Title Page:

Analgesia with Gabapentin and Pregabalin May Involve NMDA Receptors, Neurexins and Thrombospondins

Charles P. Taylor and Eric W. Harris

CPT: CP Taylor Consulting

7560 Lake Shore Dr.

Chelsea, MI 48118

USA

EWH: Cambrium Group

5236 Theys Rd.

Raleigh, NC 27606

USA

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Running Title Page:

Gabapentin Analgesia, NMDA Receptors and Thrombospondins

Charles P. Taylor* and Eric W. Harris

*Corresponding Author

CP Taylor Consulting

7560 Lake Shore Dr.

Chelsea, MI 48118

email: taylor.charles54@gmail.com

tel and fax: 734-475-2172

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Abbreviations:

$\alpha_2\delta$-1 – alpha$_2$delta subunit type 1 (Ca$_V$a2d1)

AMPA receptor - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor

Cache domain – extracellular CAlcium channels and CHEmotaxis receptor domain

CaMKII - calcium calmodulin kinase II

GPI anchor – glycosylphosphatidylinositol-anchor

FRET - fluorescent resonance energy transfer

LRP1 – low density lipoprotein (LDL) receptor-related protein-1

LTP – long-term potentiation

mEPSC – miniature excitatory synaptic current

MIDAS – metal ion dependent adhesion site common to von Willebrand domain proteins

NMDA receptor – N-methyl-D-aspartate sensitive glutamate receptor

P2X receptor - ATP-gated P2X receptor cation channel

PKC – protein kinase C

RAP – GTP binding Ras-related protein

SNAP25 - synaptosomal nerve-associated protein 25, involved in vesicle exocytosis

VWF-A domain – von Willebrand A glycoprotein adhesion domain

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Abstract

The gabapentinoid drugs gabapentin and pregabalin (Neurontin® and Lyrica®) are mainstay treatments for neuropathic pain and for preventing focal seizures. Both drugs have similar effects to each other in animal models and clinically. Studies have shown that a protein first identified as an auxiliary subunit of voltage-gated calcium channels (the alpha$_2$-delta subunit, $\alpha_2\delta$-1 or Ca_Va2d1) is the high-affinity binding site for gabapentin and pregabalin, and is required for the efficacy of these drugs. The $\alpha_2\delta$-1 protein is required for the ability of gabapentin and pregabalin to reduce neurotransmitter release in neuronal tissue, consistent with a therapeutic mechanism of action via voltage-gated calcium channels. However, recent studies have revealed that $\alpha_2\delta$-1 interacts with several proteins in addition to voltage-gated calcium channels, and these additional proteins could be involved in gabapentinoid pharmacology. Furthermore, gabapentin and pregabalin have been shown to modify the action of a subset of NMDA-sensitive glutamate receptors, neurexin-1α, and thrombospondin proteins by binding to $\alpha_2\delta$-1. Thus, these effects may contribute substantially to gabapentinoid therapeutic mechanism of action.

Significance Statement

It is widely believed that gabapentin and pregabalin act by modestly reducing the membrane localization and activation of voltage-gated calcium channels at synaptic endings in spinal cord and neocortex via binding to the $\alpha_2\delta$-1 protein. However, recent findings show that the $\alpha_2\delta$-1 protein also interacts with NMDA-sensitive glutamate receptors, neurexin-1α, thrombospondins (adhesion molecules), and other presynaptic proteins. These newly discovered interactions, in addition to actions at calcium channels, may be important mediators of gabapentin and pregabalin therapeutic effects.

