

Review

On the Role of Glutamate in Presynaptic Development: Possible Contributions of Presynaptic NMDA Receptors

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Abstract: Proper formation and maturation of synapses during development is a crucial step in building the functional neural circuits that underlie perception and behavior. It is well established that experience modifies circuit development. Therefore, understanding how synapse formation is controlled by synaptic activity is a key question in neuroscience. In this review, we focus on the regulation of excitatory presynaptic terminal development by glutamate, the predominant excitatory neurotransmitter in the brain. We discuss the evidence that NMDA receptor activation mediates these effects of glutamate and present the hypothesis that local activation of presynaptic NMDA receptors (preNMDARs) contributes to glutamate-dependent control of presynaptic development. Abnormal glutamate signaling and aberrant synapse development are both thought to contribute to the pathogenesis of a variety of neurodevelopmental disorders, including autism spectrum disorders, intellectual disability, epilepsy, anxiety, depression, and schizophrenia. Therefore, understanding how glutamate signaling and synapse development are linked is important for understanding the etiology of these diseases.

Keywords: glutamate; NMDA; presynaptic; synaptogenesis; synapse formation

1. Introduction

The molecular and cellular mechanisms by which genes and the environment interact to control circuit formation and refinement remain incompletely understood. While it is well established that sensory and neuronal activities induce structural remodeling of postsynaptic spines, axonal boutons also undergo activity-dependent growth and remodeling. Less is known about this process, but recent studies have provided important new insight. Therefore, this review will focus on activity-dependent control of presynaptic terminal development.

Pioneering work from a number of laboratories has begun to identify the cellular events in presynaptic terminal formation. In general, presynaptic terminal formation is induced by axo-dendritic contacts [1–6]. Upon stabilization of an axo-dendritic contact, synaptic vesicle (SV) and active zone (AZ) proteins are accumulated at the site of contact [1–6]. Recruitment of presynaptic proteins and structures begins rapidly, with the first components arriving on a time-scale of minutes [7,8]. In addition, specialized presynaptic structures, such as the AZ and a cluster of SVs are established at the site of contact (Figure 1A). Formation of the AZ is thought to be initiated by the fusion of AZ protein transport vesicles with the axonal surface [9]. SVs form within the nascent terminal or are acquired from preassembled clusters of SVs that are mobile within axons [7,10,11]. As a bouton continues to develop, the number of SVs within the terminal increases and the AZ expands, requiring continual recruitment of SV and AZ proteins [12,13].

Recently, we found that NMDA receptors (NMDARs) bi-directionally regulate the accumulation of SV and AZ proteins at nascent excitatory presynaptic terminals [14]. We also observed this NMDAR-dependent regulation at presynaptic terminals that did not have NMDAR-expressing postsynaptic partners (Figure 1B), and at terminals of individual neurons with impaired vesicular glutamate release in an otherwise active network (Figure 1B and [14,15]). These observations led us to hypothesize that presynaptic terminal development is facilitated by the activation of NMDARs in a cell-autonomous manner, without the need for retrograde signaling from postsynaptic partners (Figure 1C). It is not yet clear where the relevant NMDARs are localized, but one intriguing possibility is that glutamate regulates presynaptic development through local activation of presynaptic NMDARs (preNMDARs). Below, we will summarize our current understanding of preNMDARs and discuss their potential role in presynaptic development.

2. General Properties of NMDA Receptors

NMDARs are ionotropic glutamate receptors. Activation of NMDARs, and in particular calcium influx through their channels, plays critical roles excitatory synaptic transmission and synaptic plasticity. NMDARs are tetramers containing two GluN1 subunits co-assembled with GluN2 and/or GluN3 subunits. There are four GluN2 subunits (A through D) and two GluN3 subunits (A and B). While GluN1 is required for assembly of functional receptors, GluN2 subunits are necessary for glutamate-dependent activation: glutamate binds in the pocket created by the GluN2 amino terminal extracellular domain and the extracellular loop. Receptors that contain GluN2A and/or GluN2B subunits are typically blocked by magnesium unless they are activated in conjunction with depolarization, while incorporation of GluN2C/D or GluN3A/B subunits confers a low sensitivity to blockade by magnesium [16,17].

