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TOPICAL REVIEW

NMDA receptors in axons: there's no coincidence

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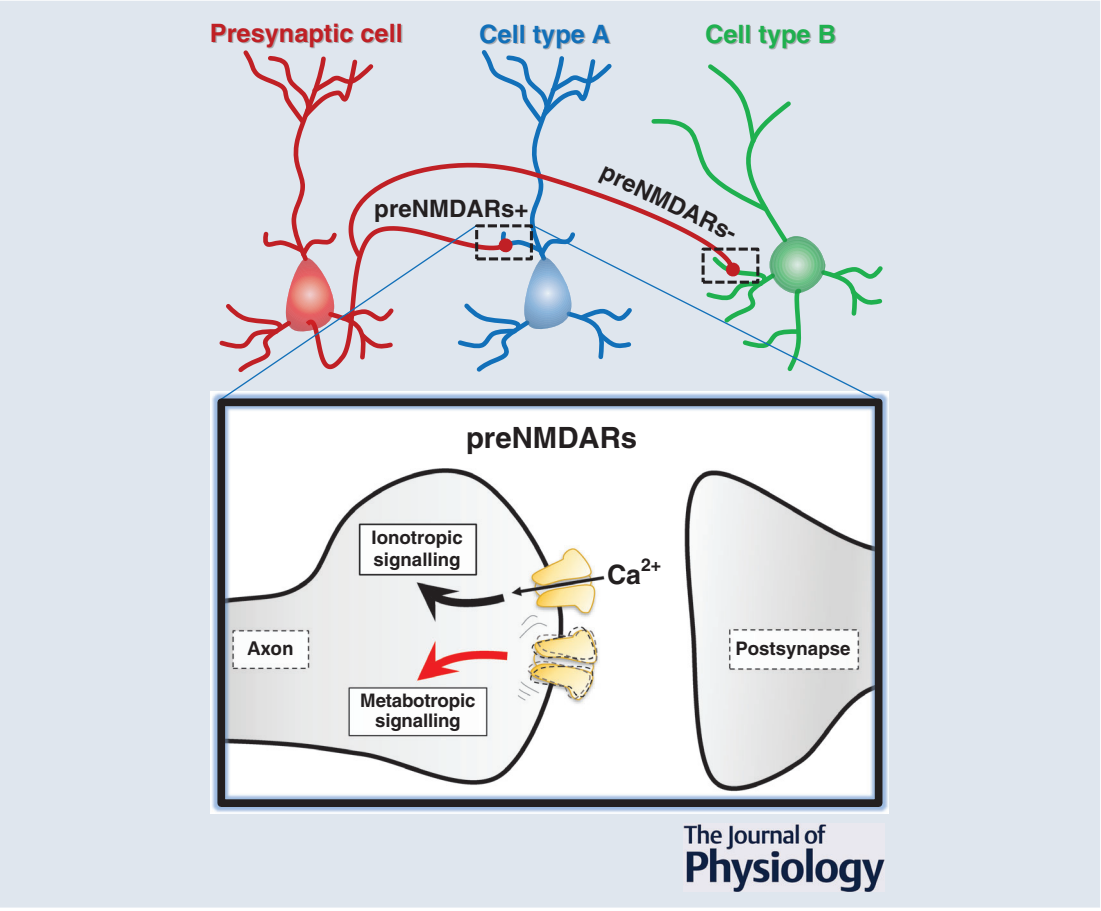

Hovy Ho-Wai Wong conducted his PhD research in Prof. Christine Holt's lab at the University of Cambridge, where he revealed how RNA trafficking and highly localized protein synthesis in axons regulate synaptogenesis *in vivo*. For his HBHL and FRQS Postdoctoral Fellowships, he joined Prof. Jesper Sjöström's lab to pursue his keen interest in presynaptic plasticity in neurotransmission. **P. Jesper Sjöström** is Associate Professor in Neuroscience at McGill University's Centre for Research in Neuroscience and holds an FRQS *Chercheur-Boursier Senior* award. His team explores plasticity in the brain using quadruple patch-clamp, two-photon microscopy, optogenetics and computer modelling. His research has unveiled plasticity learning rules, neocortical connectivity patterns and unconventional forms of NMDA receptor signalling. He is Editor-in-Chief of *Frontiers in Synaptic Neuroscience*.

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Abstract In the textbook view, *N*-methyl-D-aspartate (NMDA) receptors are postsynaptically located detectors of coincident activity in Hebbian learning. However, controversial presynaptically located NMDA receptors (preNMDARs) have for decades been repeatedly reported in the literature. These preNMDARs have typically been implicated in the regulation of short-term and long-term plasticity, but precisely how they signal and what their functional roles are have been poorly understood. The functional roles of preNMDARs across several brain regions and different forms of plasticity can differ vastly, with recent discoveries showing key involvement of unusual subunit composition. Increasing evidence shows preNMDAR can signal through both ionotropic action by fluxing calcium and in metabotropic mode even in the presence of magnesium blockade. We argue that these unusual properties may explain why controversy has surrounded this receptor type. In addition, the expression of preNMDARs at some synapse types but not others can underlie synapse-type-specific plasticity. Last but not least, preNMDARs are emerging therapeutic targets in disease states such as neuropathic pain. We conclude that axonally located preNMDARs are required for specific purposes and do not end up there by accident.

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Abstract figure legend Presynaptic NMDARs (preNMDARs) are present in axons of specific synapses to regulate different forms of neurotransmission and synaptic plasticity. PreNMDARs can signal through both ionotropic mode by fluxing calcium and metabotropic mode without calcium influx.

Introduction

In the textbook view, the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) is critical for memory formation, because it acts as a detector of coincident activity across connected neurons. According to Donald Hebb's famous postulate (Hebb, 1949), coincident activity causes information storage by strengthening neuronal connections (Sjöström *et al.* 2008; Maheux *et al.* 2016). In this classical view, NMDARs are perfectly suited as coincidence detectors in Hebbian learning because both glutamate and depolarization are required to relieve NMDARs of magnesium block so that they flux calcium, which triggers long-term potentiation (LTP) of synaptic connections (Sjöström & Nelson, 2002). As expected from their central role in Hebbian plasticity and memory formation (e.g. Nabavi *et al.* 2014b), NMDARs have also been implicated in many neuropathologies, including Alzheimer's disease and schizophrenia (Paoletti *et al.* 2013).

But to function as a Hebbian coincidence detector, NMDARs must be situated postsynaptically (Duguid & Sjöström, 2006; Bouvier *et al.* 2018), because postsynaptic NMDARs require presynaptically released glutamate and postsynaptic depolarization to open, flux calcium and trigger Hebbian learning (Sjöström *et al.* 2008; Maheux et al. 2016). Yet, neuroscientists have for decades found evidence for presynaptic NMDARs (preNMDARs) (Duguid & Sjöström, 2006; Banerjee *et al.* 2016). These preNMDARs are peculiar because both depolarization and glutamate would have to originate from the presynaptic neuron, which means they cannot function as classic Hebbian coincidence detectors (although other forms of presynaptic coincidence detection remain possible, e.g. see Humeau *et al.* 2003; Sjöström *et al.* 2003; Duguid & Sjöström, 2006). As a consequence, the functional roles of preNMDARs have been poorly understood and hotly debated (Duguid & Sjöström, 2006; Banerjee *et al.* 2016). Some have even argued that preNMDARs simply do not exist (Christie & Jahr, 2008; Christie & Jahr, 2009), although that may not be the current consensus view (Bouvier *et al.* 2018).

Furthermore, it has more recently emerged that NMDARs can also signal by a conformational change (Aow *et al.* 2015; Dore *et al.* 2015) without needing calcium influx (Nabavi *et al.* 2013). This metabotropic mode of NMDAR signalling has also been implicated in synaptic plasticity, structural plasticity, learning and disease (Kessels *et al.* 2013; Nabavi *et al.* 2013; Stein *et al.* 2015; Thomazeau *et al.* 2020), but it too is debated, because it only requires glutamate binding but not depolarization (Nabavi *et al.* 2014*a*). Like preNMDARs, metabotropic NMDAR signalling can thus not function in classic Hebbian plasticity (Dore *et al.* 2017).

Taken together, these oddities suggest that our understanding of NMDARs is incomplete and that there is much more to elucidate. Here, we focus on the preNMDAR, overviewing our current understanding of this elusive receptor type and its roles in regulating short-term as well as long-term plasticity, including how it may signal metabotropically. We argue that, although preNMDARs cannot carry out classic Hebbian coincidence detection, it is no coincidence that they are found in axonal compartments.

Subunit composition and properties of NMDARs

Before we embark on reviewing preNMDARs, we overview the basic subunit composition and properties of NMDARs. There are seven different GluN subunits – GluN1, GluN2A–D and GluN3A–B. Each NMDAR is composed of two obligatory GluN1 subunits and two GluN2/3 subunits to form a heterotetramer. When the configuration involves a pair of identical GluN2/3 subunits, a di-heterotetrameric NMDAR is formed. In contrast, a tri-heterotetrameric NMDAR is formed when two different GluN2/3 subunits are paired with the two GluN1 subunits (Stroebel *et al.* 2018) (Fig. 1*A*).