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Introduction

The amino acid drugs gabapentin (Neurontin®) and pregabalin (Lyrica®) are mainstay treatments for neuropathic pain from diabetic neuropathy, post-herpetic neuralgia or spinal cord injury. In animal models (reviewed in: Tuchman et al., 2010) and clinical trials, gabapentinoid drugs reduce several kinds of chronic, neuropathic and post-surgical pain for a short period after dosing. In addition, if given throughout injury, they reduce subsequent long-lasting pain in animal models (Field et al., 1997) and are often used during surgery to reduce long-lasting pain and opiate use (Buvanendran et al., 2010; Clarke et al., 2012). They also are approved for preventing focal seizures of epilepsy, and pregabalin is approved for fibromyalgia and for generalized anxiety disorder (in the EU). Gabapentin and pregabalin are similar structurally, pharmacologically and in side-effect profile, but differ in the extent of oral absorption. Because gabapentin is a structural derivative of GABA (gamma-aminobutyric acid, the primary inhibitory neurotransmitter), the USAN Council chose the name “gabapentin.” However, this does not indicate its mechanism: gabapentin and pregabalin are essentially inert at GABA receptors and synapses (reviewed in: Taylor et al., 2007; Taylor, 2009; Dolphin, 2016). Recently, a third, gabapentinoid compound, mirogabalin, has been studied (Domon et al., 2018). All three drugs bind with high affinity and specificity to a membrane-bound protein originally identified as an auxiliary subunit of voltage-gated calcium channels. The $\alpha_2\delta$-1 and $\alpha_2\delta$-2 isoforms (coded by CACNA2D1 and -2 genes) mediate high affinity binding of both gabapentin and pregabalin in forebrain and spinal cord (Gee et al., 1996; Bian et al., 2006). Recently, a cryo-electron microscope study of purified rabbit skeletal muscle calcium channels (Wu et al., 2016) enabled a high resolution model (Kotev et al., 2018) showing $\alpha_2\delta$-1 with a bound drug molecule (Figure 1). An artificial alanine substitution for the arginine residue at $\alpha_2\delta$-1 position 217

(R217A) greatly reduces drug binding (Wang et al., 1999) and also reduces gabapentinoid analgesic (Field et al., 2006), anxiolytic-like (Lotarski et al., 2011) and anticonvulsant actions (Lotarski et al., 2014) in mouse models. In contrast, the actions of non $\alpha_2\delta$-1 drugs were spared.

Although $\alpha_2\delta$-2 also binds gabapentin and pregabalin with high affinity, this apparently does not contribute to most clinical pharmacology, since mutations that reduce drug binding to $\alpha_2\delta$-2 failed to alter gabapentinoid anxiolytic-like or anticonvulsant action in mice, and did not alter drug effects on glutamate release (Lotarski et al., 2011; Quintero et al., 2011; Lotarski et al., 2014). Although analgesic effects of gabapentinoid drugs have not been tested in $\alpha_2\delta$-2 mutant mice, findings to date suggest that $\alpha_2\delta$-2 does not contribute much to gabapentinoid analgesia (e.g. Field et al., 2006) and $\alpha_2\delta$-1 is now widely accepted as the key to gabapentin and pregabalin drug actions for analgesia, anxiolytic action and seizure prevention. One exception shows a notable drug effect that has been attributed to action at $\alpha_2\delta$-2: a recent study shows that gabapentin acts at $\alpha_2\delta$-2 to increase corticospinal plasticity and regeneration after spinal cord injury in mice (Sun et al., 2020). This study also shows enhanced behavioral recovery, but to date, it is not known what other cellular proteins might interact with $\alpha_2\delta$-2 to stimulate growth cones and the formation of new corticospinal connections. These findings suggest that chronic treatment with gabapentinoid drugs (acting at $\alpha_2\delta$-2) could enhance recovery after spinal cord injury. Furthermore, Obermair and colleagues (Geisler et al., 2019) showed that presynaptic overexpression or ablation of one splice variant of $\alpha_2\delta$-2 altered the postsynaptic localization of $GABA_A$ receptors in cultured hippocampal neurons. Although these findings are fascinating, they may not relate directly to gabapentinoid analgesia, and so the rest of our review will focus on $\alpha_2\delta$-1. It also should be noted that $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4 provide important biological functions to interact with calcium channels in various anatomical areas (e.g. Davies et al., 2006) (reviewed in: Dolphin, 2013; Dolphin, 2016; Dolphin, 2018).

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The $\alpha_2\delta$-1 protein binds directly to the calcium channel pore protein, α_1 or $Ca_V\alpha1$ (Figure 1, Table 1). This modifies both the kinetic properties (Welling et al., 1993; Gurnett et al., 1996) and cellular localization (Heblich et al., 2008; Hendrich et al., 2008; Kadurin et al., 2016) of calcium channels (reviewed in: Heblich et al., 2008; Hendrich et al., 2008; Zamponi et al., 2015). Most investigators conclude that gabapentinoid analgesia derives from reducing the excitability of neuron networks by reduced numbers of functional calcium channels and subtly modulated functional properties. However, the molecular details of reduced transmitter release caused by gabapentinoids (reviewed in: Dooley et al., 2007) have not been clear.