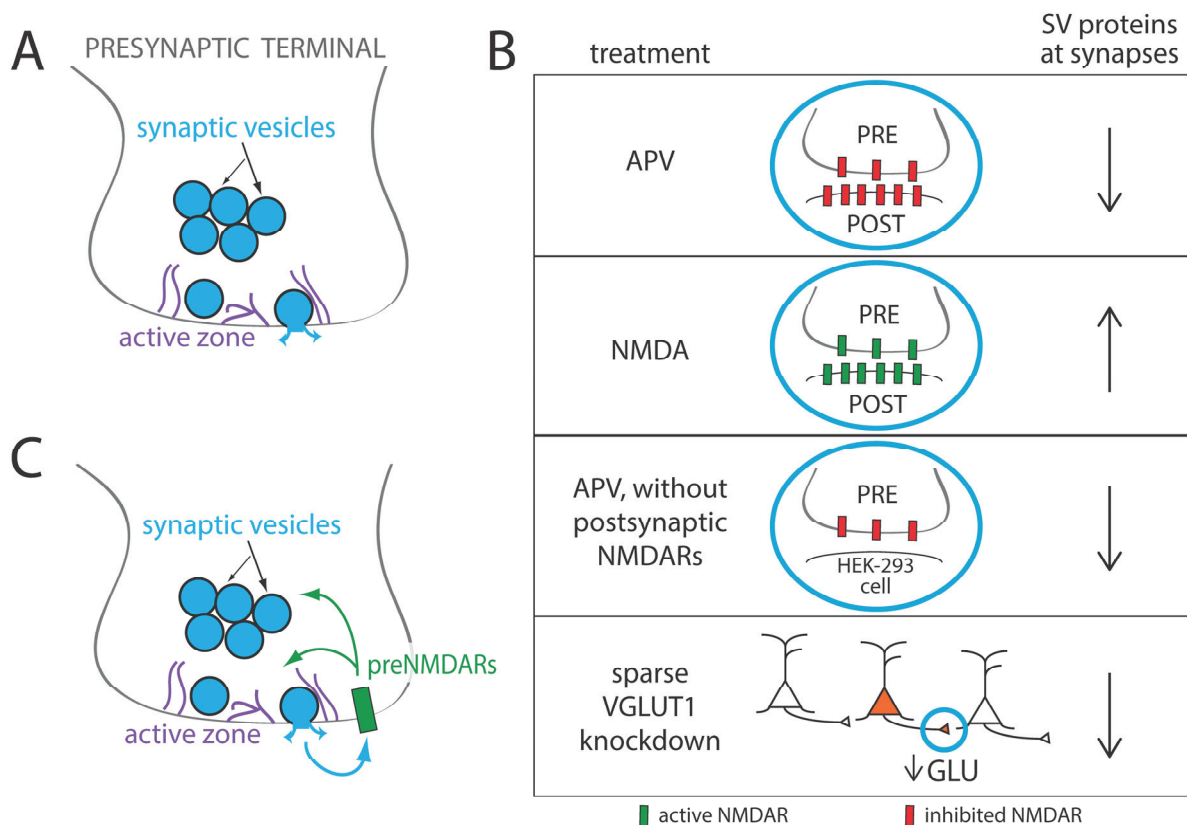


Figure 1. (A) Presynaptic terminals (or boutons) are specialized structures that form within axons at sites of synaptic contact with postsynaptic partners. Presynaptic terminals contain clusters of small synaptic vesicles that are loaded with small molecule neurotransmitters, such as glutamate and GABA. When an action potential invades the presynaptic terminal, synaptic vesicles fuse with a region of the plasma membrane known as the active zone, and a neurotransmitter is released into the synaptic cleft where it can bind to its receptors. Synaptic transmission occurs upon binding of receptors within the postsynaptic membrane; however, presynaptic neurotransmitter receptors also exist. The probability that a vesicle fuses in response to an action potential is known as the probability of release, and the postsynaptic response induced by fusion of a single vesicle is referred to as a miniature excitatory postsynaptic current, or mEPSC; (B) Presynaptic development is regulated by NMDA receptor activation, even when presynaptic terminals are formed with postsynaptic partners that lack NMDA receptors. Treatment of cortical neurons with APV, an NMDA receptor antagonist, reduced accumulation of synaptic vesicle and active zone proteins at developing synapses, while treatment with the NMDA receptor agonist NMDA did the opposite [14]. When cortical neurons formed presynaptic terminals with non-neuronal HEK-293 cells that express neuroligin but not NMDARs, treatment with APV reduced synaptic vesicle protein content at these terminals. When VGLUT1 was knocked down in individual cortical neurons in culture to reduce synaptic glutamate release, presynaptic terminals formed by those neurons had reduced synaptic vesicle protein content. This indicates that presynaptic development is regulated by glutamate release in a cell-autonomous manner [15]; (C) Based on these observations, we hypothesize that presynaptic NMDA receptors may facilitate presynaptic development.