The composition of the GluN tetramers determines NMDAR properties via the differences found in the four distinct modular domains – the agonist binding domain (ABD), N-terminal domain (NTD), transmembrane domain (TMD) and C-terminal domain (CTD) (Paoletti *et al.* 2013; Stroebel & Paoletti, 2020) (Fig. 1*B*). The ion channel gating is controlled by the ABD, which is activated by glycine or D-serine in GluN1/3 subunits and by glutamate in GluN2. However, the binding of allosteric modulators to NTD further modulates gating through fine-tuning channel open probability and closing rate (Fig. 1*C*). For example, this pivotal role in ionotropic signalling is modulated positively by polyamines (e.g. spermine and spermidine) (Mony *et al.* 2011) and negatively by phenylethanolamines (e.g. ifenprodil and Ro 25-6981) in GluN2B (Karakas *et al.* 2011). Intriguingly, both GluN2A and GluN2B undergo negative allosteric modulation by zinc ions but differ vastly in sensitivity (Choi & Lipton, 1999; Rachline *et al.* 2005) – the nanomolar affinity to GluN2A and micromolar affinity to GluN2B suggest another layer of subunit-specific control.

Postsynaptic NMDARs are well-known for typically undergoing a developmental switch from GluN2B to GluN2A (Sheng *et al.* 1994; Sjöström *et al.* 2003; Abrahamsson *et al.* 2017). In some cases, however, it appears that GluN2B-containing preNMDARs do not undergo this switch. For example, in postnatal day (P) 3–5 mouse visual cortex, connections between layer-5 (L5) pyramidal cells (PCs) contain both pre- and postsynaptic GluN2B-containing NMDARs, but at P11–P16, these are only found presynaptically (Abrahamsson *et al.* 2017).

The ion channel is housed in the centre of the GluN tetramer in the TMD, which has three membrane-spanning helices (M1, M3 and M4) and a pore loop (M2) (Karakas & Furukawa, 2014). While GluN2A/2B subunits result in higher NMDAR conductance, calcium permeability and magnesium-mediated blockade sensitivity, the converse is true for GluN2C/2D (Monyer *et al.* 1992; Stern *et al.* 1992; Ishii *et al.* 1993; Kuner & Schoepfer, 1996; Wyllie *et al.* 1996; Traynelis *et al.* 2010). These properties are further lowered when GluN3 subunits are included in the NMDAR tetramer, to the point that NMDARs are no longer capable of coincidence detection (Chatterton *et al.* 2002; Pachernegg *et al.* 2012; Zhu *et al.* 2020). Several studies have reported that preNMDARs contain GluN2C/D and/or GluN3A/B subunits (e.g. Banerjee *et al.* 2009; Larsen *et al.* 2011, 2014; Andrade-Talavera *et al.* 2016). The small calcium signals elicited by such preNMDARs may help explain why some have not found preNMDAR-mediated calcium signals at certain synapses (Christie & Jahr, 2008, 2009; Carter & Jahr, 2016), whereas others have (Buchanan *et al.* 2012; Rossi *et al.* 2012; Abrahamsson *et al.* 2017).

Intracellularly, the CTD serves as a signalling hub to regulate receptor trafficking and synaptic retention (Paoletti *et al.* 2013). Given the broad functional diversity conferred by different GluN subunits alluded to above, it is perhaps not surprising that the CTDs harbour the largest sequence variation. For instance, the CTD amino acid length ranges from ∼100 bases in GluN1 to almost 650 bases in GluN2A and GluN2B in rodents (Uniprot database of protein sequence; The UniProt Consortium, 2018). The spectrum of possible subunit composition in NMDARs is further expanded by the four CTD and four NTD isoforms of GluN1, and two CTD isoforms of GluN3A through alternative splicing (Zukin & Bennett, 1995; Paoletti *et al.* 2013). Despite little is known about whether and how NMDARs are differentially localized in axons and dendrites of the same cell, the apparent postsynapse-specific developmental switch from GluN2B to GluN2A in L5 PCs (Abrahamsson *et al.* 2017) suggests that subcellular NMDAR trafficking is precisely controlled. Therefore, it is not unlikely that neuronal compartment-specific CTD isoforms of GluN subunits will be discovered in the future. Alternatively, it is possible that compartment specificity of NMDARs is not solely conferred by the CTD protein isoforms, but instead relies on other ways such as mRNA isoform trafficking and local protein synthesis at axon terminals and postsynapses (Wong *et al.* 2017; Hafner *et al.* 2019).

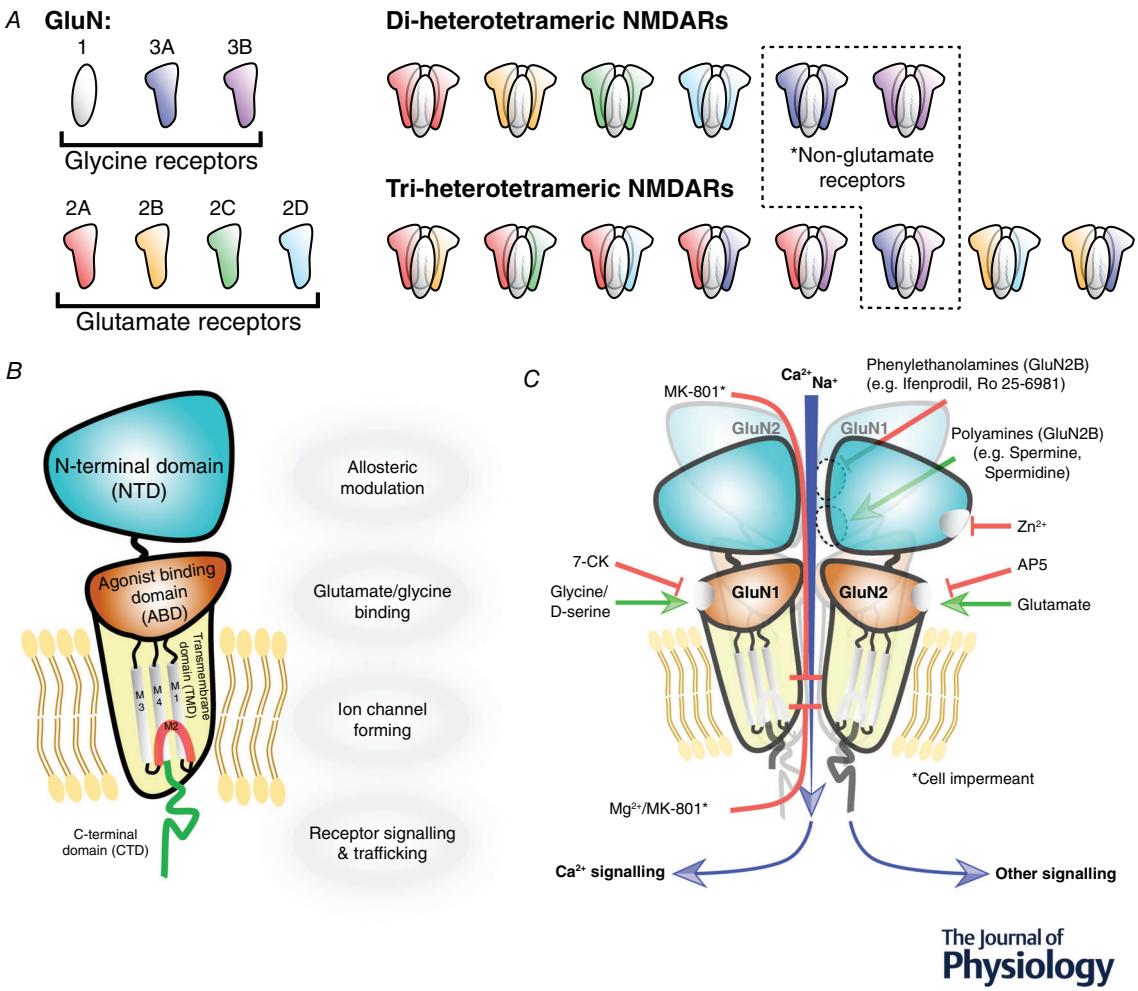

Figure 1. NMDAR composition diversity and structural domains
A, there are seven GluN subunits known to date. The GluN1/3A/3B subunits are activated by glycine and the GluN2A/2B/2C/2D subunits are activated by glutamate. Each NMDAR consists of four subunits and is hence termed 'tetrameric'. With the canonical notion that functional NMDARs result in measurable ion flux, two GluN1 subunits are thought to be obligatory in every NMDAR (Meguro *et al*. 1992; Monyer *et al*. 1992; Ishii *et al*. 1993). With the combinations of two GluN1 and two GluN2/3 subunits, NMDARs adopt a di- or tri-heterotetrameric configuration (Stroebel *et al*. 2018). As GluN1 and GluN3 are not activated by glutamate, NMDARs lacking GluN2 subunits are effectively glycine receptors (Chatterton *et al*. 2002). GluN2A/2B confers high channel conductance and magnesium-mediated blockade sensitivity, whereas GluN3A/3B results in low channel conductance and poor magnesium blockade (Pachernegg *et al*. 2012; Paoletti *et al*. 2013). *B*, each GluN subunit is made up of four structural modules – the N-terminal domain that can bind allosteric modulators, the agonist binding domain for glutamate/glycine recognition, the transmembrane domain that allows the ion channel to span the lipid bilayer of cell membrane, and the C-terminal domain that signals receptor trafficking and synaptic localization (Paoletti *et al*. 2013; Karakas & Furukawa, 2014). Agonist binding to GluN2 increases the tension of the ABD–TMD linkers to relieve ion channel gating, whereas antagonism of GluN1/2 mediates gating through relaxing the GluN2 ABD–TMD linkers (Chou *et al*. 2020). *C*, NMDAR activity can be modulated pharmacologically through multiple target sites. Competitive antagonists such as 2-amino-5-phosphonopentanoate (AP5) compete for the glutamate binding site of GluN2 subunits to inhibit NMDARs (Olverman *et al*. 1984; Jespersen *et al*. 2014). 7-Cholrokynuerenic acid (7-CK) is an antagonist that competitively inhibits the glycine/D-serine site of GluN1 subunit (Kemp *et al*. 1988). Phenylethanolamines and polyamines are allosteric modulators that bind NMDARs at the NTD interface of GluN1 and GluN2B subunits (Karakas *et al*. 2011; Mony *et al*. 2011). GluN2A and GluN2B also harbour the allosteric inhibition sites for Zn^{2+}, with binding affinity to GluN2A over three orders of magnitude higher than GluN2B (Choi & Lipton, 1999; Rachline *et al*. 2005). Uncompetitive channel blockers such as MK-801 (also known as dizocilpine) bind inside the ion channel of the NMDAR TMD (Song *et al*. 2018). As Mg^{2+} normally blocks the channel, MK-801 binding is thought to first require NMDAR activation and depolarization to release Mg^{2+} – blocking NMDARs in a use- and voltage-dependent manner. With its cell-impermeant chemical property, MK-801 has been used as both an external blocker and an internal blocker. The loading of MK-801 intracellularly allows the examination of cell-specific effects. Green arrows, positive modulation; red bar-headed lines, negative modulation.