In addition to its role as a subunit of voltage-gated calcium channels, $\alpha_2\delta$-1 acts as a cell adhesion molecule with structural homologies to integrins (Pan et al., 2016b) that also contain a von Willebrand homology (VWA) domain with a metal ion dependent adhesion site (MIDAS) (see Figure 1). The VWA domains of integrins bind to extracellular matrix proteins and deliver signals to the cytosol and also change conformation in response to cellular signals (Ginsberg et al., 2005; e.g. Li et al., 2017). The VWA domain of $\alpha_2\delta$-1 mediates the interaction between $\alpha_2\delta$-1 and calcium channels (Canti et al., 2005). In a cell adhesion role, $\alpha_2\delta$-1 in striate muscle cells promotes adhesion to collagen (Garcia et al., 2008) and increases cell motility.

Recent studies show that $\alpha_2\delta$-1 alters the function of proteins other than voltage-gated calcium channels. In particular, gabapentinoids modify certain N-methyl-D-aspartate (NMDA)-sensitive glutamate receptors (NMDA receptors), modify some actions of neurexin-1α and limit some actions of thrombospondin. These effects are unrelated to calcium channels and could contribute to the pharmacological actions of gabapentinoid drugs, but this idea remains controversial – and is the focus of this review.

Gabapentinoid Drugs Reduce Neurotransmitter Release

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Previous studies showed that gabapentinoid drugs reduce the release of excitatory neurotransmitters from neuronal tissues by acting at the $\alpha_2\delta$-1 binding site (reviewed in: Dooley et al., 2007). The prevailing thought has been that these drugs act therapeutically to reduce excitability in spinal cord sensory circuits and in neocortex by subtly reducing excitatory neurotransmitter release at many synapses at once, and glutamate synapses in many regions have abundant $\alpha_2\delta$-1 protein (Taylor and Garrido, 2008).

The amount of $\alpha_2\delta$-1 protein at synapses is remarkably up-regulated after neuronal injuries, both in the spinal cord dorsal horn (Luo et al., 2001; Luo et al., 2002; Li et al., 2004; Li et al., 2006; Bauer et al., 2009; Boroujerdi et al., 2011) and in neocortex (Andresen et al., 2014; Prince et al., 2016; Luo et al., 2018). Interestingly, this up-regulation only alters $\alpha_2\delta$-1 and not $\alpha_2\delta$-2, and the upregulated $\alpha_2\delta$-1 is of a different post-translational splice variant and different glycosylation pattern than endogenous $\alpha_2\delta$-1 in the spinal cord (Luo et al., 2001) and dorsal root ganglia (Lana et al., 2014). Electron microscopy has shown localization of $\alpha_2\delta$-1 protein both presynaptically and postsynaptically in rodent dorsal spinal cord (Bauer et al., 2009), with about twice as much immunoreactivity presynaptically as postsynaptically. Also, following neuropathic injury, $\alpha_2\delta$-1 increases only at presynaptic sites (Figure 3).

Several studies show that gabapentin and pregabalin reduce the spontaneous rate of release of vesicles from glutamate synapses, measured by miniature excitatory synaptic currents (mEPSCs), which reflect the release of individual synaptic vesicles. Unlike synaptic responses triggered by action potentials, mEPSCs are insensitive to the sodium channel blocker tetrodotoxin and the vesicles released spontaneously are regulated differently from the vesicle pool released by action potentials (reviewed in: Ramirez and Kavalali, 2011). For example, spontaneous release occurs from a separate pool of vesicles (Sara et al., 2005) and is regulated by different synaptic proteins (Groffen et al., 2010) than evoked release. A recent

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study (Ferron et al., 2018) shows that the presence of mature $\alpha_2\delta$-1 in cultured neurons promotes both synchronous and asynchronous neurotransmitter release.