GluN2B (also known as GRIN2B or NR2B) is essential for proper neural development. During development, GluN2B is widely expressed and the predominant GluN2 subunit in the cortex and hippocampus. GluN2B homozygous knockout mice die around birth, and genetically swapping GluN2B with GluN2A results in poor survival, reduced NMDAR current amplitude, abnormal homeostatic plasticity, and abnormal social behavior [18]. At around 2–3 weeks postnatal, NMDARs in the cortex and hippocampus undergo a developmental switch in their subunit composition: GluN2B levels plateau, while GluN2A levels dramatically increase, particularly within postsynaptic densities [19]. In the adult, GluN2B is expressed specifically in the forebrain, where it is found in most cortical neurons, while GluN2A is expressed throughout the central nervous system (CNS) [20].

GluN2C/D and GluN3A/B subunit expression is also temporally and spatially regulated. In the rodent cerebral cortex, GluN2D is expressed during postnatal development, but only at modest levels when compared to the brainstem [21]. GluN2C expression is first detected during the first postnatal week, and its expression is high in the adult cerebellum [19]. GluN3A is expressed at high levels throughout the forebrain, midbrain, and cerebellum during postnatal development but is down-regulated in adults, while GluN3B expression is highest in adults [17].

Activation of NMDARs requires binding of a co-agonist, either glycine or D-serine, to the GluN1 subunit [22,23]. There is abundant evidence that supports a role for endogenous glycine and D-serine in regulating NMDAR function *in vivo* [22,23]. Recent evidence suggests that whether particular NMDARs utilize glycine or D-serine may be dependent on the type of synapse examined [24] or the localization of the receptors [25]. Both D-serine and glycine are already found in the CNS at birth and remain present throughout postnatal development [24,26–29]; therefore, either or both could be important for NMDAR activation at developing synapses.

3. Regulation of Presynaptic Development by Glutamate and Synaptic Activity

The role of glutamate and glutamate receptors in presynaptic development is both complex and controversial. Although presynaptic terminals can form in the absence of synaptic transmission [30,31], it is clear that activity shapes synapse development. Activity-dependent changes in synapse density and morphology and the mechanisms of those changes appear to vary with: (i) developmental age; (ii) duration of treatment; (iii) how activity is blocked (e.g., blocking action potentials *vs.* all synaptic activity); (iv) whether activity is modified globally or locally; and possibly (v) neuron type [14,32–39].

In developing neurons, the reported effects of synaptic activity on morphological development of presynaptic terminals have been variable. In one study, when synaptic glutamate release was severely impaired due to knockout of VGLUT1, presynaptic terminals contained fewer SVs and levels of SV proteins were reduced [40]. On the other hand, synaptic silencing increased AZ length [31]. Mice which lack transmitter release due to the genetic knockout of Munc18-1, a protein that is essential for synaptic vesicle exocytosis, have decreased synapse density, a smaller percentage of synapses with docked SVs, and a reduced number of SVs per synapse [41]. Blocking action potential-driven activity with TTX increased the size of the readily releasable pool of SVs in young (8–9 DIV) neurons [32]. It is worth noting that in each of these studies, observed changes could have been a result of overall changes in network excitability.

In general, in more mature neurons (at least 14 DIV for cultured neurons or postnatal day 14 *in vivo*), the chronic blockade of action potential (AP) driven synaptic activity throughout the network results in an increase in probability of release and mEPSC frequency without a change in synapse density [33,35,36,42–46]. In contrast, decreasing AP generation in individual neurons decreases mEPSC frequency and the size and density of presynaptic terminals formed with the silenced neuron [47], though it is not known how silencing affects the presynaptic terminals of the silenced neuron.