Developmental profiles of GluN subunits

Early studies on GluN-encoding mRNA expression in rats revealed complex patterns that are developmentally regulated with brain region and GluN subunit specificity (Akazawa *et al.* 1994; Monyer *et al.* 1994; Sheng *et al.* 1994). The more recent Allen Institute initiative in profiling developmental and regional mRNA expression in mice is in broad agreement (Fig. 2). Overall, pan-GluN1 expression remains high in the brain from embryonic to adult stage, matching its obligatory role in forming functional ionotropic NMDARs. GluN2B follows a similar pattern but peaks at around 2 weeks postnatally. GluN2A and GluN2C expressions only become appreciable after around 1 week postnatally. GluN2D and GluN3B only appear to be sparsely expressed, whereas GluN3A shows a rapid developmental upregulation and peaks at around 1 week postnatally. It is worth noting that the actual functioning of these subunits is likely to be delayed due to the time required for mRNA translation, protein folding, modifications and recruitment to the sites of action. Moreover, the 2–4 days of GluN1/2B protein half-lives (Cohen *et al.* 2013; Dörrbaum *et al.* 2018) also implies some subunit-specific effects could linger even after mRNA clearance.

Unconventional NMDAR signalling

Recently, metabotropic actions that rely on structural changes without ion flux have emerged as an alternative mode of NMDAR signalling (Dore *et al.* 2017), including those found postsynaptically in hippocampal neurons (Nabavi *et al.* 2013; Aow *et al.* 2015; Dore *et al.* 2015; Stein *et al.* 2015; Thomazeau *et al.* 2020) and L2/3 barrel cortex (Carter & Jahr, 2016). However, classical determination of NMDAR subunit composition is based on the assumption of ionotropic function, and hence the experimental conclusions reflected primarily on the presence of current flux when co-expressing exogenous GluN subunits (Meguro *et al.* 1992; Monyer *et al.* 1992; Ishii *et al.* 1993) or with GluN1 genetic deletion (Carter

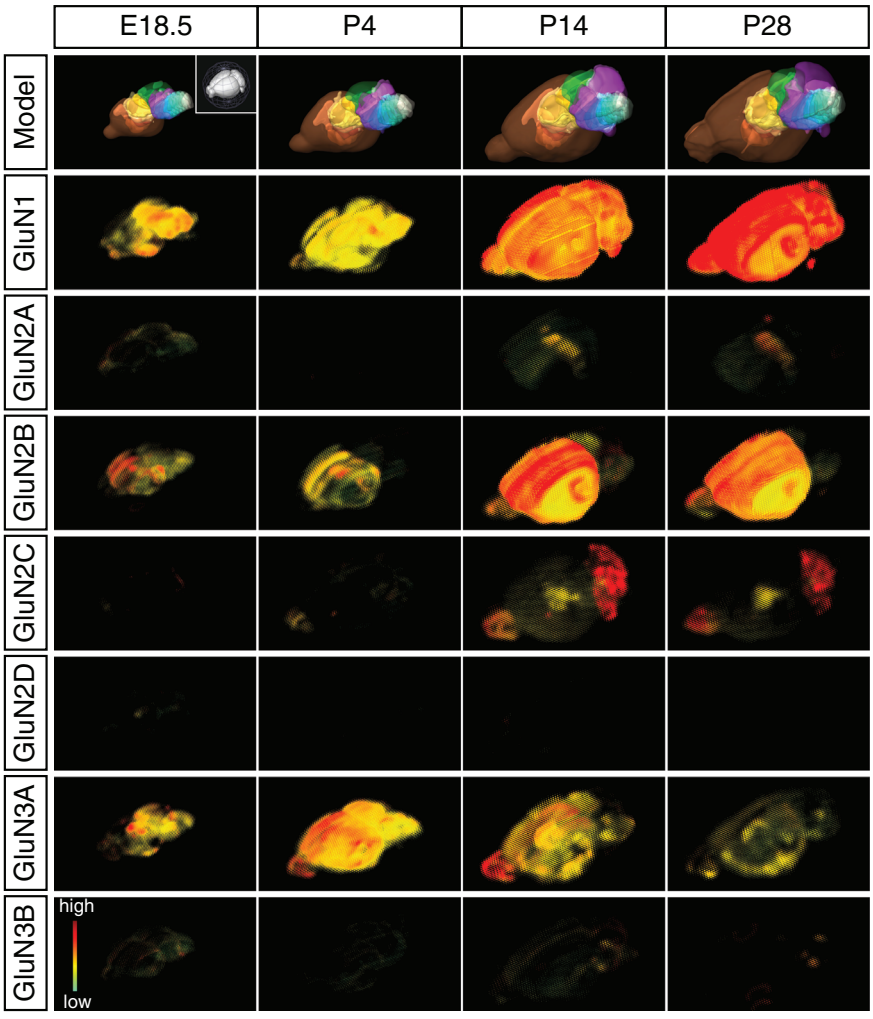

Figure 2. The expression of NMDAR subunit mRNAs is developmentally and regionally regulated
The models at top display the 3D views from the anterior-dorsolateral direction of the left hemispheres of mouse brains at embryonic day (E) 18.5, P4, P14 and P28. Brain regions are colour-coded in the models: brown, forebrain; yellow, diencephalon; green, midbrain; white, blue and purple, hindbrain. The fluorescence images represent 3D reconstructions from *in situ* hybridization images of serial brain sections (Allen Developing Mouse Brain Atlas, 2008) and demonstrate the mRNA expression profiles of GluN subunits. GluN1-encoding mRNA expression remains high in the brain from embryonic to adult stage. GluN2B follows a similar pattern but peaks at around 2 weeks postnatally. GluN2A and GluN2C expression emerge after around 1 week postnatally. GluN2D and GluN3B are only sparsely expressed, whereas GluN3A shows a rapid developmental upregulation and peaks at around 1 week postnatally. The differential expression pattern can shed light on the possible diversity and functioning time windows of NMDARs, which can be drastically different depending on the brain regions. (Images were created by Hovy H. Wong with Allen Institute for Brain Science's Brain Explorer software.)

& Jahr, 2016). Therefore, GluN subunit combinations that were previously thought to be physiologically ineffectual may require revisiting, including the interesting possibilities of an unconventional GluN1 homotetramer (Meguro *et al*. 1992) or even NMDARs lacking the supposedly obligatory GluN1 subunits (Monyer *et al*. 1992). Additionally, the enigmatic characteristics of low conductance and low calcium permeability make GluN3 subunits attractive candidates for metabotropic NMDAR actions (Chatterton *et al*. 2002; Pachernegg *et al*. 2012; Zhu *et al*. 2020). Indeed, the 3 weeks of relatively high postnatal GluN3A expression found in the cortex and hippocampus correspond well to the metabotropic NMDAR signalling described above (Wong *et al*. 2002; Pachernegg *et al*. 2012) (Fig. 2). Although the presence of axonal GluN3A staining is yet to be verified, the gross expression pattern is consistent with the finding that GluN3A-containing preNMDARs promote neurotransmitter release and spike-timing-dependent plasticity (STDP) at ascending inputs from L4 stellate cells to L2/3 PCs in visual cortex (Larsen *et al*. 2011). These raise the interesting possibility that the metabotropically signalling preNMDARs found in L5 PCs may also contain the GluN3A subunit (Abrahamsson *et al*. 2017). Since metabotropic signalling of NMDARs has only recently become more appreciated (Dore *et al*. 2017), the structural and functional diversity of both pre- and postsynaptic metabotropic NMDARs are yet to be elucidated. Importantly, it remains entirely unclear if ionotropic and metabotropic actions signal through the same or distinct pools of NMDARs.

PreNMDARs and short-term plasticity

Short-term impact on excitation. The provocative notion that NMDARs can function presynaptically to regulate release emerged more than two decades ago in the spinal cord (Liu *et al*. 1994, 1997) and in neocortex (Aoki *et al*. 1994; Berretta & Jones, 1996) (Fig. 3). Recent evidence has refined this view, showing specific and separate roles of preNMDARs in the regulation of spontaneous and evoked release (Abrahamsson *et al*. 2017; Bouvier *et al*. 2018) (Fig. 3).

In neocortex, it is well established that in early development, spontaneous release at excitatory inputs onto L2/3, L4 and L5 PCs is tonically upregulated by preNMDARs (Sjöström *et al*. 2003; Corlew *et al*. 2007; Buchanan & Sjöström, 2009; Larsen *et al*. 2011, 2014; Abrahamsson *et al*. 2017) (Fig. 3). This boosting role of preNMDARs appears to diminish around 3 weeks postnatally (Corlew *et al*. 2007), which Larsen *et al*. (2011) found coincides with lowered expression of GluN3A in visual cortex. Larsen *et al*. (2011) furthermore confirmed the involvement of GluN3A-containing preNMDARs for L4 inputs to L2/3 PCs by GluN3A deletion, which caused premature attenuation of spontaneous release regulation in P13–18 animals. Conversely, they found that GluN3A overexpression resulted in a corresponding delay of this attenuation (Larsen *et al*. 2011).