Such mEPSCs recorded in dorsal spinal cord (or trigeminal nucleus) neurons typically have rates in the range of 0.2 to 1.0 Hz, and the rate is augmented in response to experimental peripheral nerve damage (Li et al., 2014a; Li et al., 2014b; Zhou and Luo, 2015; Alles et al., 2017; Chen et al., 2018), chemotherapy-induced allodynia (Chen et al., 2019) allodynia from tolerance to repeated morphine treatment (Deng et al., 2019) or from artificial excess expression of $\alpha_2\delta$-1 genes (Zhou and Luo, 2014). In each of these studies, the drugs gabapentin or pregabalin normalize pathologically elevated rates of mEPSCs in rat or mouse neuronal tissue with synapses in spinal cord dorsal horn (Patel et al., 2000; Li et al., 2014b; Matsuzawa et al., 2014; Zhou and Luo, 2014; Zhou and Luo, 2015; Park et al., 2016; Alles et al., 2017; Chen et al., 2018; Chen et al., 2019; Deng et al., 2019). Gabapentinoids reduced the rate of mEPSCs between glutamatergic neurons in rat entorhinal cortex (Cunningham et al., 2004), in neocortex neurons after cortical freeze lesions (Andresen et al., 2014; Lau et al., 2017), between neocortex and striatal neurons (Zhou et al., 2018), at glutamate neurons in hypothalamus of spontaneously hypertensive rats (Ma et al., 2018), at the mouse calyx of Held (Di Guilmi et al., 2011) and in cortico-striatal synapses after prolonged prior stimulation of striatum (Nagai et al., 2019). Therefore, the most widely replicated effects of gabapentin and pregabalin at the cellular level are decreases in the rate of mEPSCs at excitatory synapses, particularly at synapses with pathologically enhanced activity. Interestingly, gabapentin had little effect on the rate of mEPSCs recorded in inhibitory neurons in dorsal spinal cord (Zhou and Luo, 2015; Alles et al., 2017).

Despite agreement that gabapentinoid drugs reduce the rate of spontaneous mEPSCs, and also subtly reduce bulk neurotransmitter release from whole tissues, it is not clear if these effects result from decreased current through presynaptic calcium channel α_1 subunits. In fact, one set

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of studies showed that over-expression of $\alpha_2\delta$-1 in cultured cortex neurons increases neurotransmitter release and synaptic localization of calcium channels but *decreases* presynaptic calcium influx (Hoppa et al., 2012; Hoppa et al., 2014).

Additional Binding Partners of $\alpha_2\delta$-1

Although $\alpha_2\delta$-1 was originally identified in association with voltage-gated calcium channels, it has subsequently been found to associate with proteins other than calcium channels based on several methods (see Table 1 and Figure 2). There also is evidence from fluorescent microscopy of living cells (Table 2), including the extracellular matrix proteins, collagen and thrombospondin. In addition, gabapentin binding to $\alpha_2\delta$-1 prevents both thrombospondin and NMDA receptors from augmenting the release of glutamate at synapses and also prevents thrombospondin from promoting the formation of new glutamate synapses (see sections below). The $\alpha_2\delta$-1 protein has several unusual properties that may contribute to its selectivity in interacting with other proteins. It is highly glycosylated (decorated with polysaccharides) and is found concentrated in cholesterol-rich and detergent-resistant membrane microdomains called lipid rafts (Davies et al., 2006; Dolphin, 2013; Dolphin, 2016). In addition, mature $\alpha_2\delta$-1 seems to exist in some situations bound to membranes by a glycosylphosphatidylinositol-anchor (GPI anchor), attached during post-translational protein processing (Davies et al., 2011). The GPI anchor is needed for $\alpha_2\delta$-1 interaction with calcium channel α_1 (Davies et al., 2011) but not for interactions with thrombospondin (Risher et al., 2018) or NMDA receptors (Chen et al., 2018). The GPI anchor adheres the protein to only the outer leaflet of the plasma membrane (Alvarez-Laviada et al., 2014) and favors localization in cholesterol-rich membrane areas. The putative relationships of $\alpha_2\delta$-1 with several of these known protein binding partners is shown in Figure 3. Three-dimensional structures of partner proteins have been determined, including the voltage-gated calcium channel (Wu et al., 2016), α-neurexin (Miller et al., 2011), thrombospondin

(Carlson et al., 2008), heteromeric NMDA receptors (Karakas and Furukawa, 2014) and BK calcium-dependent potassium channels (Yuan et al., 2010). The evidence for gabapentinoid drugs acting via these various proteins is discussed below, beginning with NMDA receptors, for which there is the most evidence.

