) at a site called SSB (Boucard et al., 2005; Chih et al., 2006). Specifically, Nlgn1 containing an insert in SSB binds to β-neurexins, but not α-neurexins, lacking an insert in SS4, probably because the EGFC domain in α-neurexins tightly abuts the LNS6 domain, thus sterically interfering with Nlgn1 binding if an insert in SSB is present (Tanaka et al., 2011). In contrast, Nlgn1 lacking an insert in SSB, as well as other neuroligins, binds to both α- and β-neurexins (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006). Most other ligands that bind to

Figure 3. Domain Structures, Alternative Splicing, and Selected Ligand Interactions of Neurexins

(A) Domain structures and sites of alternative splicing of neurexins. Domains are labeled above the schematics of α-neurexins, and sites of alternative splicing (labeled SS1-SS6) are indicated below α-, β-, and γ-neurexins.

(B) Expression of Nrxn1, Nrxn2, and Nrxn3 (top, shown in gray) and ratio of SS4+/SS4− splice forms of these neurexins (bottom, blue and green) as determined by qRT-PCR in selected brain regions dissected from adult mice (modified from Aoto et al., 2013).

(C) Schematic of the interactions of α- and β-neurexins with selected ligands in the context of the synapse. Requirements for neurexin splice variants are indicated; possible competition between ligands are indicated by junctions marked with a circle; Proteins are not drawn to scale (abbreviations: E, EGF-like domain; EHD, esterase homology domain; L, LNS-domain; LRRs, leucine-rich repeats; Nt and Ct, N- and C-terminal sequences surrounding LRRs; Ig, Ig-domain; F, fibronectin III domain; MAM, MAM-domain; Lc, lectin domain; O, olfactomedin-like domain; H, hormone-binding domain; GAIN, GAIN domain).

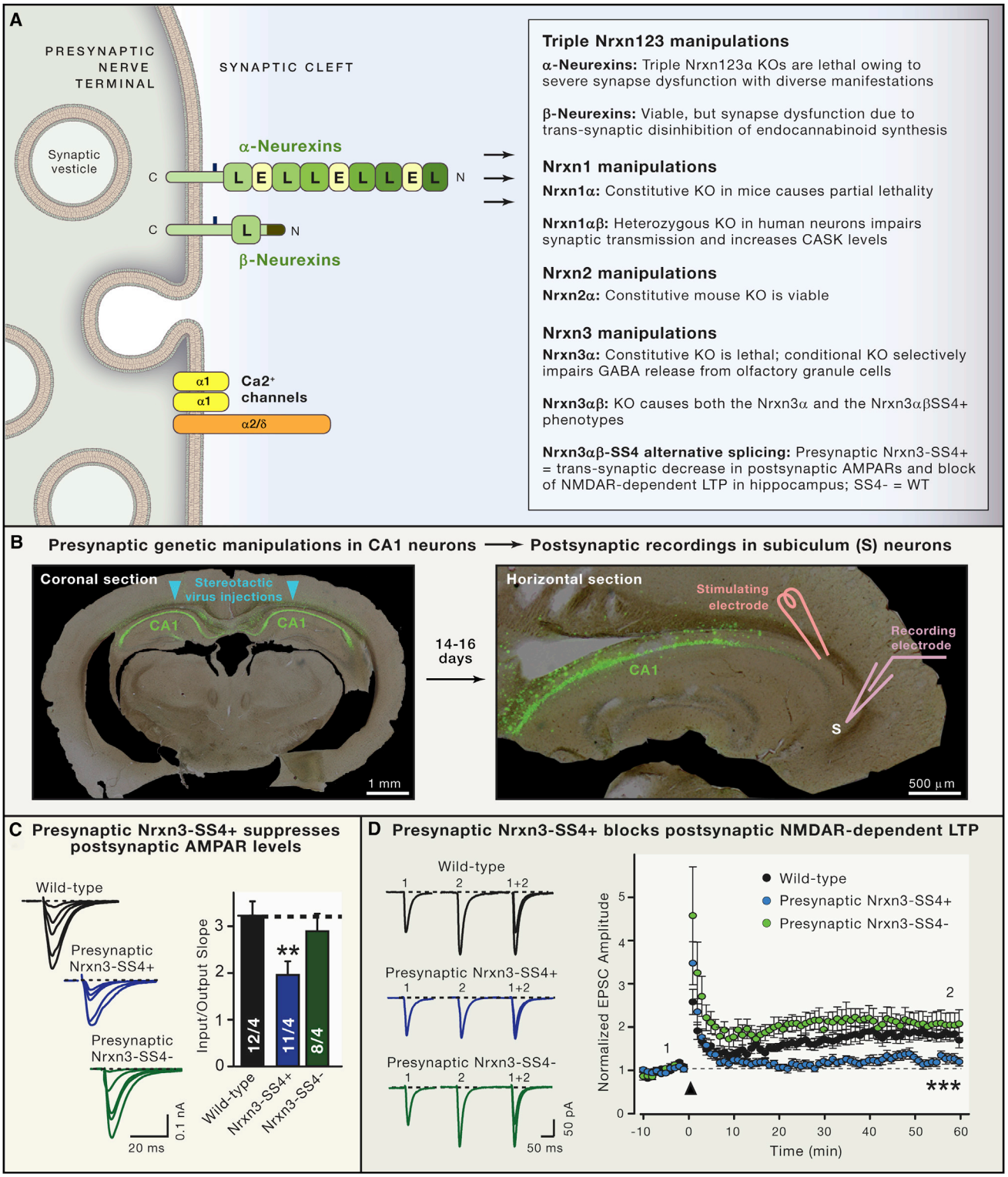

Figure 4. Phenotypes Produced by Genetic Manipulations of Neurexins, Illustrated with the Results Obtained by Genetic Control of SS4 in Nrxn3

(A) Schematic summary of the most salient phenotypes emerging from genetic manipulations of neurexins. A presynaptic terminal with surface-exposed neurexins are shown on the left, and a summary list of phenotypes observed with genetic manipulations on the right.

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both α- and β-neurexins via interacting with the LNS6 domain are also regulated by alternative splicing at SS4 (Figure 3C). In particular, LRRTMs and latrophilins only appear to bind to SS4− neurexins (Ko et al., 2009a; Siddiqui et al., 2010; Boucard et al., 2012), whereas cerebellins only bind to SS4+ neurexins (Uemura et al., 2010).

Neurexin Function

Not surprisingly given their complex interaction networks, neurexins mediate many regulatory functions. At this point, several key results have provided dramatic insights, and some general conclusions about neurexin functions may be possible.

Early studies demonstrated that constitutive KOs of individual α-neurexins impaired survival and that deletion of all three α-neurexins dramatically compromised synaptic transmission, in part by impairing Ca^{2+} influx during an action potential (Figure 4A; Missler et al., 2003). These studies—borne out by later, more sophisticated approaches—suggested that neurexins are not essential for synapse formation as such but are central regulators of synapse properties.

Subsequent experiments with conditional KOs of all three β-neurexins showed that the β-neurexin deletion also caused a major synaptic phenotype but without significantly impairing survival (Anderson et al., 2015). In synapses of cultured hippocampal neurons and in synapses formed by CA1 neurons on subiculum neurons *in vivo* (see Figure 4B), β-neurexin deletions suppressed the presynaptic release probability. Strikingly, this phenotype was, at least in part, not due to a cell-autonomous presynaptic effect but was caused by disinhibition of tonic postsynaptic endocannabinoid synthesis (Anderson et al., 2015). The increased postsynaptic endocannabinoid secretion induced by the β-neurexin deletion then activated presynaptic CB1-receptors, thereby inhibiting neurotransmitter release. These experiment revealed a *trans*-synaptic regulatory loop in which presynaptic β-neurexins regulated postsynaptic endocannabinoid synthesis, thus demonstrating that β-neurexins perform a specific function independent of α-neurexins.

Recent preliminary analyses of conditional triple KOs that ablate expression of all α- and β-neurexins extended these results, uncovering dramatic differences in the overall functions of neurexins between different types of synapses (Chen et al., 2017). Deletion of all neurexins from parvalbumin-positive interneurons in the prefrontal cortex caused a loss of synapses (∼30%) and a decrease in synaptic strength (∼50%) but no impairment in action-potential-triggered Ca^{2+} influx. In contrast, deletion of neurexins from somatostatin-positive interneurons caused no synapse loss but a large decrease in action-potential-triggered Ca^{2+} influx (∼50%) that also suppressed synaptic strength (∼50%; Chen et al., 2017). Thus, deletion of neurexins impaired synaptic transmission in two different types of synapses to the same extent but by different mechanisms, suggesting that neurexins perform distinct regulatory functions in different classes of neurons, instead of a canonical regulatory function in all classes of neurons. It is possible, however, that neurexins also have a canonical role in controlling presynaptic Ca^{2+} channels as suggested by the common phenotype observed in some synapses in pan-α-neurexin KO and pan-αβ-neurexin KO mice (Missler et al., 2003; Chen et al., 2017) and that this phenotype is occluded in other synapses by unknown redundancies, a possibility that requires further study.