Based on these findings, it may seem that preNMDARs only play an essential role up to 3 weeks postnatally. However, Larsen *et al*. (2011) also found that preNMDAR regulation of neurotransmission may persist well into adulthood. This regulation was only unmasked in low magnesium condition, implying that preNMDARs are present in young adulthood but can only be activated in conditions involving relief from magnesium blockade (Larsen *et al*. 2011). In this view, the heightened magnesium sensitivity of preNMDARs in older mice is presumably triggered by the developmental switch from GluN3A-positive to GluN3A-negative NMDARs. In keeping with this view, Kesner *et al*. (2020) found that in the mature *Xenopus* retinotectal system, the knockdown of GluN1 in presynaptic neurons also did not affect spontaneous release frequency. These similar findings across species suggest the general principle that preNMDARs play key roles in early and late development, but perhaps less so in mature animals, although this may depend on the specific brain region (Pérez-Rodríguez *et al*. 2019).

In neocortex, preNMDARs generally upregulate both evoked and spontaneous release, which has led to the notion that preNMDARs generally promote the probability of neurotransmitter release (Fig. 3). Yet, several studies have shown that preNMDAR regulation of evoked release is steeply dependent on frequency, whereas that of spontaneous release is not (Sjöström *et al*. 2003; Buchanan *et al*. 2012; Larsen *et al*. 2014; Abrahamsson *et al*. 2017). Specifically, preNMDAR control of evoked release in neocortex is engaged at a critical minimum frequency of 5–10 Hz, but spontaneous release occurs at much lower frequencies, yet is also regulated by preNMDARs (Larsen *et al*. 2014; Abrahamsson *et al*. 2017). This apparent discrepancy between preNMDAR control of evoked and spontaneous release is enigmatic and has provoked disagreement over the years.

While it is common to interpret spontaneous and evoked release as synonymous readouts for release probability, the two forms of neurotransmission are in actuality only partially overlapping and can employ distinct vesicle pools and molecular machinery (Chanaday & Kavalali, 2018). In agreement with this view, we recently found that in L5 PCs, preNMDAR-based control of spontaneous and evoked release is mediated by two divergent downstream pathways (Abrahamsson *et al*. 2017). During periods of elevated activity, preNMDARs help to sustain the vesicle replenishment rate of the readily releasable pool, thus affecting the probability of release only indirectly (Fig. 4). This requires classic

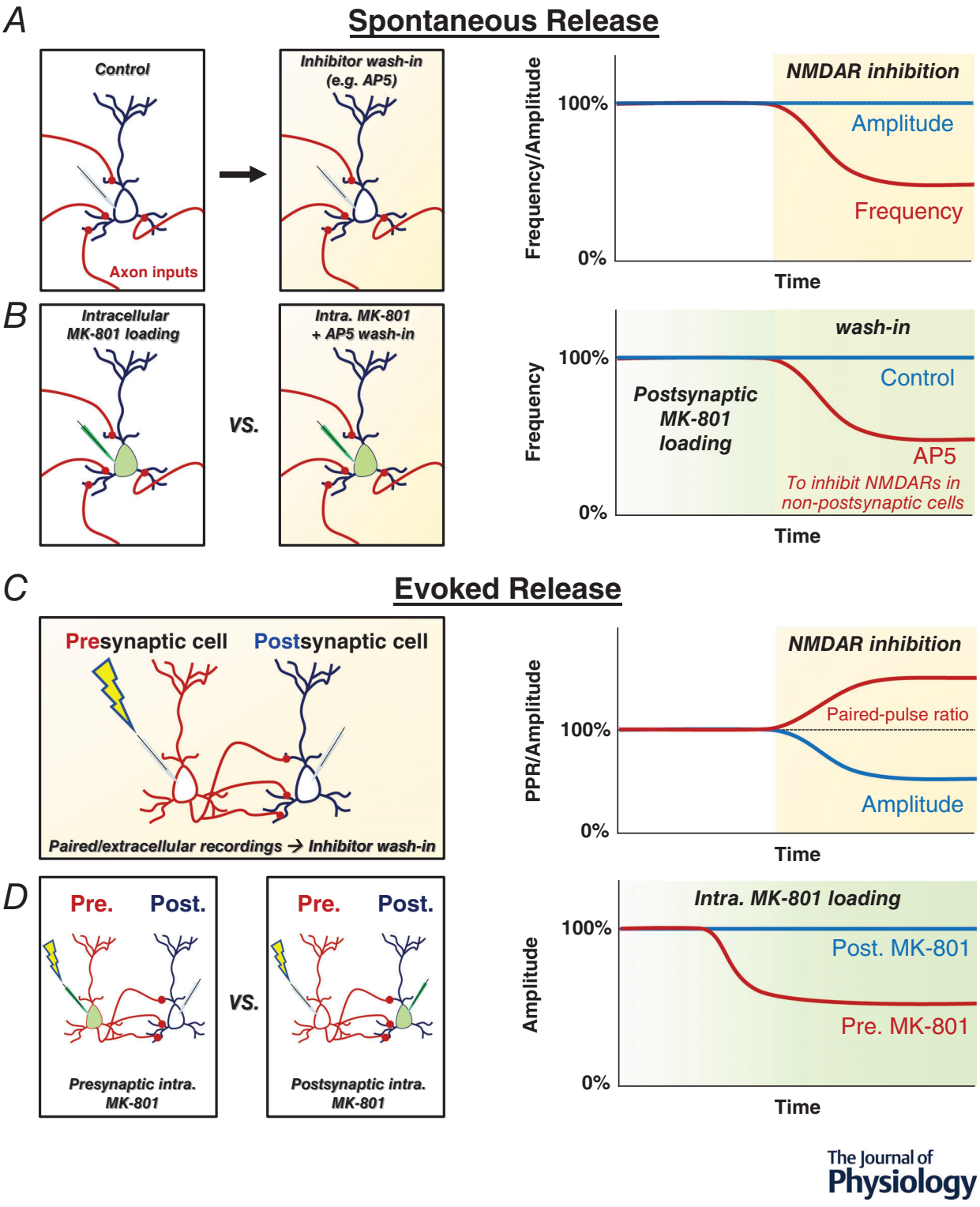

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Figure 3. Experimental approaches for probing pre- *vs.* postsynaptic NMDAR functions
The illustration shows examples of experimental designs for examining the potential contribution of preNMDARs in spontaneous and evoked release. *A*, whole cell recording of miniature postsynaptic currents in the absence of stimulation can be used as a proxy for measuring spontaneous presynaptic release. Bath application of NMDAR inhibitors such as AP5 has been shown to suppress the frequency of miniature excitatory postsynaptic currents (mEPSCs) but not the amplitude in cortical neurons, suggesting the effect stems from a presynaptic locus (e.g. Beretta & Jones, 1996; Sjöström *et al*. 2003; Corlew *et al*. 2007). *B*, to further exclude a postsynaptic contribution,

cell-impermeant NMDAR channel blockers such as MK-801 can be intracellularly loaded in the postsynaptic cell via patching pipette (green). Since this specifically targets postsynaptic NMDARs, the mEPSC frequency has been shown to be unaffected (e.g. Beretta & Jones, 1996; Abrahamsson *et al*. 2017). In contrast, when an NMDAR inhibitor is additionally bath applied to block NMDARs in non-postsynaptic cells, the reduction in mEPSC frequency seen in *A* has been recapitulated (e.g. Beretta & Jones, 1996; Brasier & Feldman, 2008; Larsen *et al*. 2011; Abrahamsson *et al*. 2017). This indicates the regulation of spontaneous release by NMDARs is non-postsynaptic and putatively presynaptic or glial. *C*, activity-dependent presynaptic release can also be evoked by stimulating the presynaptic cells in paired recordings or the afferents via field stimulation. A drop in amplitude of post-synaptic current/potential after bath application of an NMDAR inhibitor can be a result of either or both pre- and postsynaptic NMDAR inhibition. However, other matrices such as a concomitant increase in paired-pulse ratio (PPR) and/or trial-to-trial fluctuation in responses (CV analysis) have been used as proxies to reveal preNMDAR contribution (e.g. Sjöström *et al*. 2003; Brasier & Feldman, 2008; Larsen *et al*. 2011). *D*, to directly dissect pre- *vs*. postsynaptic effects of NMDARs in evoked release, intracellular loading of cell-impermeant NMDAR channel blockers in one of the two cells in the paired recording configuration can be performed (e.g. Rodríguez-Moreno & Paulsen, 2008; Buchanan *et al*. 2012). Intracellular loading of MK-801 in the presynaptic (red), but not postsynaptic (blue) cells, was shown to suppress postsynaptic responses and strongly supports the involvement of preNMDARs (e.g. Buchanan *et al*. 2012).

ion flux signalling via preNMDARs to recruit the vesicle pre-priming protein RIM1$\alpha\beta$. But preNMDARs also signal metabotropically to upregulate spontaneous release via C-Jun N-terminal kinase 2 (JNK2) (Abrahamsson *et al*. 2017). As the blockade of JNK2 alone increases spontaneous release but blocks the reduction normally observed from preNMDAR inhibition, it suggests that preNMDARs normally inhibit JNK2, which in turn downregulates spontaneous release (Fig. 4). The default state is thus a brake on spontaneous neuro-transmitter release, which can be relieved by preNMDAR activation.