Recently, we showed that knockdown of VGLUT1 in individual cortical neurons results in cell-autonomous reductions in the synaptic expression of both SV and AZ proteins [15]. In this case, it is unlikely that the observed effects were due to changes in network activity since VGLUT1 was knocked down in less than 1% of neurons. In addition, it is unlikely that these effects were due to altered action potential generation in either the knockdown neuron or its postsynaptic partner since (i) loss of VGLUT1 selectively affects glutamate release from the presynaptic terminals of the knockdown neuron and (ii) the postsynaptic neurons still receive normal input from the vast majority of their presynaptic partners. This observation points to a specific role of glutamate signaling during development of presynaptic terminals. These data are also consistent with the hypothesis that presynaptic glutamate receptors are involved in this regulation.

Interestingly, activity-dependent changes in synapse development and plasticity can be driven by spontaneous synaptic drive, independent of action potentials [48–53]. For example, control of synapse development by neuroligin and LRRTM2 depends on neuronal activity [54,55], and spontaneous activity is sufficient [38]. In developing neurons, blockade of spiking leads to either a reduction or no change in synapse density, possibly dependent on the duration of treatment [14,32,33], while blockade of both spontaneous and evoked transmitter release in young neurons appears to consistently cause a decrease in synapse density [41,56]. Interestingly, spontaneous and evoked glutamate release can activate distinct populations of NMDA receptors, even at the same synapses [57]. They can also drive different downstream signaling pathways that lead to unique effects on animal behavior [37,38,48,58,59].

4. Regulation of Synapse Formation by NMDA Receptors

Most studies on the effects of NMDARs on synapse formation and plasticity have been performed on relatively mature neurons, after the peak of synaptogenesis. In general, studies of mature synapses have shown that NMDAR blockade tends to decrease spine density or shift spine morphology to more filopodial-like structures [39,43,60–67], but this may not always correlate with a decrease in presynaptic terminal density [39,64]. Importantly, several reports suggest that NMDAR activity is important for presynaptic development and maturation [14,39,63,67–69]. At retino-collicular synapses, NMDAR blockade from birth increases synapse density in 6–10 day old animals but not in 14 day olds, while NMDA decreases synapse density at P14 but not P8 [61]. In young, cultured cortical neurons, 24–48 h exposure to NMDAR antagonists results in a reduction in the amount of SV and AZ proteins at individual synapses, while activation of NMDARs does the opposite [14]. In this case, no changes in synapse density are observed.

Differences in NMDAR-dependent regulation of presynaptic terminal development might arise from developmental changes in synaptogenic mechanisms. For example, immature synapses are highly sensitive to actin disruption, while mature synapses are resistant to actin-depolymerizing drugs [70].

Similarly, disruption of adhesive properties of cadherins leads to instability of immature, but not mature, synapses [71,72]. Finally, immature presynaptic terminal activity and synapse elimination are sensitive to protein synthesis inhibitors, while mature presynaptic terminals are fairly resistant to protein synthesis inhibitors [72].

NMDARs could signal cell-autonomously to alter presynaptic terminal development. Alternatively, activation of NMDARs on postsynaptic neurons could induce the release of a retrograde signal that in turn acts on the presynaptic partner to regulate its development. With regard to cell-autonomous effects of NMDARs, one report showed knockdown of GluN1 decreased mEPSC frequency in the hippocampus, and this was not dependent on NMDAR activity [60]. On the other hand, another study showed that Cre-mediated elimination of GluN1 in the cortex did not alter mEPSC frequency within GluN1-lacking neurons [39]. Both studies were performed on neurons >14 DIV, and neither study examined the effects of cell-autonomous removal of NMDARs on presynaptic development in GluN1-deficient neurons.

5. Presynaptic NMDA Receptors

The role of presynaptic ionotropic glutamate receptors at glutamatergic synapses has only recently been appreciated. Presynaptic NMDA receptors were first identified in cortical presynaptic terminals by electron microscopy by Aoki and colleagues in 1994 [73], while the first physiological evidence for preNMDARs in the cerebral cortex appeared in 1996 [74].

Although many studies report only postsynaptic NMDARs, both immunoperoxidase and immunogold electron microscopy studies have demonstrated NMDAR subunit immunoreactivity in axons and presynaptic boutons at a variety of synapse and neuron types. Several studies have used immuno-electron microscopy to demonstrate that NMDARs can be observed in presynaptic terminals in the cortex [73,75–78] and hippocampus [78–81], although there is some inconsistency in the details of (i) whether they are present at excitatory synapses; (ii) where they are localized within terminals; and (iii) how abundant this labeling is [75–82].