A major question that arises now, given that pan-αβ-neurexin deletions produce distinct phenotypes in different types of synapses, is whether the three different neurexins perform similar or distinct functions in these synapses. At present, this question cannot be answered because only *Nrxn3* has been examined in detail (Aoto et al., 2013, 2015). Conditional deletion of *Nrxn3* in the hippocampal CA1 region produced two dramatic phenotypes, as analyzed at synapses formed by presynaptic CA1-region neurons on postsynaptic subiculum neurons (Figure 4B): a decrease (∼40%) in AMPA-receptor- (AMPAR-) mediated excitatory responses that was caused by a loss of postsynaptic AMPARs, and a complete block of postsynaptic, NMDA-receptor- (NMDAR-) mediated LTP (Aoto et al., 2015). Conditional deletion of *Nrxn3* in the olfactory bulb, however, led to a different phenotype—namely, a decrease (∼60%) in GABA-receptor- (GABAR-) mediated inhibitory responses. The hippocampal phenotype was rescued with a Nrxn3β protein composed of the extracellular sequences tethered to the membrane via a lipidic GPI-anchor, whereas the olfactory bulb phenotype could only be rescued with full-length Nrxn3α protein (Aoto et al., 2015). Thus, in the two different brain regions examined, Nrxn3 performs distinct functions via different molecular mechanisms, echoing the theme of context-specific functions of neurexins described above.

Function of Neurexin Alternative Splicing at SS4

The alternatively spliced SS4 sequence of neurexins is encoded by a single exon with a non-canonical splice acceptor sequence. To control SS4 alternative splicing of endogenous *Nrxn3* genetically, we converted its non-canonical SS4 splice acceptor sequence into a canonical splice acceptor sequence and flanked the exon with loxP sites (Aoto et al., 2013). As a result, the SS4 exon was no longer alternatively spliced but constitutively

(B) Experimental approach for analyzing the effects of presynaptic manipulations in hippocampal CA1 neurons on synapses formed by these neurons on pyramidal neurons in the mouse subiculum. Stereotactic infection of CA1 neurons with viruses mediating genetic manipulations are performed at P21 (left). Mice are analyzed 14-16 days later by slice physiology using whole-cell recordings from subiculum neurons and extracellular stimulations of CA1 region neuron axons as indicated (right).

(C) Illustration of control of postsynaptic AMPAR levels by presynaptic Nrxn3 alternative splicing at SS4. Control mice (black) and SS4-knockin mice in which the the alternatively spliced SS4 exon is rendered constitutively included (SS4+) were analyzed; in the latter, the CA1 region of the hippocampus was injected with control or Cre-recombinase expressing virus as described in (B) to either retain Nrxn3-SS4+ in the CA1 to subiculum projection (blue symbols), or to convert Nrxn3-SS4+ into Nrxn3-SS4− in this projection (green symbols). Input/output curves were then used to determined the strength of AMPAR-mediated EPSCs, demonstrating that the decrease in AMPAR-mediated responses in Nrxn3-SS4+ mice can be fully reversed by presynaptic excistion of SS4 yielding presynaptic Nrxn3-SS4-.

(D) Illustration of the all-or-none gating of postsynaptic NMDAR-dependent LTP by presynaptic Nrxn3 alternative splicing at SS4. Experiments were performed as described in (B) and (C), except that LTP as induced by 100 Hz tetani was examined.

(B)–(D) were modified from Aoto et al. (2013).

included (SS4+) in all Nrxn3 mRNAs in the absence of Cre recombinase and constitutively excluded (SS4−) in the presence of Cre recombinase.

Analysis of hippocampal neurons revealed that constitutive Nrxn3-SS4+ expression caused a decrease in postsynaptic AMPARs and a block of NMDAR-dependent LTP that was identical to the phenotype of the *Nrxn3* KO (Figure 4C). Presynaptic excision of the SS4 exon using Cre recombinase rescued the phenotype (Aoto et al., 2013). Thus, presynaptic alternative splicing of *Nrxn3* in hippocampal neurons controlled postsynaptic AMPAR levels and postsynaptic LTP, thereby *trans*-synaptically specifying the properties of the synapses involved. Again, however, this phenotype was context dependent. The same manipulation of *Nrxn3* alternative splicing at SS4 had no effect on excitatory or inhibitory transmission in olfactory bulb neurons (Aoto et al., 2015). It is unclear whether alternative splicing of other neurexins at SS4 performs a similar *trans*-synaptic function, as suggested by experiments with SLM2 KO mice. The SLM2 KO caused an increase in SS4 exon inclusion in all neurexins and impaired LTP; this LTP was rescued by expression of Nrxn1-SS4− (Traunmüller et al., 2016), suggesting that Nrxn1-SS4− can rescue a phenotype similar to that caused by the constitutive expression of Nrxn3-SS4+. However, these experiments are indirect, and direct manipulations of SS4 in *Nrxn1* and *Nrxn2* will be needed to test this question.

Neurexins as Signaling Platforms

The characterization of neurexin functions and interactions is only beginning, but it is clear from the available information that neurexins are not molecularly or functionally monogamous; they engage in multifarious interactions and perform at least some distinct roles in different synapses. The diversity of isoforms and possible interactions is staggering. Because neurons express multiple neurexins and various neurexin ligands, neurexins likely engage in a mixture of competitive and simultaneous complexes that transmit different *trans*-synaptic signals. As a result, neurexins and their ligands likely engage in a dynamic interaction network in which neurexin-ligand interactions do not produce stationary complexes but are continuously remodeled by activity-dependent or modulatory changes in gene expression and/or alternative splicing. For example, a small activity-dependent shift in alternative splicing at SS4 of neurexins would cause a major change in their relative binding to neuroligins, cerebellins, and LRRTMs and may produce large functional changes (Aoto et al., 2013). However, the binding affinities of various neurexins and their ligands are largely unknown; thus, even if we knew the expression levels of neurexins and their ligands in a given pair of synaptically connected neurons, we would not be able to calculate the state of the neurexin-based dynamic interaction network. Again, much remains to be done.

A picture of neurexins emerges as signaling platforms that host a diverse array of *trans*-synaptic mediators. It seems likely that as signaling platforms, neurexins activate pre- and postsynaptic signal transduction pathways that are as yet uncharacterized. Presynaptically, neurexins bind to CASK, a hybrid protein composed of an unusual N-terminal protein kinase and a C-terminal domain set characteristic of MAGUKs (for membrane-associated guanylate kinases) (Hata et al., 1996; Mukherjee et al., 2008), to Mints (Biederer and Südhof, 2000), and to FERM-domain proteins such as protein 4.1 (Biederer and Südhof, 2001). It is possible that these interactions mediate intracellular signaling, an exciting possibility that remains to be explored. Postsynaptically, neurexin ligands presumably perform their different functions via diverse signaling pathways. In the following, I will discuss selected neurexin ligands to illustrate the diverse output pathways activated by *trans*-synaptic neurexin-based interactions.

Neuroligins

In vertebrates, four genes encode neuroligins, of which *Nlgn1*, *Nlgn2*, and *Ngln3* are highly conserved, whereas *Nlgn4* varies between rodents and humans and is expressed only at low levels in mice (Ichtchenko et al., 1995, 1996; Bolliger et al., 2008). Neuroligins are type 1 membrane proteins that are composed of a single large extracellular domain consisting of a constitutively dimeric, enzymatically inactive esterase-homology domain, a TMR, and a short cytoplasmic tail. Neuroligins are differentially targeted to synapses. Nlgn1 is localized to excitatory synapses (Song et al., 1999) and Nlgn2 to inhibitory, dopaminergic, and cholinergic synapses (Graf et al., 2004; Varoqueaux et al., 2004; Uchigashima et al., 2016; Takács et al., 2013), possibly because dopaminergic and cholinergic synapses use GABA as a co-transmitter. Nlgn3 is found in both excitatory and inhibitory synapses (Budreck and Scheiffele, 2007), and Nlgn4 is found in glycinergic synapses (Hoon et al., 2011).

Nlgn1 contains two sites of alternative splicing (SSA and SSB). SSA in Nlgn1 and Nlgn3 involves the independent inclusion or exclusion of two alternatively spliced exons that are separated by large introns, whereas SSA in Nlgn2 includes only the second alternatively spliced exon, and Nlgn4 lacks either alternatively spliced (Comoletti et al., 2006). SSB consists of the variable insertion of an eight-residue sequence that is only found in Nlgn1 (Boucard et al., 2005). Nlgn1-SSB+ binds only to Nrxn1β, but not Nrxn1α, lacking an insert in SS4, whereas Nlgn1-SSB− and all other neuroligins bind to Nrxn1β independent of SS4 (Comoletti et al., 2006). Strikingly, Nlgn2 and Nlgn3 exhibited a significantly lower affinity for Nrxn1β than Nlgn1 (Comoletti et al., 2006; Elegheert et al., 2017). Although these data are limited by the lack of analysis of Nrxn1α and of other neurexins, they reveal dramatic differences in the relative biochemical properties of neuroligins.