NMDA Receptor Interaction with $\alpha_2\delta$-1

Early in the investigation of the mechanism of action of gabapentin, it was suggested that NMDA receptors in brain and spinal cord were mediators of its pharmacology (Oles et al., 1990; Singh et al., 1996; Jun and Yaksh, 1998; Yoon and Yaksh, 1999), but this suggestion was generally overlooked. Results with gabapentinoid drugs in models of NMDA receptor function are summarized in Table 3. The early studies showed that the analgesic, anticonvulsant, and anxiolytic actions of gabapentin in rodent models were prevented by prior intra-cerebroventricular injection with the NMDA receptor glycine site agonist D-serine, but not its inactive stereoisomer L-serine (D-serine injections alone did not alter behavioral responses). These findings with animal models resembled those with HA-966 (Singh et al., 1990), an experimental drug with partial agonist actions at the NMDA receptor glycine site. In addition, an unpublished doctoral thesis showed that isolated rat striatal neurons have electrical responses to NMDA plus glycine that were reduced by gabapentin, and gabapentin alone behaved like a glycine-site partial agonist (Sprosen, 1991). However, these authors noted that gabapentin did not displace radiolabeled glycine binding at NMDA receptors, and D-serine did not interact with gabapentin radioligand binding at $\alpha_2\delta$-1, so they concluded that the gabapentin-NMDA receptor interaction, if real, must be indirect (Singh et al., 1996). Pregabalin also was later shown not to interact with binding sites on NMDA receptors (Li et al., 2011). Once it was shown that drug binding to $\alpha_2\delta$-1 was required for the pharmacology of gabapentin and pregabalin, the early findings regarding gabapentin and NMDA receptor responses were mostly forgotten. However,

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it is now clear that, in addition to calcium channels, $\alpha_2\delta$-1 interacts with NMDA receptors and several other proteins.

NMDA Receptor Proteins Bind Directly to $\alpha_2\delta$-1

NMDA receptors consist of a tetramer of both NR1 and NR2 subunits (NR1/NR2A and NR1/NR2B are most common in brain, with occasional NR1/NR3) and they are located both presynaptically and postsynaptically in spinal cord and brain (Traynelis et al., 2010). Chen and coworkers from the H.L. Pan lab (Chen et al., 2018) showed with co-immunoprecipitation that $\alpha_2\delta$-1 interacts with the NMDA receptors of GluN1/2A and GluN1/2B types by binding that requires a specific sequence on the GluN1 subunit. This physical interaction between proteins also requires complete multimeric NMDA receptor proteins, because single subunits (GluN1, GluN2A or GluN2B) did not interact with $\alpha_2\delta$-1. The interaction occurs between the membrane spanning C-terminal region of $\alpha_2\delta$-1 and an unidentified region of NMDA receptor heteromers that includes the NR1 subunit. These interactions between $\alpha_2\delta$-1 and NMDA receptors do not require the widely studied von Willebrand domain of the $\alpha_2\delta$-1 protein, in contrast to the interaction between $\alpha_2\delta$-1 and thrombospondin (Eroglu et al., 2009) or between $\alpha_2\delta$-1 and calcium channel α_1 subunits (Canti et al., 2005). Furthermore, studying fluorescent resonance energy transfer (FRET) between tagged $\alpha_2\delta$-1 proteins and tagged NMDA receptors in model cells, Chen et al. showed that FRET (which is absent unless the two proteins are physically adjacent) is absent in the presence of gabapentin (Chen et al., 2018). Additional data suggests that gabapentin decreases the physical interaction between $\alpha_2\delta$-1 and NMDA receptors in this system and also disrupts traffic of both $\alpha_2\delta$-1 and NMDA receptors from the cytosol to the cell membrane. In addition, experimental co-expression of $\alpha_2\delta$-1 with GluN1/2A alters NMDA receptor properties such that much more inward current occurs at membrane potentials between -80 mV and -20 mV; that is, $\alpha_2\delta$-1 reduces magnesium block of the NMDA receptor

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channel that ordinarily prevents current from flowing at resting potentials. Magnesium block ordinarily prevents NMDA receptors from functioning at resting membrane potentials and causes NMDA receptors to function as coincidence detectors, only active with simultaneous glutamate activation and cell membrane depolarization (Traynelis et al., 2010). The reduced magnesium block was not seen when $\alpha_2\delta$-1 was co-expressed with GluN1/2B receptors, but only with GluN1/2A receptors (Chen et al., 2018). Importantly, the effect of $\alpha_2\delta$-1 on magnesium block was completely absent in the presence of gabapentin. These results suggest that association of NMDA GluN1/2A receptors with $\alpha_2\delta$-1 would cause more than the normal amount of glutamate-gated current near resting membrane voltages, but this augmentation of NMDA receptor responses would be reduced by gabapentinoid drugs.