Additional evidence supports the existence of preNMDARs. Using ultrasynaptic fractionation, we showed that presynaptic NMDARs that contain GluN1 and GluN2B are present within the AZ membrane [83], where SV fusion and subsequent glutamate release occurs. At the light microscopic level, NMDARs have been examined in axonal growth cones of young hippocampal neurons, with varying results [84–87]. Using fluorescence imaging, we recently demonstrated that GluN1 is clustered at many (but not all) presynaptic terminals of cultured cortical neurons [83]. GluN1 puncta occur at a lower density in axons than dendrites [83] and are smaller and dimmer than dendritic GluN1 puncta in the same neuron. Presynaptic clustering of GluN1 occurs early in synapse development [83]. The early appearance of GluN1 at presynaptic terminals is consistent with a role for presynaptic NMDARs in synapse development.

In developing neurons, NMDARs located within presynaptic neurons are responsible for acute regulation of presynaptic plasticity. NMDARs in presynaptic neurons are essential for LTP at cortico-striatal synapses [88]. Within the cortex, activation of NMDARs in presynaptic neurons can facilitate spontaneous and evoked neurotransmitter release and is required for induction of timing-dependent LTD and pattern-dependent LTD [74,76,89–94]. The responsible receptors appear to contain GluN2B and GluN3A [21], but their subcellular localization was unclear until recently.

Recent evidence suggests that, at least at some synapses, preNMDARs are indeed responsible for presynaptic plasticity. An elegant study showed that axonal, but not dendritic, NMDARs are necessary for the induction of timing-dependent LTD in cortical layer 4-layer 2/3 synapses [93,94]. These effects of presynaptic NMDARs are observed at layer 4-layer 2/3 synapses but not at layer 2/3-layer 2/3 synapses formed with the same postsynaptic neuron [94]. In the cerebral cortex [91] and hippocampus [79], calcium transients can be imaged in presynaptic boutons upon focal uncaging of NMDA or glutamate. In addition, activation of NMDARs in a given neuron selectively regulates glutamate release at a subpopulation of synapses formed by that neuron [91]. This observation argues that the relevant NMDARs are activated locally, at or near presynaptic terminals.

Interestingly, preNMDARs appear to be restricted to a subset of synapses formed by the same neurons. In the cerebral cortex, layer five pyramidal neurons form presynaptic terminals with a range of postsynaptic partners, including other cortical pyramidal neurons, somatostatin-positive Martinotti cells (a type of GABAergic interneuron), and parvalbumin-positive basket cells (another type of GABAergic interneuron). Buchanan and colleagues showed that synapses formed between two pyramidal neurons and those formed between pyramidal and Martinotti neurons possess functional presynaptic NMDARs [91]. However, functional preNMDARs were only found at a subset of synapses formed between pyramidal neurons and parvalbumin-positive GABAergic neurons. Those synapses that appeared to have preNMDARs were formed with a distinct subtype of parvalbumin-positive cells that were defined by a unique axonal morphology [91].

The down-stream mechanisms by which preNMDARs regulate presynaptic plasticity are not yet clear. PreNMDARs can pass calcium and some of their roles may depend on this calcium influx [74,79,91,95–98]; however, this is not always the case (Figure 2). Surprisingly, preNMDAR-dependent facilitation of spontaneous neurotransmitters does not require calcium influx from extracellular space or the release of calcium from intracellular stores [99]. It does depend on extracellular sodium, probably to depolarize the presynaptic terminal, but other roles for sodium influx are possible [99]. In addition, preNMDAR-dependent regulation of spontaneous glutamate release depends on protein kinase C (PKC), but the relevant phosphorylated target(s) of PKC have not yet been identified [99].

Presynaptic regulation of LTD may utilize distinct down-stream mechanisms (Figure 2). Timing-dependent LTD appears to depend on postsynaptic metabotropic glutamate receptors, coincident activation of presynaptic CB1 receptors and preNMDARs, and astrocyte release of glutamate [89,100–102]. In contrast, pattern-dependent LTD does not require metabotropic glutamate receptors, CB1 receptors, glia, postsynaptic calcium, or G-protein signaling [103]. However, pattern-dependent LTD does depend on the activity of presynaptic calcineurin [103].