Functions of Neuroligins

Similar to neurexins, neuroligins perform multiple synapse-specific functions that cannot be subsumed under a single theme. Compelling initial evidence for a synaptic function of neuroligins emerged from the dramatic effects of overexpression experiments that revealed increases in synapse density and synaptic transmission in transfected neurons (Scheiffele et al., 2000; Chubykin et al., 2007). Although informative, these experiments were indirect. The neuroligin-induced increase in synapse density was activity dependent (Chubykin et al., 2007), suggesting that it differs from physiological initial synapse formation, which is largely activity independent (Verhage et al., 2000; Sando et al., 2017; Sigler et al., 2017). Moreover, the original conclusion that neuroligin overexpression increases spine density was based on comparing expression of labeled neuroligins with a diffusible marker; careful recent quantifications suggested that

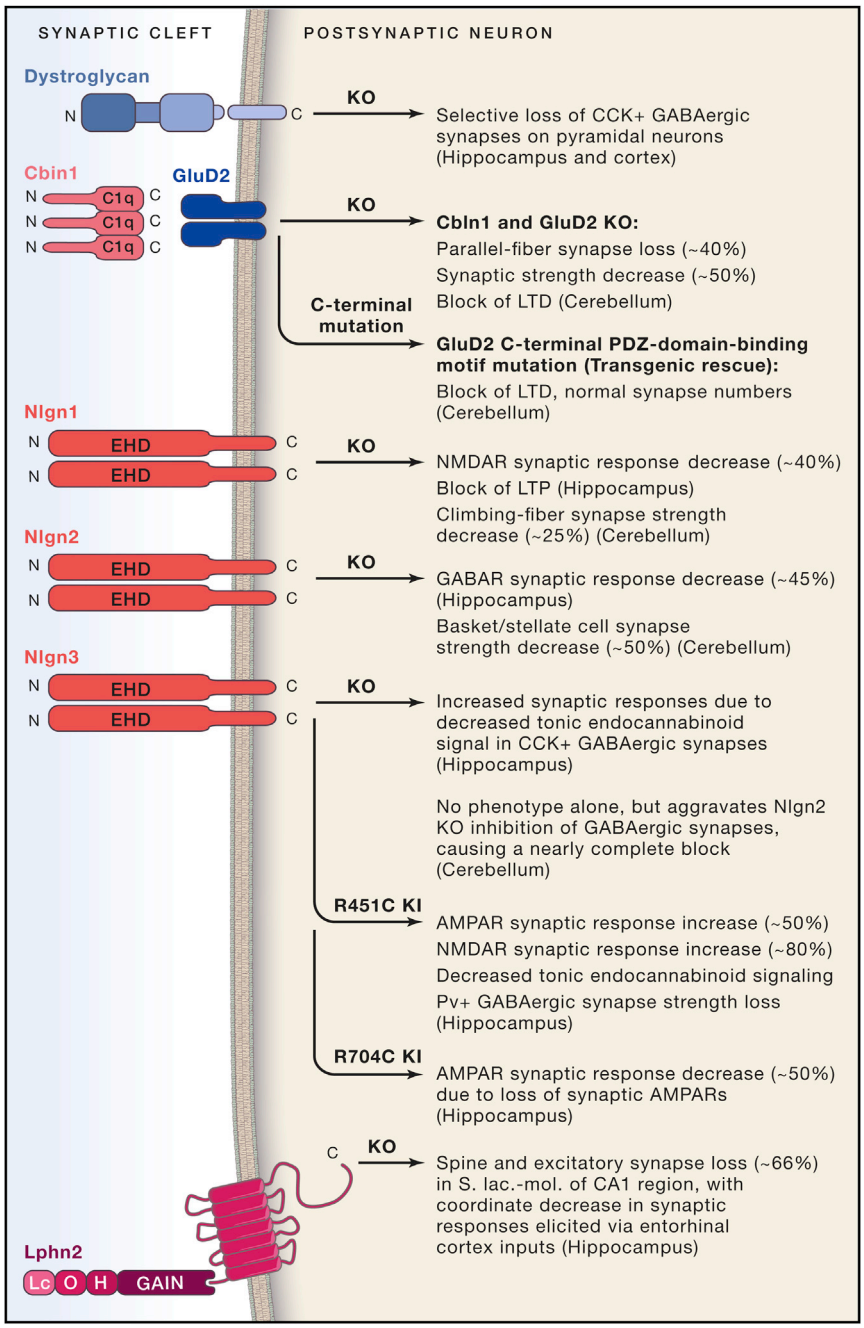

Figure 5. Phenotypes Produced by Genetic Manipulations of Selected Neurexin Ligands
Salient effects of specific manipulations are shown next to the diagrams of the respective proteins. For references, see text.

overexpressed neuroligins do not increase spine numbers but only synapse numbers (Chanda et al., 2017). Similarly, while Nlgn3 overexpression in wild-type neurons increased synapse numbers, Nlgn3 overexpression in neurons lacking other neuroligins did not (Chanda et al., 2017), indicating that overexpressed Nlgn3 is unable to promote synapse formation when heterodimer formation with endogenous Nlgn1 or Nlgn2 is excluded. Puzzlingly, Nlgn4 overexpression decreased excitatory synaptic strength even though it also increased synapse density in transfected neurons (Zhang et al., 2009), suggesting that Nlgn4 overexpression induced production of non-functional synapses and additionally interfered with synaptic transmission in pre-existing synapses. Nlgn1 overexpression increases synapse numbers in neurons even when it is mutated to abolish dimerization, suggesting that Nlgn1 can act as a monomer (Ko et al., 2009b). Note that a later study concluded that neuroligin dimerization is important (Shipman and Nicoll, 2012), but that in these experiments, a mutation was used that may denature the protein, making it difficult to interpret the results. Thus, although neuroligin overexpression continues to be a valuable approach, conclusions need to be interpreted as gain-of-function effects that depend on the neuroligin isoform overexpressed and the endogenous neuroligins present.

Genetic loss-of-function studies have revealed dramatic but diverse effects of neuroligin deletions (Figure 5). Constitutive Nlgn123 triple-KO mice died at birth and exhibited no changes in synapse numbers or synapse ultrastructure but displayed major impairments in synaptic transmission (Varoqueaux et al., 2006). Similarly, conditional triple-KO of Nlgn123 in cerebellar Purkinje cells impaired both excitatory and inhibitory synaptic transmission without major decreases in synapse numbers (Zhang et al., 2015). However, only excitatory climbing-fiber synapses, but not parallel-fiber synapses, were affected, presumably because parallel-fiber synapses are maintained by cerebellin-neurexin complexes instead of neuroligin-neurexin complexes (Uemura et al., 2010). Conditional triple KO of Nlgn123 confirmed that even when neuroligins were deleted in a sparse subset of neurons, no change in synapse numbers occurred, whereas synaptic transmission was impaired (Chanda et al., 2017).

Deletion of only *Nlgn1* decreased both NMDAR- and AMPAR-mediated excitatory synaptic responses in cultured neurons, with a greater effect on NMDAR responses than AMPAR responses and without an effect on inhibitory responses (Chubykin et al., 2007; Chanda et al., 2017). No direct interaction of Nlgn1 with NMDARs or AMPARs is known, but these may be cuptured indirectly by Nlgn1 via the common binding of Nlgn1,

AMPARs and NMDARs to PSD95 (Irie et al., 1997; Mondin et al., 2011). In acute hippocampal slices, sparse conditional deletion of *Nlgn1* only decreased NMDAR-mediated, but not AMPAR-mediated, synaptic responses, as monitored at Schaffer-collateral synapses (Jiang et al., 2017). In addition, the *Nlgn1* deletion completely blocked postsynaptic, NMDAR-dependent LTP. Even though called NMDAR-dependent LTP, this type of LTP is also efficiently induced by increases in postsynaptic Ca^{2+} in the absence of NMDAR activation (Kullmann et al., 1992; Weisskopf et al., 1999; Kato et al., 2009). The *Nlgn1* deletion also blocked LTP induced by this NMDAR-independent protocol, demonstrating that Nlgn1 performs independent functions in maintaining normal NMDAR-mediated responses and in enabling LTP (Jiang et al., 2017), with the function of Nlgn1 in LTP possibly related to that of Nrxn3 in LTP (Aoto et al., 2013). The *Nlgn1* deletion also impaired extrasynaptic NMDAR-mediated, but not synaptic AMPAR-mediated, responses in cerebellar stellate cells, further supporting a role for Nlgn1 in regulating NMDAR levels in neurons (Zhang and Südhof, 2016).

Different from *Nlgn1* deletions but commensurate with its localization to inhibitory synapses, *Nlgn2* deletions impaired inhibitory but not excitatory synaptic responses (Figure 5) (Chubykin et al., 2007; Gibson et al., 2009; Poulopoulos et al., 2009; Zhang et al., 2015; Liang et al., 2015). In cortex, *Nlgn2* deletions selectively decreased synaptic transmission mediated by fast-spiking (presumably parvalbumin-positive) interneurons without affecting synaptic transmission mediated by somatostatin-positive interneurons (Gibson et al., 2009). In cerebellar Purkinje cells, *Nlgn2* deletions caused a decrease in inhibitory inputs that was greatly augmented by simultaneous deletion of *Nlgn3*, which in itself had little effect (see below). Intracellularly, Nlgn2 binds to the inhibitory-synapse-specific molecules gephyrin, collibystin, GARLH3, and GARLH4, which may recruite Nlgn2 to synapses (Poulopoulos et al., 2009; Yamasaki et al., 2017). However, some of these interactions may also apply to other neuroligins, and at present, it is unclear how Nlgn2 function is targeted to particular types of inhibitory synapses.