In cases where it was tested, the ability of gabapentin and pregabalin to reduce spontaneous vesicle release in chronic pain models was absent if NMDA receptors were blocked in spinal cord dorsal horn neurons (Chen et al., 2018; Chen et al., 2019; Deng et al., 2019). Similarly, pregabalin reduced spontaneous vesicle release (measured by release of fluorescent dye from synaptic vesicles) from synapses in cultured hippocampal neurons (Micheva et al., 2006), but not with NMDA receptors blocked. Since $\alpha_2\delta$-1 is expressed mostly presynaptically, it is likely that presynaptic membranes are an important anatomical site of $\alpha_2\delta$-1 interactions with NMDA receptors as suggested experimentally (Chen et al., 2018; Zhou et al., 2018; Chen et al., 2019; Deng et al., 2019). However, it will require additional studies to conclusively show that presynaptic NMDA receptors are uniquely sensitive to gabapentinoids. In summary, it is likely that gabapentin and pregabalin reduce spontaneous (miniature) synaptic release in several regions of brain a manner that requires $\alpha_2\delta$-1-linked presynaptic NMDA receptors.

Although considerable evidence links gabapentinoid drugs to some NMDA receptors, the anatomical distribution of immunostained NMDA receptors in brain (Petralia et al., 1994) is quite different from that for immunostained $\alpha_2\delta$-1 (Taylor and Garrido, 2008). For example, NMDA

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receptor immunostaining is most dense in cell body layers of hippocampus (where $\alpha_2\delta$-1 immunostaining is sparse) with denser $\alpha_2\delta$-1 immunostaining in dendritic layers. Furthermore, a survey of gabapentinoid actions on NMDA receptor-dependent processes (Table 3) shows both inhibitory effects and negligible effects in different preparations. Therefore, it is clear that $\alpha_2\delta$-1 proteins and gabapentinoid drugs interact only with a subset of NMDA receptors.

Analgesia Produced by Gabapentinoids Compared to Known NMDA Antagonists

NMDA antagonists acting at all NMDA receptors have pharmacological profiles very different form the gabapentinoids and are far from ideal analgesic, antiseizure or anxiolytic drugs. To date, no broad-spectrum NMDA antagonist drug has proven useful for treating epilepsy or chronic pain in humans, likely because broad spectrum NMDA antagonists have undesirable properties consisting of feeling dissociated from present time and surroundings (dissociative effects) (van Schalkwyk et al., 2018), memory disruption, confusion, agitation, nausea and sometimes psychosis, particularly at high dosages. This contrasts with gabapentin and pregabalin, which do not notably cause dissociative effects, disrupt memory nor cause agitation or psychosis in clinical use. Although ketamine (the most widely studied NMDA antagonist for treating pain) is used primarily for perisurgical pain (Kreutzwiser and Tawfic, 2019) and increasingly for treatment-resistant depression (Murrough et al., 2013; van Schalkwyk et al., 2018), it is not used for chronic neuropathic pain (Gilron, 2007), and it has strong psychotomimetic properties (Sos et al., 2013).

In contrast to recent findings that pregabalin reduces NMDA receptor responses in some regions (e.g. Zhou et al., 2018), studies show that gabapentin and pregabalin have variable effects on long-term potentiation of glutamate synapses (LTP, Table 3), with most studies showing either no effect or a very modest action of these drugs on the formation of LTP, which is widely accepted to require activation of postsynaptic NMDA receptors.

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Despite these findings, the H.L. Pan lab recently showed that in vitro, gabapentin completely prevents NMDA receptor-dependent LTP of neocortical afferents to the dorsal striatum (Zhou et al., 2018) by an action on $\alpha_2\delta$-1 that modulates both presynaptic and postsynaptic NMDA receptors. It was previously shown that cortico-striatal LTP requires presynaptic NMDA receptors (Park et al., 2014) and it has become clear that presynaptic NMDA receptors have different properties from the more widely-studied postsynaptic NMDA receptors of neocortical and hippocampal neurons (Banerjee et al., 2016; Dore et al., 2017; Bouvier et al., 2018). Other findings suggest that gabapentin may reduce presynaptic NMDA receptor activity in area CA1 of rat hippocampal slices (Suarez et al., 2005) and in entorhinal cortex slices, where gabapentin reduced the occurrence of spontaneous miniature synaptic events (Cunningham et al., 2004). Furthermore, both gabapentin and pregabalin inhibit cortical spreading depression in vitro (Cain et al., 2017), which might result from inhibiting presynaptic NMDA receptors (Zhou et al., 2013). In summary, there is solid evidence of an interaction between $\alpha_2\delta$-1 drugs and NMDA receptor function, but it appears that inhibition is restricted to only a few brain locations and/or at a molecular subset of NMDA receptors (Chen et al., 2018; Luo et al., 2018; Ma et al., 2018; Zhou et al., 2018).