Since NMDARs require a co-agonist for activation, it would be interesting to know whether preNMDARs are co-activated by glycine, D-serine, or both. GluN2B-containing NMDARs have a higher affinity for glycine than GluN2A-containing receptors, while GluN2B-containing NMDARs have a lower affinity for D-serine than NMDARs with GluN2A [22]. This suggests that glycine may be the preferred co-agonist of preNMDARs; however, either or both co-agonists could contribute to preNMDAR activation.

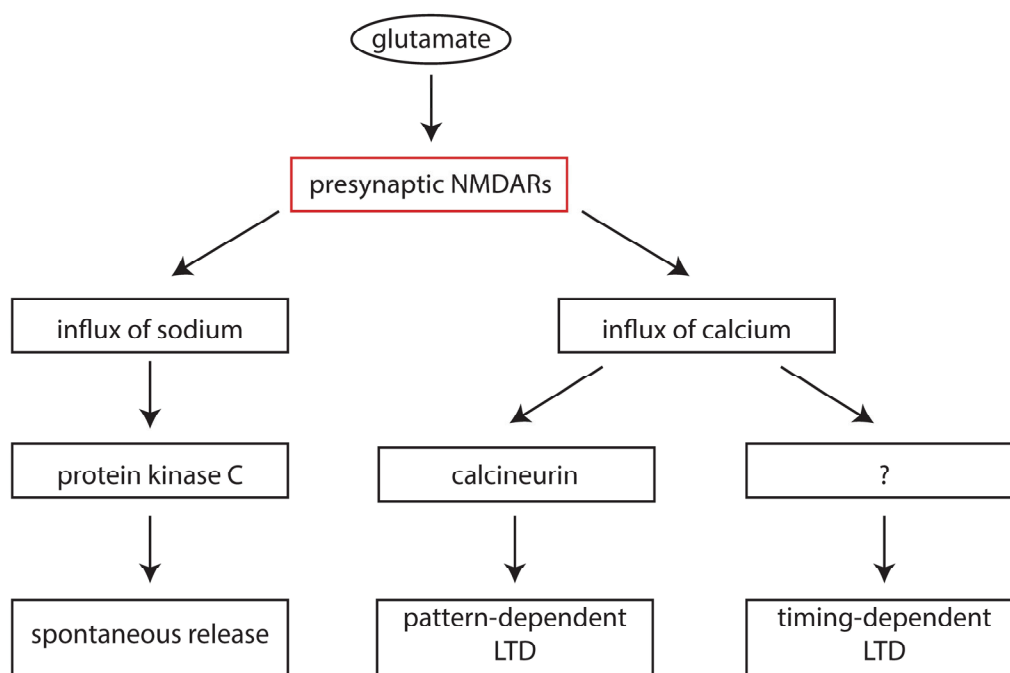


Figure 2. Signaling mechanisms downstream of presynaptic NMDA receptor activation. Presynaptic NMDARs control spontaneous glutamate release, timing-dependent LTD and pattern-dependent LTD through three distinct signaling pathways. Facilitation of spontaneous release does not depend on calcium influx. Timing-dependent LTD appears to require coincident activation of presynaptic CB1 receptors and glutamate release from astrocytes.

6. Presynaptic NMDA Receptors in Development

The role of axonal NMDARs appears to be developmentally regulated. NMDARs are observed in axons and axonal growth cones of hippocampal neurons during the first postnatal week, but expression in distal axons and growth cones is dramatically decreased later in postnatal development [83–87]. Similarly, ultrastructural evidence in the visual cortex indicates that the fraction of presynaptic terminals with identified NMDARs decreases significantly after the critical period for receptive field plasticity [76]. In addition, there is no apparent effect of preNMDARs on neurotransmitter release after postnatal day 23 [76], and the presence of NMDARs in biochemically isolated presynaptic membranes is reduced in mature animals when compared to young animals [83].