Nlgn3 deletions overall produced a much weaker phenotype than *Nlgn1* and *Nlgn2* deletions but significantly enhanced the phenotypes caused by these deletions in some synapses. *Nlgn3* deletions had no effect on excitatory synaptic transmission in hippocampus (Etherton et al., 2011a) and caused a small selective decrease in climbing-fiber synaptic responses in cerebellum without altering parallel-fiber synaptic transmission or mGluR5-dependent LTD (Zhang et al., 2015). The most profound and informative effect of Nlgn3 deletions, however, was detected in inhibitory synapses in the hippocampus and striatum. In the hippocampus, *Nlgn3* deletions produced a selective disinhibition of tonic endocannabinoid signaling in CCK-positive synapses (Földy et al., 2013). This phenotype, which resembles the effect of β-neurexin deletions in hippocampal synapses (Anderson et al., 2015), is consistent with a discrete context-dependent role of Nlgn3. In the striatum, *Nlgn3* deletions selectively impaired inhibitory inputs onto N. accumbens medium spiny neurons (Rothwell et al., 2014). Note that the autism-associated R351C and R704C point mutations of Nlgn3 cause more severe phenotypes than *Nlgn3* deletions (see below), probably because the point mutations produce gain-of-function effects (Tabuchi et al., 2007; Etherton et al., 2011a, 2011b; Földy et al., 2013).

Apart from its localization to glycinergic synapses (Hoon et al., 2011) and the puzzling overexpression effects described above (Zhang et al., 2009; Chanda et al., 2016), little is known about the functions of Nlgn4.

Overall, the functional sanalyses show that neuroligins perform synaptic regulatory functions that are surprisingly isoform specific and, at least in the case of Nlgn1, are mediated by multiple mechanisms. As described below, these features of neuroligins could at least in part be explained by their potential interactions with additional ligands such as MDGAs and other proteins that remain to be identified.

MDGAs: Novel Neuroligin Regulators

A fascinating recent discovery is the binding of the cell-adhesion molecules MDGA1 and MDGA2 to neuroligins in competition with neurexins (Lee et al., 2013; Pettem et al., 2013; Connor et al., 2016; Kim et al., 2017; Gangwar et al., 2017). MDGA1 and MDGA2 are composed of six N-terminal immunoglobulin (Ig) domains followed by single fibronectin type III and MAM domains and a glycosylphosphatidylinositol anchor that attaches them to the plasma membrane (Figure 3C; Litwack et al., 2004). MDGAs bind to Nlgn1 and Nlgn2 in a *cis*-configuration with high affinity and to Nlgn3 and Nlgn4 with a 10- to 20-fold lower affinity (Connor et al., 2016; Gangwar et al., 2017; Kim et al., 2017; Elegheert et al., 2017). Crystal structures of MDGA1/Nlgn1 and MDGA1/Nlgn2 complexes revealed that the two N-terminal Ig domains of MDGA1 straddle the Nlgn1 or Nlgn2 homodimer, such that each neuroligin homodimer binds two MDGA1 molecules (Gangwar et al., 2017; Kim et al., 2017; Elegheert et al., 2017). Specifically, the Ig1 domain of MDGA1 interacts with the neurexin-binding site on one of the neuroligins in a homodimer, whereas the Ig2 domain binds to the opposite side of the other neuroligin subunit.

Heterozygous constitutive deletion of MDGA2 causes a small increase (~10%) in excitatory synapse numbers and discrete behavioral changes (Figure 5) (Connor et al., 2016), consistent with a physiological role of MDGA2 in regulating synapses by disrupting interactions of neuroligins with neurexins. Different from constitutive Nlgn1 or Nlgn2 KOs that are not by themselves lethal (Varoqueaux et al., 2006), however, homozygous MDGA2 deletions are lethal, possibly because MDGAs perform major functions earlier in development during neurogenesis and neuronal migration (Takeuchi and O'Leary, 2006; Ishikawa et al., 2011; Ingold et al., 2015). Since neuroligins do not have known functions in early brain development, MDGAs likely engage in additional cell-adhesion interactions whose impairment could also have contributed to the synaptic phenotype of heterozygous MDGA2 KO mice. Thus, at present, the physiological significance of the interaction of MDGAs and neuroligins—for example, whether this interaction impairs or promotes synapse function—remains unclear. Notably, even after binding to neuroligins via their Ig1 and Ig2 domains, MDGAs retain large potential interacting surfaces via their other domains and appear poised for additional molecular activities. Conditional double MDGA1 and MDGA2 KO experiments are required to assess the relative importance of the interactions of MDGAs with neuroligins; ideal would be conditional knockins

that impair specifically the binding of MDGAs to neuroligins without altering the binding of neurexins to neuroligins.

Neuroligin Puzzles

Current results establish the general importance of neuroligins for synaptic function, as well as the surprising diversity of these functions. Although the fundamental synaptic roles of neuroligins have thus now been established, at least in part, the mechanisms involved remain enigmatic. To mention a few salient issues, what other effectors do neuroligins bind to besides neurexins and MDGAs? Does neurexin- or MDGA-binding activate neuroligins, and if so, does this activation trigger a signal-transduction cascade that involves intracellular neuroligin interactions (e.g., with PSD-95; Irie et al., 1997) or an extracellular *cis*-interaction? At present, the only evidence for a role of neuroligin cytoplasmic sequences in a physiological function that does not involve overexpression is the gain-of-function effect of the R704C Nlgn3 mutation that produces an unexplained large decrease in AMPAR-mediated, but not NMDAR-mediated, synaptic responses (Etherton et al., 2011b; Chanda et al., 2013). Similarly, it is unclear by what mechanism Nlgn1 is required separately for NMDAR-level maintenance and for postsynaptic LTP, a continuation of the puzzle posed by the requirement for Nrxn3 in LTP. Another major question regards synapse specificity: given that neuroligins are so homologous and that their cytoplasmic sequences appear to bind to the same intracellular proteins at least *in vitro*, how are the different neuroligins sorted to distinct types of synapses in a given neuron, and by what mechanisms do they mediate their distinct functions? Despite decades of work, much remains to be learned.

Cerebellins

Cerebellin-1 (Cbln1) was identified as a hexadecapeptide that is now considered a likely breakdown product of physiologically active full-length Cbln1 (Urade et al., 1991). Vertebrates express four homologous cerebellins (Cbln1-Cbln4), which are small secreted proteins (~21 kDa) that are composed of an N-terminal cysteine-rich sequence and a C-terminal C1q-domain. Cerebellins resemble two other secreted C1q-domain proteins, adiponectin and C1qls, except that adiponectin includes a different, collagen-like N-terminal sequence and Cq1ls contain both the N-terminal cysteine-rich sequence of cerebellins and the collagen-like sequence of adiponectin (Martinelli et al., 2016). Cerebellin C1q domains assemble into trimers similar to other C1q domains, and their N-terminal cysteine-rich sequence dimerizes the C1q-domain trimers into hexamers (Lee et al., 2012). Cbln1, Cbln2, and Cbln4 autonomously assemble into homohexamers, whereas Cbln3 requires Cbln1 for proper assembly and secretion (Bao et al., 2006), suggesting that Cbln1 and Cbln3 form heteromultimers and that other cerebellins co-expressed in a neuron could also assemble into heteromultimers.

Despite their name, cerebellins are not cerebellum-specific. Only Cbln3 is present exclusively in cerebellum, while the other cerebellins are broadly expressed throughout brain in discrete and characteristic patterns (Miura et al., 2006; Seigneur and Südhof, 2017). For example, the entorhinal cortex expresses high levels of both Cbln1 and Cbln4, whereas the hippocampus proper lacks detectable cerebellin expression; in the olfactory bulb, similarly, Clbn2 is detectable at high levels only in mitral cells, whereas Clbn4 is expressed only in inhibitory neurons (Seigneur and Südhof, 2017).

Interactions of Cerebellins

Cbln1 and Cbln2, but not Cbln4 (little is known about Cbln3), bind to the N-terminal domains of GluD1 and GluD2, which are homologous to ionotropic glutamate receptors but function as adhesion molecules instead of glutamate receptors (Matsuda et al., 2010; Wei et al., 2012; Yasumura et al., 2012). Cbln1 and Cbln2 bind to all neurexins with high affinity but only to neurexins containing an insert in SS#4 (Uemura et al., 2010; Matsuda and Yuzaki, 2011; Joo et al., 2011). These interactions form *trans*-synaptic complexes composed of GluD1 or GluD2, Cbln1 or Cb1n2, and Nrxn1 or Nrxn2 or Nrxn3. In these complexes, tetrameric GluD2 interacts with two hexameric Cbln1 molecules that each in turn bind to two neurexin molecules. As a result, one postsynaptic tetrameric GluD molecule captures four presynaptic neurexin molecules (Lee et al., 2012). Interestingly, Cbln4 binds to the netrin-receptor DCC (for deleted-in-colon-cancer) and its homolog, neogenin (Wei et al., 2012; Haddick et al., 2014). However, it is unclear whether DCC and neogenin are pre- or postsynaptic and what "other" binding partners in the opposite synaptic membrane might be involved—in fact, it is unclear whether Cbln4, DCC, and neogenin are synaptic at all.