It is tempting to speculate that preNMDAR metabotropic signalling is widespread across presynaptic terminals of different types, thereby constituting a major mode of action for low-conductance NMDAR channels composed of GluN2C/2D/3A/3B subunits. This could help reconcile why calcium influx has been readily observed in some axonal compartments – e.g. L5 PCs (Buchanan *et al*. 2012; Abrahamsson *et al*. 2017) and cerebellar molecular layer interneurons (Rossi *et al*. 2012) – but not others, e.g. in boutons at L4–L2/3 connection (Carter & Jahr, 2016) where metabotropic signalling and GluN3A-containing preNMDARs may

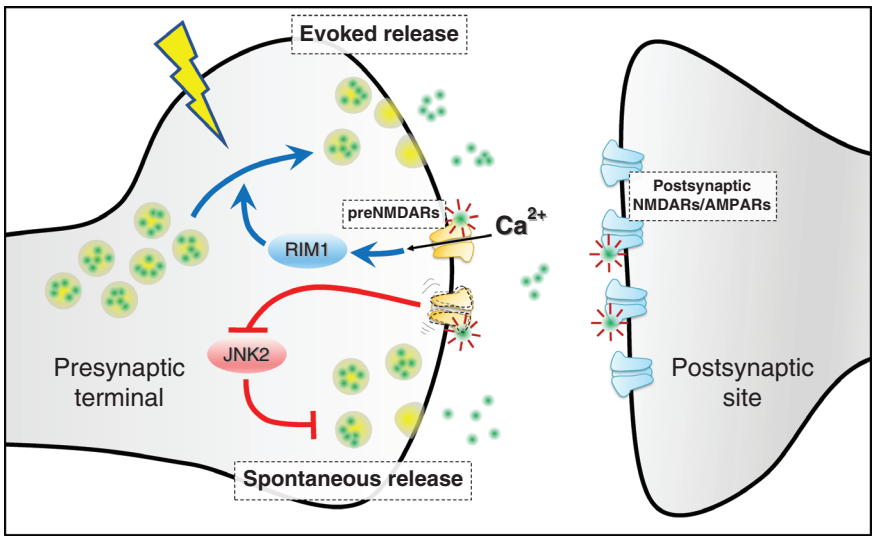

Figure 4. Evoked and spontaneous forms of release are differentially regulated by preNMDARs
At L5 PC-to-PC excitatory connections, preNMDARs regulate evoked and spontaneous release through non-overlapping pathways (Abrahamsson *et al*. 2017). During evoked release above a critical presynaptic frequency of ~8 Hz, calcium influx through preNMDARs activates the vesicle pre-priming protein RIM1$\alpha\beta$ resulting in an upregulation of vesicle replenishment rate (blue arrows). In contrast, without the need of calcium influx, metabotropic action of tonically active preNMDARs inhibits JNK2 and leads to disinhibition of spontaneous release (red bar-headed lines). Note that there may be considerable overlap between the two vesicle pools (Atasoy *et al*. 2008), even though they are illustrated separately here. The dual functionality of preNMDARs implies a specific functional significance of spontaneous release, e.g. maintaining synapses during low activity periods or regulation of synaptic strength (McKinney *et al*. 1999; Chanaday & Kavalali, 2018).

dominate (Larsen *et al*. 2011). However, Savtchouk *et al*. (2019) found that at medial perforant-path inputs to the dentate gyrus, GluN3A-containing preNMDARs regulate both spontaneous and evoked release. This suggests that metabotropic preNMDAR signalling does not necessarily preclude classic ionotropic signalling by the same receptors. Precisely how this works mechanistically remains unclear and will require additional research.

Interestingly, Savtchouk *et al*. (2019) also discovered that ionotropically signalling preNMDARs were involved in plasticity at the medial, but not lateral, perforant path. This is analogous to what we found in L5 of the visual cortex, where preNMDARs in PC axons regulate short-term plasticity at connections to neighbouring PCs and Martinotti cells, but not to basket cells (Buchanan *et al*. 2012). Similarly, Brasier & Feldman (2008) found preNMDARs at ascending L4-to-L2/3 connections, but not at horizontal L2/3-to-L2/3 or L4-to-L4 connections (also see Banerjee *et al*. 2014). It may thus be a general principle that preNMDARs are expressed at certain excitatory synapse types but not others. This synapse-type-specific expression of preNMDARs may additionally help explain why some studies have not been able to image preNMDAR-mediated calcium signals (Christie & Jahr, 2008, 2009; Carter & Jahr, 2016), whereas others have (Buchanan *et al*. 2012), since heterogeneity may lead to lack of experimental control. More generally, these expression patterns may provide a mechanistic explanation for STSP (Maccaferri *et al*. 1998; Tóth & McBain, 2000; Blackman *et al*. 2013; Larsen & Sjöström, 2015), i.e. the notion that plasticity is systematically specified not just by cell type but by synapse type.

Dissimilar to the preNMDARs typically found in the cortex, the GluN3A-containing preNMDARs at perforant-path connections to the dentate gyrus do not appear to be tonically active under basal conditions (Savtchouk *et al*. 2019). The preNMDARs can nonetheless be exogenously activated by NMDA puff application (Savtchouk *et al*. 2019). Whether they signal metabotropically or ionotropically to regulate spontaneous release under physiological conditions remains to be explored. Prius-Mengual *et al*. (2019) recently reported that at Schaffer collateral inputs to CA1 PCs, tonically active preNMDARs are sensitive to GluN2C/2D pharmacology, but not to blockade of the GluN2A subunit that confers high ionic conductance. This lends further support to the idea that preNMDARs may not always rely on ionotropic action, but have the potential for operating via metabotropic signalling.

In conclusion, it is becoming increasingly clear that, whether ionotropically or metabotropically, preNMDARs play an integral part in regulating excitatory neurotransmission (Bouvier *et al*. 2018). However, as we shall see in the next section, the regulatory reach of preNMDARs extends beyond excitatory terminals to the influence of inhibitory synapses.

Short-term impact on inhibition. The inhibitory neurotransmitter GABA is mainly released from interneurons and underpins both feed-forward and feedback inhibition in local circuits (Isaacson & Scanziani, 2011). GABAergic activity is crucial for maintaining excitation–inhibition balance in the CNS and dysfunctional inhibitory transmission has consequently been linked to several neuropathologies, including epilepsy, autism and schizophrenia (Yizhar *et al*. 2011; Sohal & Rubenstein, 2019). Since preNMDARs are glutamatergic, it is reasonable to assume that they do not directly govern inhibitory neurotransmission. However, as we will describe in more detail below, it surprisingly turns out that preNMDARs are also found in axonal compartments of inhibitory cells, where they play important roles in controlling inhibition in local circuits (Glitsch & Marty, 1999; Duguid & Smart, 2004; Crabtree *et al*. 2013; Pafundo *et al*. 2018).

PreNMDARs have for decades been detected at GABAergic terminals in the cortex (DeBiasi, 1996; Pafundo *et al*. 2018), cerebellum (Glitsch & Marty, 1999; Duguid & Smart, 2004), as well as deeper structures such as the thalamus and hypothalamus (Paquet & Smith, 2000; Crabtree *et al*. 2013). However, it appears that a subset of these preNMDARs may not always be functional (Bidoret *et al*. 2015). Nevertheless, the existence of functional glutamatergic receptors such as preNMDARs in axonal compartments of inhibitory cells suggests that there is crosstalk from excitatory neurotransmission to inhibitory release machinery. This could be a feedback mechanism to maintain the excitation–inhibition balance within local circuits. Neocortical and hippocampal astrocytes have also been demonstrated to release glutamate (Parpura *et al*. 1994; Angulo *et al*. 2004), which may act on preNMDARs at L4–L2/3 excitatory synapses in the barrel cortex (Min & Nevian, 2012). Whether glial glutamate is an important source for generally activating preNMDARs at inhibitory synapses is a compelling avenue to pursue. There is evidence to suggest that the existence of glutamatergic receptors in inhibitory axons may in fact generalize to receptor types other than NMDARs such as kainate receptors (Rodríguez-Moreno *et al*. 1997, 2000; Engelman & MacDermott, 2004; Lourenço *et al*. 2010; Pressey & Woodin, 2020), although here we focus on preNMDARs.

In the cerebellum, molecular layer interneurons (MLIs) form GABAergic synaptic connections with other MLIs and with Purkinje cells (Häusser & Clark, 1997; Rieubland *et al*. 2014). Consequently, MLIs contribute to inhibition and disinhibition of Purkinje cells (Brown *et al*. 2019) – the sole output of the cerebellar cortex – which is crucial for

development and fine-tuning of motor circuits (Astorga *et al*. 2017; Gaffield & Christie, 2017). At MLI terminals, pharmacological activation of preNMDARs increases the frequency of spontaneous GABA release without changing the amplitude of the postsynaptic response (Glitsch & Marty, 1999; Duguid & Smart, 2004; Rossi *et al*. 2012). This presynaptic effect is thought to be mediated by Ca^{2+} entry through preNMDARs (Glitsch, 2008), depolarizing the presynaptic bouton, which allows a second wave of Ca^{2+} entry through voltage-gated Ca^{2+} channels and/or release from intracellular stores. Interestingly, chronic preNMDAR activation in cerebellar cultures from early stages of development increases GABAergic bouton size and enhances evoked GABA release in more developed neurons (Fiszman *et al*. 2005). This suggests a key role of preNMDARs in circuit formation at early stages of development.