Some recent authors (Chen et al., 2018) have proposed that an $\alpha_2\delta$-1 / NMDA receptor interaction is required for analgesic actions of gabapentin and pregabalin in animal models and in clinical use. However, this has only been demonstrated in the sensory nerve ligation model of neuropathic pain with spinal reflex-like tactile responding in mice. Analgesia with gabapentin or pregabalin for pain-related responses in animals that are not reflex-like and that require the forebrain have not been studied to see if they rely upon $\alpha_2\delta$-1 interactions with NMDA receptors. See for example (Bannister et al., 2017).

Furthermore, it is clear that NMDA receptors are not required for all of the actions of gabapentin or pregabalin. For example, gabapentin rapidly reduces the frequency of spontaneous miniature

excitatory synaptic potentials in dorsal horn neurons of sensory nerve ligated mice (an apparently presynaptic action), even in the presence of an NMDA antagonist (Zhou and Luo, 2015). Nevertheless, it is quite interesting that gabapentin alters NMDA receptor function in animal models of acute focal ischemia (Luo et al., 2018) and at corticostriate glutamate afferent inputs to the striatum (Zhou et al., 2018) including LTP of the corticostriate pathway.

In summary, NMDA receptors newly incorporated into neuronal membranes following ischemia (Luo et al., 2018), neuropathic pain stimulation (Chen et al., 2018; Chen et al., 2019) or spreading depression (Cain et al., 2017) may be particularly sensitive to gabapentin and pregabalin, as are certain native NMDA receptors located in brain regions such as the striatum (Zhou et al., 2018).

$\alpha_2\delta$-1 Proteins Interact with only Certain NMDA Receptors

Presynaptic NMDA receptors (Banerjee et al., 2016; Abrahamsson et al., 2017; Bouvier et al., 2018) have been studied between nearby pyramidal neurons of neocortex, on cortico-amygdalar glutamatergic endings, and in long-term depression in the hippocampus that is timing-dependent. These receptors are important for use-dependent facilitation of glutamate release (Woodhall et al., 2001; Li et al., 2008; Li et al., 2009) and presynaptic NMDA receptors have specific protein subunits that differ from those of the more widely studied postsynaptic NMDA receptors. For example, mature mice have predominantly GluN1/GluN2B subunits at presynaptic NMDA receptors between neocortical and hippocampal neurons (Woodhall et al., 2001; Larsen et al., 2011) while postsynaptic GluN1/GluN2A and GluN1/GluN2B receptors both contribute to LTP In hippocampus (Liu et al., 2004; Berberich et al., 2005). Glutamate receptors consisting of GluN1/GluN2B subunits also are required for long-term depression in hippocampus (Liu et al., 2004). Although details are not yet very clear, it is likely that gabapentinoids and NMDA antagonists differ functionally by acting at distinct subpopulations of

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NMDA receptors. Previous studies suggested that some cellular actions of gabapentin and pregabalin require activation of protein kinases (Gu and Huang, 2001; Maneuf and McKnight, 2001; Fehrenbacher et al., 2003), and it is clear that enhanced NMDA receptor function from phosphorylation contributes to neuropathic and chronic pain (Salter and Kalia, 2004; Salter and Pitcher, 2012). It has not yet been studied whether alternative splicing (Sengar et al., 2019) of NMDA receptors alters the interaction with $\alpha_2\delta$-1 and modulation by gabapentinoid drugs. However, a recent paper (Huang et al., 2020) indicates that increased NMDA receptor phosphorylation in spinal cord enhances the amount of $\alpha_2\delta$-1 bound NMDA receptor protein and also increases the gabapentin sensitivity of NMDA receptors.