Functions of Cerebellins

Cbln1, Cbln2, and Cbln4 KO mice are viable; thus, individual cerebellins are not essential for survival (Hirai et al., 2005; Rong et al., 2012; Wei et al., 2012; Haddick et al., 2014). Only Cbln1 KO mice have been analyzed in detail and exhibit a phenotype that is identical to that of their receptor GluD2 (Hirai et al., 2005; Otsuka et al., 2016; Kusnoor et al., 2010; Ito-Ishida et al., 2008, 2014). The fact that Nrxn1 and Nrxn3 are essential for survival (Missler et al., 2003; Aoto et al., 2015) while cerebellins and GluD2 are not may be due to the interactions of neurexins with many other different postsynaptic partners. In contrast, Cbln1 and Cbln2 and GluD2 appear to only bind to each other and to neurexins and thus only mediate part of the overall functions of neurexins.

In cerebellum—the only brain region extensively analyzed—Cbln1 and GluD2 KO mice exhibit a decrease in parallel-fiber synapses formed by granule cells on Purkinje cell spines, with a loss of presynaptic terminals and the appearance of "naked spines" (Figure 5) (Kashiwabuchi et al., 1995; Hirai et al., 2005; Rong et al., 2012). Importantly, the loss of parallel-fiber synapses is only partial (~30%–50%, depending on the study) and primarily found on distal dendrites of Purkinje cells and is not associated with a decrease in spine density (hence the naked spines; Hirai et al., 2005). The remaining parallel-fiber synapses in Cbln1 and GluD2 KO mice—still the majority—are impaired: their postsynaptic specializations are larger than their presynaptic active zones, synaptic transmission is inefficient, and importantly, LTD is blocked (Hirai et al., 2005; Ito-Ishida et al., 2008; Uemura et al., 2007; Kakegawa et al., 2008). Since most parallel-fiber synapses and most synaptic transmission remains in Cbln1 and GluD2 KO mice but LTD is blocked completely, the *trans*-synaptic neurexin-Cbln1-GluD2 complex may be quantitatively more important for long-term synaptic plasticity than for synaptic transmission or synapse stability. This conclusion mirrors observations made for Nrxn3 and Nlgn1 discussed above

(Aoto et al., 2015; Jiang et al., 2017). The Cbln1 KO phenotype is not developmentally fixed but rapidly reversed by simple addition of recombinant Cbln1 to cultured cerebellar neurons or acute cerebellar slices (Ito-Ishida et al., 2008).

The greater depth of analysis of GluD2 than of Cbln1 allows further conclusions about the functions of the Cbln1-GluD2 complex. Acute application of an antibody to the N-terminal ligand-binding domain of GluD2 induced AMPAR endocytosis, decreased synaptic strength, and blocked LTD at parallel-fiber synapses, suggesting a signaling function of the neurexin-cerebellin-GluD complex (Hirai et al., 2003). Conditional deletion of GluD2 in adult mice caused an increasing mismatch between pre- and postsynaptic specializations with a broadening of the distribution of AMPARs and a progressive loss of synaptic parallel-fiber contacts, consistent with a synapse-stabilizing effect of the neurexin-cerebellin-GluD complex (Takeuchi et al., 2005). Strikingly, even though the GluD2 KO induced synapse loss and blocked LTD, it increased the content of AMPARs in the remaining parallel-fiber synapses, again suggesting a role in shaping synapse properties (Yamasaki et al., 2011). GluD2 levels in parallel-fiber synapses are activity dependent, suggesting that activity shapes synapse properties not only by altering presynaptic neurexin SS4-alternative splicing, but also by changing postsynaptic GluD2 levels (Hirai, 2001). In GluD2 KO mice, parallel-fiber synapses initially form normally but then become partly eliminated, demonstrating that the GluD2 deletion does not impair initial synapse formation (Kurihara et al., 1997). Importantly, the entire GluD2 KO phenotype was rescued with transgenic expression of mutant GluD2 that carries substitutions in its presumptive channel, proving that GluD2 does not act as an ion channel in its parallel-fiber synapse functions (Kakegawa et al., 2007). Moreover, only the synapse-loss phenotype, but not the LTD block was rescued in GluD2 KO mice by expression of mutant GluD2 that lacked a C-terminal intracellular PDZ-domain-binding sequence (Uemura et al., 2007; Kakegawa et al., 2008), demonstrating that LTD and synapse stabilization are mechanistically distinct functions of the neurexin-Cbln1-GluD2 complex.

Cbln1 as a Cerebellar Signaling Molecule

Overall, current data suggest that the *trans*-synaptic neurexin-Cbln1-GluD2 complex is not involved in the initial formation of parallel-fiber synapses (i.e., is not synaptogenic) but performs two related, mechanistically distinct functions mediated by *trans*-synaptic signaling: specification of synapses and enabling LTD. The loss of the first function partly destabilizes synapses, leading to the observed loss of synapses, whereas the loss of the second function is caused by the ablation of cytoplasmic GluD2 signaling. How the neurexin-Cbln1-GluD2 complex signals, however, remains enigmatic. Although partial crystal structures of the complex are available, they provide few clues to its mechanism of action (Elegheert et al., 2016; Cheng et al., 2016).

Cerebellins are expressed in many neurons throughout the brain, but little is known about their functions outside of the cerebellum. In striatum, Cbln1-deficient thalamic axons exhibited an increase in synaptic spine density instead of a synapse loss, consistent with a general signaling function of cerebellins (Kusnoor et al., 2010). The loss of Cbln1 signaling may cause distinct downstream, region-dependent effects on synapse stability, accounting for the different phenotypes of Cbln1-deficient cerebellum and striatum. Moreover, Cbln1 was shown to be essential for hippocampal learning by an unknown mechanism (Otsuka et al., 2016). Thus, our understanding of Cbln1 function is incomplete, and even less is known about the functions of Cbln2 and Cbln4.

Latrophilins

Latrophilins are three adhesion GPCRs (Lphn1-Lphn3) that contain large extracellular sequences, a seven-TMR-domain typical of GPCRs, and a relatively long intracellular tail (Sugita et al., 1998). The extracellular sequences of latrophilins include N-terminal lectin- and olfactomedin-like domains, followed by a serine- and threonine-rich sequence, a hormone-binding domain (that has no known hormone-binding activity), and an autoproteolytic GAIN domain that is characteristic of all adhesion GPCRs (Araç et al., 2011). Like Nrxn1, Lphn1 was initially purified from brain homogenates as a candidate α-latrotoxin receptor by binding to immobilized α-latrotoxin (Krasnoperov et al., 1997; Lelianova et al., 1997). Latrophilins bind to at least three cell-adhesion molecules in a *trans*-configuration: neurexins (Boucard et al., 2012), teneurins (Silva et al., 2011; Boucard et al., 2014), and FLRTs (O'Sullivan et al., 2014).

Constitutive Lphn1 KO mice exhibit a partial loss of α-latrotoxin binding sites and a decrease of α-latrotoxin-triggered glutamate release from cortical synaptosomes, consistent with a role of latrophilins as presynaptic α-latrotoxin receptors (Figure 5) (Tobaben et al., 2002). However, a presynaptic localization for latrophilins was difficult to reconcile with their trans-interaction with neurexins, which as presynaptic proteins would have to interact with postsynaptic latrophilins (Boucard et al., 2012). Recently, conditional Lphn2 KO mice showed that postsynaptic, but not presynaptic, deletion of Lphn2 in CA1-region pyramidal neurons causes a severe decrease in excitatory synapse density and excitatory synaptic transmission (Anderson et al., 2017). In these CA1-region neurons, Lphn2 was localized preferentially to postsynaptic spines in the S. lacunosum-moleculare. *In vivo* deletion of Lphn2 from CA1-region pyramidal neurons caused a selective loss only of spines and synapses in the S. lacunosum-moleculare but not the S. oriens or S. radiatum as analyzed morphologically and electrophysiologically (Anderson et al., 2017). These experiments suggest that latrophilins may function as postsynaptic recognition molecules for incoming axons in a defined dendritic domain. Even as postsynaptic cell-adhesion molecules, latrophilins could still function as α-latrotoxin receptors, as they could act by recruiting α-latrotoxin to synapses for subsequent presynaptic insertion of the α-latrotoxin molecule, which would then induce neurotransmitter release (Ichtchenko et al., 1998).

Dystroglycan

Dystroglycan is a cell-adhesion molecule that is broadly expressed and that, in muscle, anchors the intracellular actin cytoskeleton to the extracellular matrix (Ervasti and Campbell, 1993). Extracellularly, dystroglycan binds to laminin, neurexins, and other LNS-domain containing proteins with high affinity (Ervasti and Campbell, 1993; Sugita et al., 2001). Laminin and neurexin binding to dystroglycan requires dystroglycan glycosylation by dedicated glycosyltransferases called LARGE

(Inamori et al., 2012). Dystroglycan binds to the second LNS-domain of α-neurexins in an SS2-dependent manner, and additionally to the sixth LNS-domain in an SS4-dependent manner (Figure 3; Sugita et al., 2001; Reissner et al., 2014). Intracellularly, dystroglycan interacts with dystrophin, thereby linking extracellular interactions to the intracellular cytoskeleton (Ervasti and Campbell, 1993).