However, the Jahr team was not able to detect preNMDAR calcium signals in MLI axonal compartments with two-photon microscopy and therefore argued against the existence of preNMDARs in MLI terminals, instead proposing that the relevant NMDARs might actually be in the dendrites of presynaptic cells (Christie & Jahr, 2008; Pugh & Jahr, 2011). Moreover, another study found that while preNMDARs could be detected at MLI terminals, activation of these receptors did not alter spontaneous GABAergic release (Bidoret *et al*. 2015), suggesting that these preNMDARs might not be functional. On the other hand, direct patch-clamp recordings of actual GABAergic terminals in cerebellar culture provided concrete evidence for the existence of preNMDARs in MLI axons (Fiszman *et al*. 2005). A parsimonious interpretation is perhaps that these contradictory findings are due to experimental design, e.g. calcium imaging as an indirect measure of neuronal activity, since these receptors may not flux calcium well and may signal metabotropically (Dore *et al*. 2017; Bouvier *et al*. 2018). This controversy will require further study to be resolved.

Interestingly, it appears that MLI preNMDARs have a distinct subunit composition that differ from the classic dendritic and somatic NMDARs (Dubois *et al*. 2016). For example, GluN1/GluN2B/GluN2D preNMDARs in stellate cells mediate LTP of inhibitory transmission, but these triheteromeric NMDARs could not be detected in the stellate cell dendrite or soma (Dubois *et al*. 2016). Investigating preNMDAR structure and subunit composition will likely reveal more about their precise contribution to plasticity.

Some studies have suggested interaction between preNMDARs and cytokine receptors in hippocampal synaptosomes (Di Prisco *et al*. 2016; Olivero *et al*. 2019). Inflammatory experimental conditions in the acute slice preparation may thus alter preNMDAR function, which may potentially help to explain disagreements across laboratories.

Less is known about preNMDARs at GABAergic terminals in brain regions other than the cerebellum. PreNMDARs have been detected in putative GABAergic terminals in the somatosensory cortex using electron microscopy (DeBiasi *et al*. 1996), but their precise functional role remains unclear. PreNMDARs have also been found at parvalbumin interneuron terminals in the prefrontal cortex where they regulate PC inhibition (Pafundo *et al*. 2018). Outside neocortex, one study found GluN2B-containing preNMDARs at GABAergic terminals in the hypothalamus, thalamus and basal forebrain, although their precise functional role was not explored (Paquet & Smith, 2000). However, another study reported that preNMDARs in the thalamus may regulate inhibitory control of thalamic output towards the cortex (Crabtree *et al*. 2013).

Taken together, the evidence reveals that preNMDARs are expressed at inhibitory synapses across several brain regions, although only at specific GABAergic terminal types. It appears that, as a consequence, far from all interneuron axons possess preNMDARs. These findings are in good agreement with the principle that preNMDARs have synapse-specific roles discussed above in the context of excitatory synapses. This may again mechanistically help to explain the existence of synapse-type-specific forms of plasticity (Tóth & McBain, 2000; Blackman *et al*. 2013; Larsen & Sjöström, 2015). Since findings may depend on how this heterogeneity is experimentally addressed, it could also explain disagreements in the field (Bouvier *et al*. 2018). Finally, it remains to be determined to what extent preNMDARs generally regulate inhibitory transmission and what impact this regulation has at the network level.

PreNMDARs and long-term plasticity

In addition to regulating basal transmission and short-term plasticity, preNMDARs are also involved in several forms of long-term synaptic plasticity at both excitatory and inhibitory synapses in different brain areas, including LTP and long-term depression (LTD). These are activity-dependent long-lasting changes in synaptic strength that are often expressed in both pre- and postsynaptic elements (Costa *et al*. 2017) and that are widely believed to underlie learning and memory in the brain (Sjöström & Nelson, 2002; Sjöström *et al*. 2008).

PreNMDARs in homosynaptic plasticity. The axonal location of preNMDARs close to the active zone is ideal for autoreceptor function to sense presynaptic release and thereby control homosynaptic plasticity, i.e. regulation specific to an individual activated synapse (Fig. 5). In a pioneering pair of studies, Casado *et al*. (2000, 2002) first described how preNMDARs are necessary for the induction of postsynaptically expressed LTD

at parallel fibre (PF) synapses onto cerebellar Purkinje cells. The mechanism for this non-Hebbian form of plasticity involved preNMDAR-mediated calcium flux and anterograde nitric oxide (NO) signalling acting on the postsynaptic compartment, without affecting the probability of neurotransmitter release (Casado *et al.* 2002; Bidoret *et al.* 2009).

There were, however, some disagreements about the origin of the NO. In 2005, Shin and Linden suggested a complementary and alternative interpretation to the original finding by Casado *et al.* (2000; 2002). Shin & Linden (2005) proposed that the NMDAR-dependent NO cascade involved in this form of LTD is not actually localized to PFs, but resides in interneuron axon terminals. In this alternative view, preNMDARs are activated by glutamate spillover from PFs, leading to the release of NO from interneurons, which diffuses to Purkinje cells to help elicit LTD.

PreNMDARs have also been consistently implicated in STDP, which is a temporally asymmetric form of Hebbian learning induced by millisecond-scale temporal correlations between the spikes in pre- and postsynaptic neurons. Although it varies with synapse type (Abbott & Nelson 2000; Sjöström *et al.* 2008), in the canonical form of STDP, timing-dependent LTP (tLTP) is induced when presynaptic spiking is repeatedly and persistently followed by postsynaptic spiking within 10 ms or so. Timing-dependent LTD (tLTD), on the other hand, is elicited with the opposite temporal order (Markram *et al.* 1997; Bi & Poo, 1998; Debanne *et al.* 1998).

Sjöström *et al.* (2003) provided the first evidence of an involvement of preNMDARs in neocortical endocannabinoid (eCB)-dependent tLTD at visual cortex L5 PC-to-PC synapses. These findings were confirmed and extended by others at L4–L2/3 synapses in visual cortex (Corlew *et al.* 2007; Larsen *et al.* 2011, 2014), the somatosensory cortex (Bender *et al.* 2006; Rodriguez-Moreno & Paulsen, 2008), barrel cortex (Banerjee *et al.* 2009; Rodriguez-Moreno et al. 2011; Min & Nevian, 2012) and hippocampus (Andrade-Talavera *et al.* 2016; Pérez-Rodríguez *et al.* 2019). Although the precise details of the mechanism are still debated, e.g. the exact location of the eCB receptors (see below; Min & Nevian, 2012), a majority of studies are in agreement that preNMDARs are critically needed for this form of tLTD (although see Carter & Jahr, 2016).

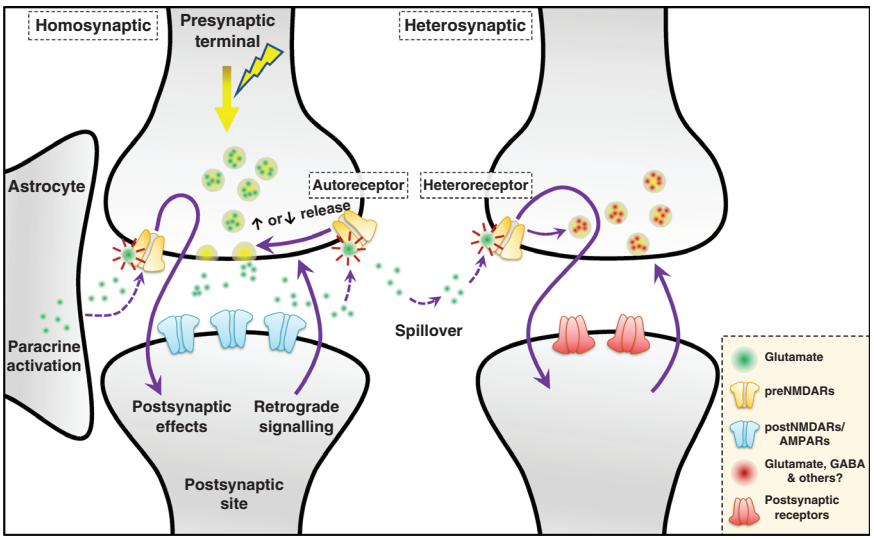

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Figure 5. PreNMDARs in homo- and heterosynaptic long-term plasticity
In homosynaptic plasticity, the effects of preNMDAR activation are by definition restricted to the activated synapse (lightning symbol). PreNMDAR can have presynaptic effects regulating vesicle release (e.g. Sjöström *et al.* 2003; Bender *et al.* 2006; Rodríguez-Moreno & Paulsen, 2008; Andrade-Talavera *et al.* 2016), or postsynaptic effects leading to long-lasting increase or decrease in AMPA receptor mediated synaptic gain (Casado *et al.* 2000, 2002). Postsynaptic activity can also trigger retrograde signalling, e.g. by release of endocannabinoids, nitric oxide, etc. from the postsynaptic site (e.g. Sjöström *et al.* 2003, 2007) and/or neighbouring astrocytes (Min & Nevian, 2012) to alter vesicle release presynaptically. In addition, glutamate spillover can cause heterosynaptic preNMDAR signalling in neighbouring glutamatergic (Humeau *et al.* 2003) or GABAergic terminals (Shin & Linden, 2005; Lien *et al.* 2006; Liu & Lachamp, 2006).

Several lines of evidence indicate that this form of eCB-dependent tLTD is presynaptically induced and expressed: firstly, tLTD is abolished when preNMDARs are blocked by internal MK-801 in paired recordings of L4 and L2/3 cells (Rodríguez-Moreno & Paulsen, 2008); secondly, an increase in paired-pulse ratio is observed after an tLTD protocol (Bender *et al*. 2006); thirdly, the coefficient of variation (CV) analysis that assesses trial-to-trial fluctuation in postsynaptic responses is consistent with presynaptic expression of tLTD, with a lowered probability of release underlying the reduction in synaptic strength (Rodríguez-Moreno & Paulsen, 2008; Andrade-Talavera *et al*. 2016; Brock *et al*. 2020). PreNMDAR involvement in tLTD was shown *in vivo* in L4–L2/3 connections of the barrel cortex, where activation of L4 inputs during *Up* states led to LTD, which strengthens the biological relevance of this form of tLTD in particular and of STDP in general (González-Rueda *et al*. 2018).