Since the period of highest NMDAR expression coincides with the period of intense synapse formation and an elevated probability of release has been proposed to enhance synapse formation, it has been hypothesized that activation of axonal NMDARs might facilitate synapse formation [104]. However, this hypothesis has not yet been directly tested. Formation of an individual synapse occurs over a time course of hours, and initial synaptogenesis occurs over a period lasting for several weeks in rodents, but the effects of chronic activation or inactivation of preNMDARs have not yet been studied. This is largely due to the difficulty of differentiating presynaptic and postsynaptic effects and because there is no method for selectively inhibiting, activating, or removing preNMDARs without also affecting postsynaptic NMDARs.

If preNMDARs were involved in synaptogenesis, activation of preNMDARs would provide a feedback mechanism that could ensure that functional presynaptic terminals are preferentially made

and maintained, even in the absence of postsynaptic responses. PreNMDAR-dependent regulation of presynaptic bouton development could be especially important during circuit and sensory map development since many synapses in the early postnatal cortex are postsynaptically silent [66,105,106]. In contrast, preNMDARs are thought to be active [104,107], possibly due to depolarization of the presynaptic terminal.

Recent studies have demonstrated that preNMDARs are regulated by sensory input in both the somatosensory [108] and visual [109] cortex, providing an intriguing possible link between experience and synapse and circuit development. The converse may also be true: control of presynaptic development by glutamate and NMDARs could ultimately play a role in establishing receptive field properties. For example, coincident with the period of intense synapse formation and high expression of preNMDARs in the visual cortex [76], several important aspects of visual cortical receptive fields are established, including retinotopy, ocular dominance, and orientation selectivity. Glutamatergic signaling via NMDARs contributes to the establishment of these receptive field properties [110–112].

7. Relevance to Neurodevelopmental Disease

Impaired synapse formation and elimination is thought to contribute to pathogenesis of numerous neurodevelopmental and psychiatric disorders, including autism spectrum disorders (ASD), intellectual disability (ID), epilepsy, anxiety, depression, and schizophrenia [113]. For example, ASD is typically diagnosed before age three, during a period of intense synapse formation [114,115]. Analysis of human genetics, postmortem brain histopathology, and mouse models have all pointed to a role for synapse formation in ASD pathogenesis [114,116–123]. In addition, early life emotional experience both correlates with altered synapse density [124,125] and predicts anxiety and depression later in life [126–128]. Therefore, the likelihood of developing anxiety disorders has been proposed to be established during development of neural circuits [129].

Abnormal synaptic glutamate signaling has also been proposed to contribute to ASD, ID, epilepsy, schizophrenia, and depression. Mutations in NMDAR subunits have been linked to pathogenesis of several neurodevelopmental diseases—including ASD, ID, schizophrenia, and epilepsy [116,117,130–134]. Furthermore, changes in glutamate signaling and abnormal NMDAR expression and function have been observed in the brains of ASD patients [135,136] and several distinct mouse models of ASD [137–142]. In addition, NMDAR antagonists cause cognitive and behavioral changes that mimic those observed in ASD [131,143–145]. Additionally, lead is thought to dramatically increase the risk for ID in infants and children through antagonism of NMDARs [146–150]. Together, these data suggest that disturbances in NMDAR signaling have profound effects on neuronal development in humans, but the mechanisms that link NMDARs to developmental disorders are still being elucidated. Therefore, it is essential that we understand the links between glutamate signaling and synapse development.

Here, we have focused on the role of glutamate signaling via NMDARs in the control of presynaptic development. We have proposed that preNMDARs may contribute to glutamate-dependent presynaptic development. In the future, it will be interesting to know the extent to which glutamate and preNMDAR-dependent presynaptic development is disrupted in neurodevelopmental disorders.

8. Conclusions

It is now clear that NMDARs are found within presynaptic terminals of a variety of neurons, While these receptors have been demonstrated to regulate neurotransmitter release, their developmentally-regulated expression raises the question of what role these receptors play in both normal and abnormal neuronal development. Because molecular genetic techniques are not yet available to knockout only presynaptic receptors while leaving postsynaptic receptors intact, new methods for selectively controlling the expression or activity of presynaptic receptors on timescales of days to weeks will be needed to understand the developmental role of preNMDARs. This will likely require a better understanding of which proteins selectively interact with preNMDARs and/or control their expression and localization. In addition, it will be important to further delineate the downstream signaling pathways that mediate the effects of preNMDAR activation. While there is much to be done, this represents an exciting new direction for investigation into both NMDARs and presynaptic development.

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Author Contributions

Karlie N. Fedder and Shasta L. Sabo wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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