In the developing brain, dystroglycan-binding to its ligands is essential for the migration of nascent cortical neurons, as evidenced by the dramatic "cobblestone lissencephaly" observed in patients with mutations in LARGE glycosyltransferases (Zhang et al., 2017). In mature brain, dystroglycan is broadly expressed in glia and neurons and localizes, among others, to astrocytic endfeet on the basement membrane of blood vessels and to somatic synapses on pyramidal neurons formed by CCK+ GABAergic basket cells (Knuesel et al., 2000). Postsynaptic deletion of dystroglycan from pyramidal neurons selectively ablates CCK+ synapses, suggesting that dystroglycan acts as a postsynaptic cell-adhesion molecule (Früh et al., 2016). Whether this function of dystroglycan involves neurexin binding, however, is unknown. An attempt to test this was made using a disease-relevant dystroglycan mutation (T190M; Früh et al., 2016), but it is unclear whether this mutation alters neurexin binding, and the physiological significance of the interaction neurexins with dystroglycan remains to be tested.

LRRTMs

Four LRRTM genes (*LRRTM1-4*) express homologous proteins that are composed of 10 extracellular N-terminal leucine-rich repeats surrounded by flanking sequences typical for leucine-rich repeat domains and followed by a single TMR and a 70-residue cytoplasmic tail (Laurén et al., 2003). LRRTM1-3 bind to α- and β-neurexins with high affinity, but only when these neurexins lack an insert in SS4 (Ko et al., 2009a; de Wit et al., 2009; Siddiqui et al., 2010; Um et al., 2016). LRRTM4 binds to glypicans via their heparan sulfate proteoglycan modification, and may also bind to neurexins (Siddiqui et al., 2013; de Wit et al., 2013; Ko et al., 2015). LRRTM4 was additionally identified as a stoichiometric component of AMPAR complexes, and other LRRTMs probably also bind to AMPARs (Schwenk et al., 2012).

Despite extensive studies on LRRTM-mediated protein interactions and on the effect of LRRTM overexpression in cultured neurons, the functions of LRRTMs are unclear (Figure 5). Individual LRRTM1, LRRTM3, and LRRTM4 deletions in mice produced significant but small decreases (~10%) in excitatory synapse density and evoked synaptic transmission in neurons with high expression levels for a given isoform (Takashima et al., 2011; Siddiqui et al., 2013; Um et al., 2016). This lack of a major KO phenotype may be due to a function unrelated to synaptogenesis or to redundancy among LRRTM isoforms. For example, LRRTM1 and LRRTM2 are co-expressed in CA1 neurons and LRRTM3 and LRRTM4 in dentate gyrus granule cells, but double KOs have not yet been analyzed for these proteins. The hypotheses that LRRTMs are redundant and/or perform functions unrelated to synaptogenesis are supported by the observation that combined RNAi-mediated knockdown of LRRTM1 and LRRTM2 *in vivo* caused a dramatic loss of NMDAR-dependent LTP and a decrease in AMPAR-mediated responses without changing synapse numbers (Soler-Llavina et al., 2011). However, interpretation of these results is limited by the potential off-target effects of RNAi, as documented by the many phenotypes observed with RNAi at least in cultured neurons that could not be reproduced *in vivo* using genetics. At present, LRRTMs thus remain interesting but incompletely characterized postsynaptic adhesion molecules complexed to AMPARs that form *trans*-synaptic complexes with neurexins and heparan sulfate proteoglycans and that are likely to be important for synapses in an as-yet-unknown manner.

Neurexophilins

Neurexophilins are small secreted proteins that are expressed from four genes in vertebrates and that resemble neuropeptides without exhibiting homology to any known protein (Petrenko et al., 1996; Missler and Südhof, 1998). Neurexophilins bind to the LNS2 domain of α-neurexins independent of alternative splicing at SS2 (Missler et al., 1998). *In situ* hybridization and RNA sequencing (RNA-seq) data suggest that neurexophilins are adundantly expressed in specific classes of interneurons (e.g., Földy et al., 2016), but little is known about their functions. Constitutive deletion of neurexophilin-1 caused changes in spontaneous mini-release and in short-term plasticity at inhibitory synapses in the reticular nucleus of the thalamus (Born et al., 2014); these changes were occluded by addition of a $GABA_B$-receptor inhibitor CGP-55845, suggesting that the neurexophilin-1 deletion may have inhibited $GABA_B$-receptor function. However, the mechanisms involved and the general functions of neurexophilins remained unexplored—even simple questions, such as which neurexophilins bind to which neurexins, are unanswered. It seems likely that the different neurexophilin-neurexin complexes bind to a specific postsynaptic target analogous to neurexin-cerebellin binding to GluDs and that the neurexin-neurexophilin-target complex performs a specific regulatory function at a subset of inhibitory synapses, but at this point, no direct evidence for these hypotheses is available.

Calsyntenins

Calsyntenin-1 to -3 are evolutionarily conserved type 1 membrane proteins whose extracellular sequences include two cadherin domains and one LNS domain. Two different functions have been proposed for calsyntenins in mammals. First, calsyntenins were shown to bind to kinesins and were proposed to function as adaptors linking transport vesicles to kinesin (Konecna et al., 2006). A beautiful confirmation of this activity was provided in *C. elegans*, in which the single calsyntenin homolog CASY-1 mediates the presynaptic kinesin-dependent transport of a specific splice variant of the insulin receptor DAF2, thereby enabling activity-induced synaptic changes during associative learning (Ohno et al., 2014). Second, calsyntenins were shown to bind to neurexins with an affinity that is only slightly lower than that of neurexins and were proposed to function as postsynaptic cell-adhesion molecules (Pettem et al., 2013; Um et al., 2014; Lu et al., 2014). In support of this hypothesis, calsyntenin is synaptogenic in the artificial synapse formation assay (Pettem et al., 2013; Um et al., 2014), and calsyntenin knockouts exhibit synaptic phenotypes, albeit very modest ones (Pettem et al., 2013; Ster et al., 2014;

Lipina et al., 2016). Although at present these two disparate strings of experimental results appear incompatible, it is possible that they can be reconciled, i.e., that a postsynaptic function of calsyntenins may involve a role in kinesin-mediated transport. Further experiments, especially using conditional mouse mutants and more extensive electrophysiology, may resolve this question.

C1qls

Similar to cerebellins, C1qls (expressed in three isoforms, C1ql1-3) contain an N-terminal cysteine-rich sequence and a C-terminal C1q-domain, but different from cerebellins, C1qls also include a central collagen-like sequence (Martinelli et al., 2016). The C1q domains of C1qls assemble into trimers with a similar atomic structure as other C1q domains (Ressl et al., 2015), while their complex N-terminal sequences appear to mediate formation of hexamers of trimers (i.e., decaoctamers containing 18 subunits; Shimono et al., 2010). Like cerebellins, C1qls are expressed broadly in brain in specific subpopulations of neurons (Iijima et al., 2010; Martinelli et al., 2016).

C1qls were initially linked to synapses by the observations that all three C1qls bind to the adhesion-GPCR BAI3 with high affinity and that addition of recombinant C1qls to cultured neurons alters synapse numbers (Bolliger et al., 2011). Subsequent pioneering *in vivo* experiments revealed that postsynaptic BAI3 physiologically functions as a C1ql1 receptor in climbing-fiber synapses in cerebellum and that deletion of either BAI3 or C1ql1 causes a loss of climbing-fiber synapses (Kakegawa et al., 2015; Sigoillot et al., 2015), proving a role for C1qls in synapse formation. Similarly, C1ql3 deletion from amygdala neurons was shown to suppress the number of synapses formed by C1ql3-expressing amygdala neuron on prefrontal cortex neurons, confirming a synaptic role for C1qls (Martinelli et al., 2016).

Recent surprising results expanded this view with the discovery that C1qls bind with high affinity to postsynaptic kainate-type GluK2 and GluK4 and to AMPAR GluA1 glutamate receptors (but not to other glutamate receptors) and to presynaptic Nrxn3 containing a particular alternatively spliced sequence at SS5 (Matsuda et al., 2016). C1ql2 and C1ql3 are co-expressed in presynaptic dentate gyrus granule cells that form mossy-fiber synapses on postsynaptic CA3 pyramidal neurons. Deletion of both proteins from the granule cells, but not individual deletions of either protein, severely decreased postsynaptic kainate-receptor-mediated synaptic responses at the mossy-fiber synapses without changing synapse numbers (Matsuda et al., 2016). These results suggest that in some synapses, C1qls are Nrxn3 ligands that mediate *trans*-synaptic regulation of postsynaptic kainite receptors in an SS5-dependent manner.