Several NMDAR subunits, with different magnesium sensitivity and calcium permeability, have been implicated in tLTD depending on the brain region and synapse type: GluN2B (Sjöström *et al*. 2003) and the low-magnesium-sensitivity GluN3A (Larsen *et al*. 2011) in visual cortex, the low-magnesium-sensitivity GluN2C/D in barrel cortex (Banerjee *et al*. 2009) or in hippocampus (Andrade-Talavera *et al*. 2016), and GluN2A in cerebellum (Bidoret *et al*. 2009). In the perirhinal cortex, GluN2A-containing preNMDARs are also involved in a timing-independent form of LTD, induced by low-frequency stimulation of inputs from lateral nucleus of the amygdala paired with postsynaptic depolarization of L2/3 neurons (Laing & Bashir, 2015). In general, tLTD appears to depend on postsynaptic metabotropic glutamate receptors (mGluRs), coincident activation of presynaptic cannabinoid receptor type 1 receptors (CB_1Rs) and preNMDARs, and potentially also astrocyte-released glutamate or D-serine in the neocortex (Sjöström *et al*. 2003, Bender *et al*. 2006, Nevian & Sakmann, 2006, Min & Nevian, 2012) and hippocampus (Andrade-Talavera *et al*. 2016; Pérez-Rodríguez *et al*. 2019).

In L5 of the visual cortex, preNMDARs drive the induction of tLTD together with eCB signalling. In an early working model of tLTD, Sjöström *et al*. (2003) proposed that the postsynaptic spike triggers eCB release while the presynaptic spike evokes glutamate release, thus resulting in coincident activation of presynaptic CB1 and NMDA receptors to elicit LTD even at low frequency. In agreement, tLTD has also been shown to depend on preNMDARs and eCB signalling cascade in L2/3 of the somatosensory cortex (Bender *et al*. 2006) and in the barrel cortex (Min & Nevian, 2012). However, the latter study showed that the postsynaptic production of eCBs triggers glutamate release from astrocytes that in turn activates preNMDARs (Min & Nevian, 2012), revealing non-autoreceptor functions of preNMDARs.

Astrocyte signalling has also been shown to participate in preNMDAR-dependent tLTD in the hippocampus (Andrade-Talavera *et al*. 2016, Pérez-Rodríguez *et al*. 2019). At CA3–CA1 synapses, tLTD is dependent on postsynaptic calcium, L-type voltage-dependent calcium channels, mGlu5R activation, phospholipase C, postsynaptic inositol 1,4,5-trisphosphate-receptor (IP3)-mediated calcium release from internal stores, retrograde eCB signalling, astroglial signalling, the D-serine preNMDAR coagonist and presynaptic calcineurin (Andrade-Talavera *et al*. 2016; Pérez-Rodríguez *et al*. 2019). In contrast, a non-Hebbian neocortical pattern-dependent LTD does not require mGluRs, CB_1Rs, glia, postsynaptic calcium, or G-protein signalling. However, it does depend on presynaptic calcineurin (Rodriguez-Moreno et al. 2013). Without postsynaptic pairing, high-frequency presynaptic stimulation also promotes the induction of LTD in the hippocampus via preNMDARs, which decreases the release probability (Padamsey *et al*. 2017).

A dissociation between tLTD and tLTP has been reported for neocortical (Sjöström *et al*. 2003, 2007; Rodriguez-Moreno & Paulsen, 2008; Rodriguez-Moreno *et al*. 2011) and hippocampal (Andrade-Talavera *et al*. 2016) synapses, showing that the induction of tLTD and tLTP is largely determined by preNMDARs and postsynaptic NMDARs, respectively. In other words, tLTP but not tLTD at L5 PC-to-PC connections can be induced in the presence of GluN2B-specific blockers that abolish preNMDAR signalling (Sjöström *et al*. 2003). However, preNMDARs are also involved in LTP regulation in various brain areas. Bouvier *et al*. (2016) showed that – in addition to determining cerebellar LTD (Casado *et al*. 2000, 2002) – preNMDARs are involved in a form of LTP that shares mechanisms with LTD. To our knowledge, this is the only example of preNMDARs being involved in both LTP and LTD at the same synapse. Here, the input firing pattern determines the outcome of synaptic plasticity.

PreNMDARs also trigger a presynaptically expressed form of homosynaptic LTP in the central nucleus of amygdala that occurs after high-frequency stimulation of thalamic inputs (Samson & Paré, 2005) as well as at CA1 terminals to burst-firing neurons of the subiculum (Wozny *et al*. 2008; Roggenhofer *et al*. 2010). This form of plasticity relies on presynaptic calcium signalling and requires the activation of protein kinase A (Roggenhofer *et al*. 2010). Different forms of preNMDAR-dependent forms of homosynaptic LTP have also been described in other brain regions. At cortico-striatal synapses, preNMDARs trigger LTP through activity-induced calcium influx and presynaptic BDNF secretion (Park *et al*. 2014; Zhou *et al*. 2018). In the hippocampus, GluN3A-containing preNMDARs activated by astrocytic gliotransmitters regulate LTP at medial perforant path to

granule cell synapses (Savtchouk *et al*. 2019), indicating that preNMDARs do not always function as autoreceptors. This study also found that this form of LTP was specifically found at this synapse type. Indeed, as preNMDAR expression is often specific to synapse type (see above; Corlew *et al*. 2007; Brasier & Feldman, 2008; Buchanan *et al*. 2012), it follows that preNMDAR regulation of synaptic plasticity is synapse type specific as well, as discussed earlier (Humeau *et al*. 2003; Banerjee *et al*. 2014; Larsen *et al*. 2014; Savtchouk *et al*. 2019).

PreNMDARs in heterosynaptic plasticity. PreNMDARs can act as spillover detectors to surveil glutamate release from adjacent excitatory terminals, allowing them to participate in heterosynaptic plasticity (Fig. 5). An early study by Humeau *et al*. (2003) described a presynaptically expressed form of plasticity in the amygdala that depends on the activation of preNMDARs in cortical inputs by glutamate released by thalamic afferents. By coincidently stimulating converging inputs to the amygdala from the thalamus and cortex, Humeau *et al*. (2003) observed associative LTP only at the cortical afferents. As postsynaptic blockade of NMDARs using intracellular loading of MK-801 did not prevent LTP, the NMDARs required for this form of LTP were putatively located in presynaptic axonal compartments. Here, preNMDARs may thus presumably act as coincidence detectors, although not in the Hebbian sense.

For example, it has been shown that glutamate released by cerebellar parallel fibers induces a long-lasting increase in both evoked and spontaneous release of GABA from cerebellar stellate cells via calcium entry through preNMDAR (Liu & Lachamp, 2006; Dubois *et al*. 2016). This form of heterosynaptic LTP of inhibition relied on tri-heteromeric preNMDARs containing GluN2D and GluN2B subunits (Dubois *et al*. 2016). Interestingly, the lasting enhancement of GABA release relied on presynaptic protein kinase A signalling and the active-zone protein RIM1α (Lachamp *et al*. 2009). This is in contrast to the preNMDAR-dependent mechanism controlling glutamatergic spontaneous release onto neocortical L5 PCs, which appears to be independent of RIM1 (Abrahamsson *et al*. 2017).

Another example of heterosynaptic LTD of inhibitory connections mediated by preNMDARs comes from an elegant study in the tadpole optic tectum, which showed that preNMDARs are also required for LTD at GABAergic synapses triggered by visual stimuli (Lien *et al*. 2006). This study thus revealed that these preNMDARs may function as detectors of coincident activity in neighbouring glutamatergic and GABAergic neurons. This study furthermore provided some of the first evidence for the involvement of preNMDARs in presynaptic coincidence detection and synaptic plasticity *in vivo*.

Unconventional signalling of preNMDARs in long-term plasticity. Since preNMDARs are not sensitive to postsynaptic voltage, their presynaptic location means preNMDARs cannot carry out classic Hebbian coincidence detection (Fig. 5). PreNMDAR signalling is in other words not in agreement with the textbook view on NMDAR function and has consequently been termed unconventional (Dore *et al*. 2017). Additionally, NMDAR signalling can be unconventional in other ways, e.g. it can signal metabotropically, during magnesium block and without ion flux (Nabavi *et al*. 2013; Aow *et al*. 2015; Dore *et al*. 2015). This unconventional manner of NMDAR signalling has been implicated in both functional (Kessels *et al*. 2013; Nabavi *et al*. 2013; Carter & Jahr, 2016) and structural synaptic plasticity (Stein *et al*. 2015, 2020; Thomazeau *et al*. 2020).

The findings that postsynaptic NMDARs can signal metabotropically in long-term plasticity suggest that preNMDARs may in principle also be able to do so. As discussed above, the Sjöström lab already found that preNMDARs may signal metabotropically via JNK2 to regulate spontaneous release independent of frequency, whereas RIM1$\alpha\beta$-dependent regulation-evoked release is abolished at low frequencies due to magnesium blockade of ionotropic preNMDAR signalling (Fig. 4) (Abrahamsson *et al*. 2017). This means preNMDARs can signal metabotropically to regulate release in general, but what are the implications for metabotropic preNMDAR signalling in long-term plasticity? Since tLTD at visual cortex L5 PC-to-PC connections is similarly independent of firing frequency (Sjöström *et al*. 2003) – which has been a long-standing enigma given the known magnesium dependence of these GluN2B-containing preNMDARs (Duguid & Sjöström, 2006) – it is tempting to speculate that this form of tLTD at least in part relies on metabotropic rather than ionotropic preNMDAR signalling. Further work is required to determine whether the preNMDAR metabotropic signalling cascades also underlie tLTD at cortical synapses.