Viewed together, the current experiments suggest that C1qls may perform two different synaptic roles: first, to mediate the establishment or maintenance of synapses analogous to cerebellins, by binding to postsynaptic BAI3 and to an unknown presynaptic receptor, thereby creating a *trans*-synaptic complex via binding; second, to concentrate kainate receptors at synapses via a Nrxn3-C1ql-GluK complex. Questions abound, however; given that these two roles are so different, further studies will be needed to probe these proposed functions more deeply.

Neurexin Complexes in Neuropsychiatric Disorders

Recent advances in microarray and DNA sequencing technologies enabled successive waves of sophisticated studies on the genetic architecture of neuropsychiatric disorders at an increasingly larger scale. These advances were further fueled by better access to patients and their families and were facilitated by efforts of foundations, such as the Simons Foundation. Among others, these studies led to three major surprising discoveries. First, the human genome is remarkably fluid. Especially large-scale deletions and duplications of genomic DNA (termed CNVs for copy-number variations) appear to commonly occur during every generation, creating a genetic dynamic that was unexpected (Sebat et al., 2004; Iafrate et al., 2004). Second, mutations in many different genes predispose to neuropsychiatric disorders, possibly hundreds for schizophrenia and autism. Third, for most mutations, the clinical presentations differ greatly among affected individuals. As discussed below, these three discoveries are particularly important for *NRXN1* mutations.

Although the advances in the genetics of neuropsychiatric disorders are impressive, methodological issues occasionally cloud their interpretation. Most studies are based on disease categories, not neuropsychiatric disorders in general. Often, a mutation is discovered to be associated with one disease, and its importance is considered less important if it is also associated with another disease, whereas arguably, the fact that many mutations are associated with different diseases is—if anything—evidence for their significance. Moreover, major studies generally examine a disease with one approach, such as microarray analyses to detect CNVs or exome sequencing to detect point mutations, instead of systematically trying to assess the entire genetic landscape for a patient population with multiple approaches. As a result, successive waves of studies using different techniques are difficult to relate to each other. On top of that, many of the genes identified in current studies have no known function, resulting in the construction of theoretical biological contexts that try to assess the significance of the mutations and the relation of affected genes to each other without direct data. Although such bioinformatics analyses can be helpful, the biological information is limited even for a well-studied gene such as *NRXN1*. It may be more appropriate to simply accept that gene lists are only the first step toward a genetic understanding and that conclusions about biological significance need to be postponed until the various genes have been studied.

As discussed below, *trans*-synaptic neurexin complexes have been implicated in neuropsychiatric disorders by multiple independent mutations (Figure 2). In view of space constraints and of the limitations outlined above, I will discuss below only those findings involving neurexin complexes that are linked to neuropsychiatric disorders by overwhelming evidence (Figure 6).

Neurexins

CNVs deleting expression of *NRXN1* have been significantly associated with schizophrenia, Tourette syndrome, intellectual disability, epilepsy and ASDs (Rees et al., 2014; Møller et al., 2013; Schaaf et al., 2012; Huang et al., 2017; Marshall et al.,

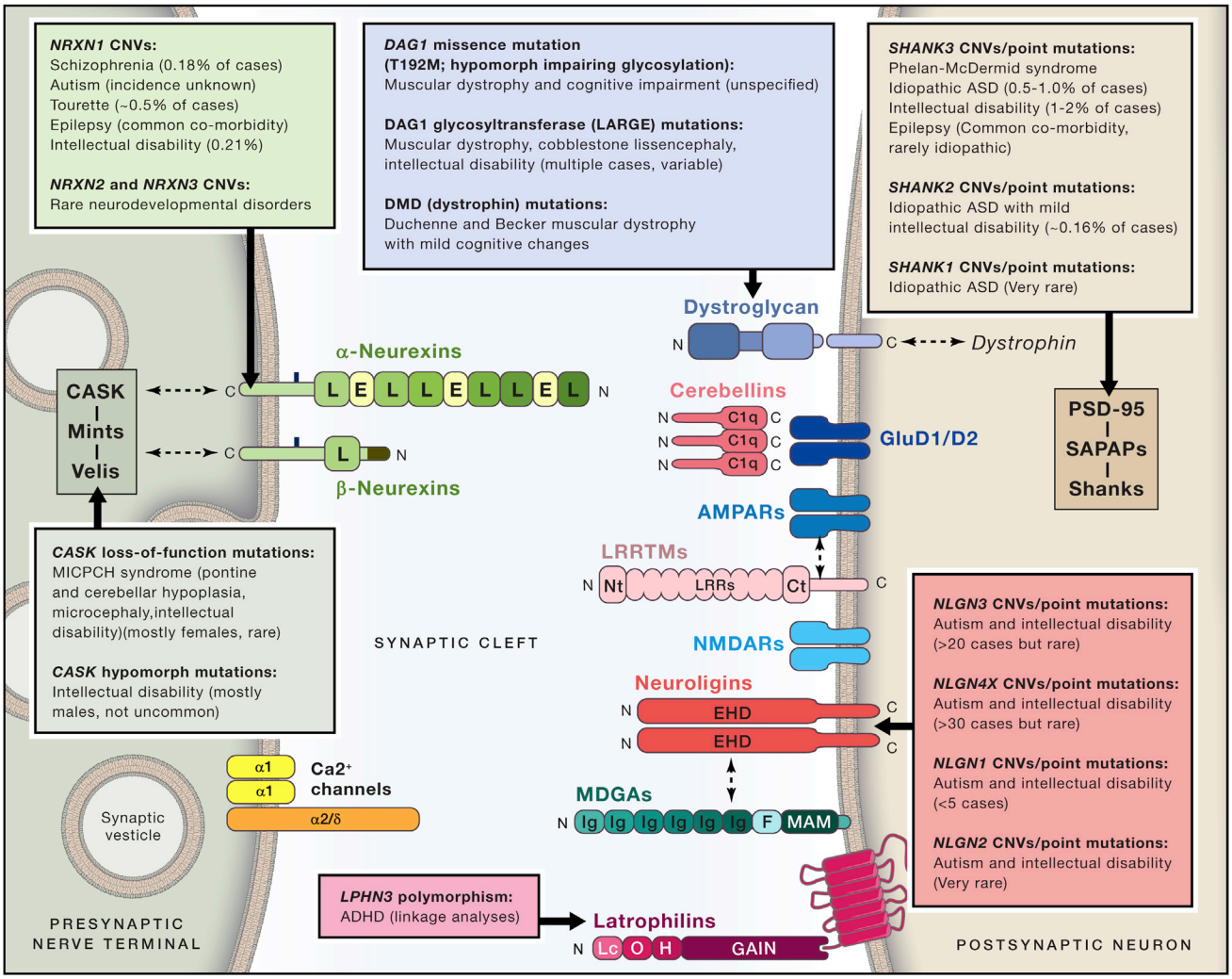

Figure 6. Mutations in Selected Genes Related to Neurexin Complexes that Are Implicated in Neuropsychiatric Disorders
Images show descriptions of the phenotypes associated with mutations in a specific gene superimposed on the schematic diagram of the neurexin-based complexes from Figure 3C. For details, see text.

2017). The large human *NRXN1* gene includes introns of more than 500 kilobases, with many repetitive elements but no known other coding or non-coding transcription units; thus, *NRXN1* gene CNVs are single-gene mutations that likely affect only NRXN1. *NRXN1* CNVs are not fully penetrant. Although *NRXN1* CNVs are extremely rare in the general population, the incidence of *NRXN1* CNVs is increased in apparently normal relatives of affected individuals (Todarello et al., 2014). Due to the constraints described above, *NRXN1* CNVs are often not classified as *NRXN1* mutations and are missed in exome sequencing studies, and the absolute incidence of NRXN1 mutations is incompletely understood. Hundreds of cases were described; in schizophrenia and Tourette syndrome, NRXN1 CNVs appear to account for 0.18% and 0.5% of cases (Rees et al., 2014; Huang et al., 2017), suggesting that based on the overall incidence of these disorders worldwide, the NRXN1 CNV patient population exceeds 100,000. In addition to *NRXN1* CNVs, rare mutations have been observed in *NRXN2* and *NRXN3* genes (Gauthier et al., 2011; Vaags et al., 2012).

The puzzling diversity of clinical presentations associated with *NRXN1* CNVs, and occasional presence of such CNVs in individuals without an apparent disorder, raised the question of whether such CNVs, as heterozygous *NRXN1* loss-of-function mutations, can credibly be considered pathophysiologically significant. This cannot be easily tested in mouse neurons because a particular gene may have subtly different functions in mouse and human neurons, and gene dosage effects may differ. One approach to address this problem is to generate conditional mutations in human neurons that resemble mutations observed in patients. In this manner, different from analyzing neurons derived from iPS cells that were isolated from patients, a mutation is studied in isolation independent of the patients' genetic background and of the clonal variation of iPS cells that can introduce significant experimental bias.

In human neurons carrying conditional heterozygous *NRXN1* loss-of-function mutations, heterozygous NRXN1 inactivation produced a significant synaptic impairment and caused an increase in the levels of CASK, which binds to the cytoplasmic sequences of Nrxn1 (Pak et al., 2015). The nature of the synaptic impairment suggested a dysregulation of neurotransmitter release, not a global loss of synaptic strength. These results do not explain why patients become ill and why they manifest with such a diverse range of presentations, but they confirm the pathological significance of the *NRXN1* CNVs.

Mouse models, finally, have also shed light on the importance of *NRXN1* mutations (Figure 5). Homo- and heterozygous Nrxn1α KO mice exhibited an array of behavioral impairments, including impaired nest building, decreased pre-pulse inhibition, and social interaction deficits (Etherton et al., 2009; Grayton et al., 2013; Dachtler et al., 2015; Esclassan et al., 2015), that may be related to neuropsychiatric disorders and support the notion that *NRXN1* deletions in human patients significantly contribute to the development of symptoms.

Neuroligins

Although mutations in *NLGN3* and *NLGN4* were the first to be linked to idiopathic autism, they are less common than *NRXN1* CNVs, with 50–100 cases reported, but seem to be more penetrant (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). *NLGN1* mutations are even rarer, with only five missense mutations and some CNVs reported to date (Nakanishi et al., 2017), and only a single report describes a family with mutations in *NLGN2* (Parente et al., 2017). Moreover, although neuroligin mutations are not associated with a specific syndrome but a range of clinical presentations, this range is narrower and involves ASDs more often than the range of presentations associated with *NRXN1* mutations.

Neuroligin mutations have not yet been studied in human neurons, but are arguably the ASD-associated mutations that have been best characterized in mouse models (Figure 5). Studies of knockin (KI) mice carrying the NLGN3 R451C point mutation that was identified in brothers with ASDs (Jamain et al., 2003) revealed dramatic synaptic and behavioral impairments, confirming the significance of these mutations (Tabuchi et al., 2007). Behaviorally, it is difficult to relate mouse models directly to ASDs because core features of ASDs—such as restricted interests, difficulty in communicating at verbal and non-verbal levels, and stereotypic activities—are not directly translatable to mice. One approach to circumnavigate this intrinsic problem without anthropomorphizing mice is to examine mouse behaviors that are not direct correlates of ASD-linked impairments but can be considered proxies for ASD-relevant behaviors. Using this approach, Nlgn3-R451C KI mice (the first neuroligin-mutant mice examined) exhibited a number of behavioral abnormalities that might serve as ASD proxies, such as impairments in social behaviors and increased ability to learn a repetitive motor task in a rotarod assay, and that were correlated with physiological impairments (Tabuchi et al., 2007; Pizzarelli and Cherubini, 2013; Rothwell et al., 2014; Jaramillo et al., 2014; Speed et al., 2015; Burrows et al., 2015, 2017; Burrows et al., 2017; note that one study did not detect major behavioral changes apart from an improved performance on the rotarod [Chadman et al., 2008]).

However, it was observed that most, but not all, behavioral and physiological phenotypes of Nlgn3-R451C KI mice differed dramatically from those of *Nlgn3* KO mice, demonstrating that the R451C mutation produces a gain-of-function effect (Etherton et al., 2011a). For example, in synapses formed by CCK+ interneurons on CA1 pyramidal neurons of the hippocampus, the Nlgn3 KO and the *Nlgn3* R451C KI caused the same disinhibition of tonic endocannabinoid signaling. In contrast, in adjacent synapses formed by Pv+ interneurons on the same neuron, the *Nlgn3* KO produced no phenotype, but the R451C almost blocked synaptic transmission (Földy et al., 2013). A common behavioral phenotype of *Nlgn3* KO and *Nlgn3* R451C KI mice was observed in their increased performance on a repetitive motor learning task in the rotarod assay (Rothwell et al., 2014), a phenotype that the *Nlgn3* mutant mice shared with *Nrxn1* mutant mice (Etherton et al., 2009). This gain-of-function phenotype of *Nlgn3* loss-of-function mutations was caused by a specific synaptic impairment in a particular circuit, namely a decrease in inhibitory synaptic inputs in the N. accumbens of the striatum, which led to a shift in the inhibitory/excitatory balance on striatal neurons (Rothwell et al., 2014). Indeed, artificially changing the excitability of these neurons in a manner mimicking the decrased inhibitory/excitatory balance reproduced the gain-of-function motor learning phenotype, confirming the circuit mechanism of this particular behavioral ASD proxy. In addition to these studies, Nlgn1 KO mice exhibited major behavioral changes, including increased repetitive behaviors (Blundell et al., 2010). Moreover, recent elegant studies on Nlgn1 KI mice with an ASD-associated point mutation (P89L) revealed a similarly dramatic behavioral phenotype that further supports the notion that diverse neuroligin mutations may, by altering synapses, predispose affected individuals to ASDs and related neuropsychiatric disorders (Nakanishi et al., 2017).

CASK

CASK binds to neurexins via its central PDZ-domain that is part of its MAGUK domain set and is preceded N-terminally by an unusual N protein kinase (Hata et al., 1996; Mukherjee et al., 2008). CASK phosphorylates neurexins, and heterozygous *NRXN1* deletions in human neurons cause an increase in CASK levels (Pak et al., 2015), suggesting that CASK physiologically interacts with neurexins. Hundreds of mutations in the X-linked CASK gene have been reported, with a range of symptoms (Hackett et al., 2010; Moog et al., 2015). Pathogenic loss-of-function variants are observed in females suffering from microcephaly with pontine and cerebellar hypoplasia (MICPCH); presumably, the same mutation would be lethal in hemizygous male patients. Less severe CASK mutations, with a higher incidence, represent one of the most frequent causes of X-linked intellectual disability in male patients (Hackett et al., 2010).

Latrophilins

Different from neurexins, neuroligins, and CASK, the association of latrophilins with neuropsychiatric disorders, or more specifically of LPHN3 with attention deficit/hyperactivity disorder (ADHD), is based not on larger deletions or coding-region mutations, but rather on linkage analyses in multigenerational families and in populations (Arcos-Burgos et al., 2010). The association of LPHN3 with ADHD was independently replicated (Ribasés et al., 2011; Bruxel et al., 2015), and functional

analysis of polymorphisms used for the linkage studies revealed a disease-associated sequence polymorphism in a conserved enhancer element that decreased LPHN3 expression ~40% (Martinez et al., 2016). Although it is unclear how decreases in Lphn3 expression predispose to ADHD (indeed, the function of Lphn3 has not yet been explored), collectively, these findings suggest a paradigmatic mechanism by which mutations in non-coding regions are linked to neuropsychiatric disorders.

Outlook: Neurexins in the Molecular Logic of Neural Circuits

Even after winnowing down candidates based on rigorous evidence, tens of genes encoding thousands of protein variants are likely essential for normal synapse formation, a broad term as described in Figure 1B. Can we envision a synthetic approach that integrates all of these molecules into a single concept? Based on current data, as outlined above, we suggest to abandon two popular paradigms in biology that seem useless for understanding synapse formation: the notion of central master regulators, for example like synaptotagmins in neurotransmitter release (Südhof, 2013), or the concept of a hierarchy of signaling molecules, for example like the MAP kinase cascade. Instead of trying to adopt and adapt these paradigms, we suggest that diverse molecules act in parallel "slots" to contribute different facets to the overall process of synapse formation, often independent of each other. This concept suggests that we can dissect different facets of synapse formation separately and that simply looking at whether or not a synapse forms normally is insufficient. We need catalogs of molecules and their mechanisms of action, which involves a comprehensive and quantitative analysis of synaptic proteins and not simply a study of the downstream effects of their loss of function.

There is evidence in support of this concept of parallel machines in synapse formation for neurexins, which contribute different functions depending on which specific neurexin genes and splice isoforms are expressed, isoforms that in turn program different types of interactions. The large effects produced by altering expression of specific neurexin variants on diverse synapse properties, such as postsynaptic receptor content or presynaptic release probability, illustrate the profound control of synaptic transmission by these molecules, effects that dramatically alter the properties of circuits in which the corresponding synapses reside.

However, the fact remains that at present, we do not yet understand how some of the most fundamental synapse properties are determined. For example, how does a postsynaptic neuron determine, after a synapse's formation is initiated, whether to recruit excitatory or inhibitory neurotransmitter receptors to the postsynaptic specialization, and how is the receptor composition specified? Whereas the work on neurexins provided a partial answer to the second question, no answer for the first question is available. Although it is known that the homologous Nlgn1 and Nlgn2 molecules that both bind to neurexins are specifically targeted to excitatory and inhibitory synapses, it is unknown by what mechanism this targeting occurs. These molecules do not actually mediate recruitment of excitatory or inhibitory receptors as such because their deletions cause only partial phenotypes in these recruitments, but they critically contribute to the functioning of these synapses.

To meet the challenges of understanding synapse formation in the broad sense defined in Figure 1, we propose that synapse formation is mediated by multiple molecular machines that operate in parallel, with and without lateral interactions. The diversity of these machines that is required to encode the diversity of synapses arises from combinatorial expression of a limited number of genes and their different alternatively spliced mRNAs. Neurexin-based complexes are a central component of many of these machines, but others are likely equally important. Unraveling the action of these machines will require a fundamental understanding of their composition and interactions, an understanding that will advance insight not only into how synapses are built and conditioned in neural circuits, but also into how synapse function is compromised in neuropsychiatric diseases.

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