PreNMDAR-mediated plasticity is tightly developmentally regulated. The fact that synaptic plasticity learning rules vary with synapse types has consequences for circuit refinement during development (Larsen & Sjöström, 2015; Kesner *et al*. 2020), which is especially important considering that preNMDAR function is tightly developmentally regulated (Corlew *et al*. 2008). For example, neocortical tLTD requires preNMDARs in early stages of experience-dependent refinement but subsequently depends on postsynaptic NMDARs in more mature animals (Corlew *et al*. 2007; Banerjee *et al*. 2009; Rodríguez-Moreno *et al*. 2013). Similarly, hippocampal tLTD disappears in the fourth week of development, through mechanisms involving adenosine receptors (Pérez-Rodríguez *et al*.

2019). Although this tight developmental regulation of preNMDAR-dependent plasticity consistently appears across studies, it remains unclear precisely why and how it is important in development. An intriguing possibility is that this developmental regulation preNMDAR-mediated plasticity is somehow linked to the closing of critical periods (Larsen *et al*. 2014). To address these questions, studies linking circuit plasticity to receptive field formation and behaviour are required (Kesner *et al*. 2020).

The clinical relevance of preNMDARs

As we outline in this section, several recent studies have provided evidence that preNMDAR hypo- or hyper-function may contribute to neuropathology. PreNMDARs are thus potential therapeutic targets.

NMDARs in the dorsal horn of the spinal cord have long been known to play key roles in spinal plasticity and neuropathic pain generation (Yamamoto & Yaksh, 1992; Deng, *et al*. 2019). Dorsal horn NMDARs are molecular targets of clinically used neuropathic pain therapeutics such as ketamine, and although classically thought of as exerting their effects postsynaptically, there is emerging evidence for a presynaptic involvement (Yan *et al*. 2013). PreNMDARs in the dorsal horn are quiescent under physiological conditions during nociception, yet are under certain neuropathic pain conditions likely to take on a pivotal role in shaping plasticity as their synaptic expression, localization and activity increase (Zeng *et al*, 2006; Chen *et al*. 2019).

Chemotherapy-induced neuropathic pain is a debilitating phenomenon with potentially lethal consequences, which can be induced by chemotherapeutic agents such as paclitaxel. Paclitaxel is known to tonically activate preNMDARs in the dorsal horn to potentiate glutamate release, while having no effect on their postsynaptic counterparts (Xie *et al*. 2017). Recently, some of the molecular events underlying this plasticity were uncovered, as paclitaxel treatment in rats was seen to induce upregulation of the voltage-gated calcium channel subunit $\alpha 2\delta$-1, which forms a complex with spinal preNMDARs (Chen *et al*, 2019). PreNMDAR–$\alpha 2\delta$-1 complex formation and synaptic trafficking were markedly increased in the neuropathic pain condition, and disruption of these events by genetic ablation, knockdown, or administration of complex-formation-interfering compounds attenuated paclitaxel-induced pain in mice (Chen *et al*. 2019). The elucidation of the molecular events underlying preNMDAR-associated chemotherapy-induced neuropathic pain may thus help to enable the development of targeted therapeutic strategies. Using agents to specifically target spinal preNMDARs or their interacting proteins that become abnormally upregulated in neuropathic pain conditions will likely form the basis of future treatments, which avoid the deleterious adverse effects of indiscriminately targeting NMDARs (LoGrasso & McKelvy, 2003).

Excitotoxicity is a hallmark of various neuropathologies such as epilepsy, cerebral ischemia and degenerative disorders, resulting from excessive excitatory transmitter release (Lau & Tymianski, 2010). Aberrant activation of NMDARs is known to exacerbate excitotoxicity in the pathological environment (Parsons & Raymond, 2014). Nonetheless, therapeutic compounds that promiscuously target a majority of NMDARs with little or no specificity have thus far proven ineffective in either treatment or prevention, and have therefore not reached clinical use. These are likely due to their broad and toxic effects on normal physiological neurotransmission (Ikonomidou & Turski, 2002).

A favourable neuroprotective strategy may be to specifically target preNMDARs, necessitating a deeper understanding of associated intracellular signalling and molecular events. In cortex, preNMDAR-dependent glutamate release is regulated by receptor interaction with JNK2, subsequently interfering with synaptic release machinery (Nisticò *et al*, 2015; Abrahamsson *et al*, 2017). It was recently shown that syntaxin-1a (STX1a), a component of the SNARE membrane fusion complex, is a key molecular player in the regulation of preNMDAR-dependent glutamate release (Marcelli *et al*, 2019). Deficits in STX1a phosphorylation have also been observed in people with schizophrenia (Castillo *et al*, 2010). Recently, inhibiting preNMDAR signalling via the use of the novel small peptide 'JGRi1' to block JNK–STX1a interaction was met with success both *in vitro* and *ex vivo* (Marcelli *et al*, 2019). With the ability to widely diffuse throughout the brain following intraperitoneal administration, similar peptide-based strategies can serve as promising therapeutic tools to correct excitotoxic glutamatergic overflow, while avoiding the damaging effects of non-specific NMDAR blockade.

Dysregulated glutamate signalling and impaired synapse development have been proposed to contribute to neurodevelopmental and psychiatric disorders. Mutations in NMDAR subunits have been linked to pathogenesis of autism spectrum disorders (ASD), intellectual disability, epilepsy, anxiety, depression and schizophrenia (Endele *et al*. 2010; Hamdan *et al*. 2011; Tarabeux *et al*. 2011; O'Roak *et al*. 2012). Changes in glutamate signalling and aberrant NMDAR expression and function have been observed in the brains of individuals with ASD (Blatt *et al*. 2001; Purcell *et al*. 2001) and in different mouse models of ASD (Etherton *et al*. 2011; Eadie *et al*. 2012; Sceniak *et al*. 2016). Furthermore, NMDAR antagonists result in cognitive and behavioural changes similar to those

reported in ASD (Chez *et al*. 2007; Kron *et al*. 2012) and increase the risk for intellectual disability in infants and children (Alkondon *et al*. 1990; Ujihara & Albuquerque, 1992; Neal *et al*. 2011). As mentioned before, the early expression of GluN1 at presynaptic terminals reveals a role for preNMDARs in synapse development. Moreover, NMDARs located within presynaptic neurons during development acutely regulate presynaptic plasticity. Taken together, these suggest that disruption of preNMDAR signalling might have considerable impact on neuronal development in humans. However, to be able to harness preNMDAR signalling for new therapies, we must first elucidate the mechanisms that link preNMDARs to neurodevelopmental and psychiatric disorders.

Conclusions and future directions

The preNMDAR research field has come a long way. Originally, studies reporting on the existence of preNMDARs were sparse and not always favourably received, which sparked controversy and raised more questions than they answered. In our opinion, there were several reasons for this. Perhaps first and foremost, it is perplexing to comprehend why nature would instruct the NMDAR – the canonical Hebbian coincidence detector – to localize to the presynapse, where it cannot fulfil its job. Hence, these findings were met with resistance from scientists who rightfully demanded extraordinary evidence for extraordinary claims. Second, much of the initial evidence was indirect, relying on, for example, interpretation of electrophysiological experiments in the absence of two-photon or confocal microscopy to visualize preNMDARs in axons. Despite early electron microscopy evidence (e.g. Aoki *et al*. 1994), findings of NMDAR subunits in the axon could be dismissed as mislocalized and non-functional. Last but not least, much of the early published evidence for preNMDARs was puzzling and seemingly not self-consistent. For example, how could preNMDAR control of evoked release exhibit a critical frequency arising from magnesium blockade while the preNMDAR control of spontaneous release occurred at rates orders of magnitude below this threshold frequency (e.g. Sjöström *et al*. 2003)? And why was it so difficult to find preNMDAR-mediated calcium signals even with highly sensitive two-photon microscopes (e.g. Christie & Jahr, 2009)?

Since then, a multitude of studies have helped to resolve apparent discrepancies such as these (Bouvier *et al*. 2018). That preNMDARs often contain unusual subunits that confer high magnesium blockade sensitivity and low ionic flux could explain why preNMDAR calcium transients were poorly resolved even with the best of microscopes (e.g. Larsen *et al*. 2011; Larsen *et al*. 2014). That preNMDARs are only expressed at some bouton types but not others (e.g. Buchanan *et al*. 2012) clarified why averaging across all of them yielded seemingly insignificant results at the population level. That preNMDARs signal ionotropically to control evoked release but metabotropically to control spontaneous release (e.g. Abrahamsson *et al*. 2017) explained why the magnesium blockade seemingly mattered in some scenarios but not others.

Although the preNMDAR research field has matured considerably, much remains to be explored. As a prediction for the future, we would like to argue that a key new direction is to connect preNMDAR function to circuit refinement and animal behaviour. For instance, the recent study by Kesner *et al*. (2020) has broken considerable new ground, by showing how pre- and postsynaptic NMDARs differentially regulate the development of visual circuits in the *Xenopus* tadpole tectum, leading to specific changes in receptive fields. Another important future direction is the on-going effort to link preNMDAR function to disease states. This prospect is especially exciting considering the fact that preNMDARs are expressed with synapse-type specificity and are often made up of unusual subunit combinations, which could help achieve specificity of drug action in therapies. Here, we identified spinal cord and neuropathic pain as a promising first therapeutic target, but many others exist.

Although predictions are famously difficult to make, especially about the future, we anticipate that few will argue that preNMDARs are an epiphenomenon or an artefact of misexpression. Although preNMDARs may not be able to carry out classic Hebbian coincidence detection like their dendritically located counterparts, we argue that preNMDARs are critically needed for specific purposes and that there is no coincidence that they are found in axons.

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Additional information

Competing interests

None declared.

Author contributions

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