Up-Regulation of $\alpha_2\delta$-1 and NMDA Receptor Function Following Neuropathic Injury

It has been known for 20 years that spinal $\alpha_2\delta$-1 protein is markedly upregulated after peripheral nerve injury and this upregulation causes neuropathic pain symptoms in animal models (Luo et al., 2001; Boroujerdi et al., 2011; Gong et al., 2018). More recently, electron microscopy has revealed that the change in $\alpha_2\delta$-1 density occurs primarily in presynaptic sensory neurons rather than postsynaptic dendrites (Bauer et al., 2009) (Figure 3). That change is accompanied by increased spinal neuron responses to application of NMDA and also by increases in the frequency of glutamate-dependent miniature synaptic potentials in dorsal horn sensory neurons (Chen et al., 2018). The increased NMDA receptor-mediated responses and increased NMDA receptor-mediated miniature synaptic potentials both are blocked by acute application of gabapentin or pregabalin. Therefore, in at least some models of neuropathic pain there is both up-regulation of $\alpha_2\delta$-1 protein and also up-regulation of NMDA receptor function in the spinal cord.

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Neurexin-1α as an Additional Target of Gabapentinoids

Neurexins are a family of presynaptic proteins that protrude from presynaptic terminals into the extracellular space and that have diverse functions in different neuron types. The neurexins are expressed both as full-length membrane-spanning protein (α-neurexin) and truncated (β-neurexin) versions. Neurexins form an important part of the trans-synaptic network that modulates synapse functions (Sudhof, 2017). Neurexins prominently bind to postsynaptic proteins of the neuroligin family (Miller et al., 2011) to stabilize and spatially align pre-and post-synaptic elements. Both proteins interact with a large number of other synaptic scaffolds, postsynaptic receptors and presynaptic proteins (Biederer et al., 2017; Sudhof, 2017).

Cell cultures of neurons lacking native neurexins have reduced synaptic calcium influx and transmitter release that is restored by α-neurexin expression. Surprisingly, this effect of α-neurexin to enhance synaptic function appears to be mediated by a weak interaction between neurexin and $\alpha_2\delta$-1, at least in one model system (Brockhaus et al., 2018). Interestingly, these effects of $\alpha_2\delta$-1 on neurexin function did not appear to be mediated by a stable protein-protein interaction, but rather by a somewhat weak and transient interaction (Brockhaus et al., 2018).

More recently, it was shown that the soluble extracellular domain of neurexin-1α reduces radioligand binding of [^{3}H]-gabapentin to recombinant $\alpha_2\delta$-1, indicating a direct interaction between the two proteins (San Segundo et al., 2019). Functional studies of this interaction were done with a model synapse system cultured in vitro. Microcultures of individual rat superior cervical autonomic neurons form monosynaptic acetylcholine synapses back onto themselves when grown in this manner. These “autaptic” synapses were studied using electrophysiology and presynaptic calcium imaging of synaptic boutons. Application of pregabalin (30 μM) reproducibly and reversibly reduced neurotransmitter release by about 50%, as measured by postsynaptic current. The pregabalin effect was particularly pronounced in response to rapid

trains of presynaptic action potentials, and these measurements indicated that pregabalin reduced the size of the "readily releasable pool" of synaptic vesicles (Rosenmund and Stevens, 1996; Kaeser and Regehr, 2017). Importantly, the effect of pregabalin was entirely independent of changes in the function of presynaptic voltage-gated calcium channels, since presynaptic calcium influx (measured by a fluorescent indicator) was unchanged. Finally, it was shown that extracellular application of a soluble fragment of neurexin-1α reduced neurotransmitter release to the same extent as pregabalin and occluded the effects of pregabalin, suggesting that pregabalin and neurexin alter synapse function through the same molecular pathway.

This investigation (San Segundo et al., 2019) was aided by several technical advantages over other studies of gabapentinoids. The hardiness of autonomic ganglion neurons allowed long-term recordings of synaptic currents without much postsynaptic receptor plasticity (a complicating factor at hippocampal synapses, for example). This allowed estimation of the time course of drug effects (onset time of about 5 min and washout time constant of about 50 min) and straightforward estimation of readily releasable pool size. The use of a genetically coded presynaptic calcium indicator was possible since only synapses from a single cell were present in each microculture. These results show that, at least in this model system, pregabalin reduces synaptic strength by reducing the readily releasable pool via an interaction between $\alpha_2\delta$-1 and presynaptic α-neurexin, without any change in the function of presynaptic calcium channels. It will require additional work to establish whether the changes mediated by neurexin are relevant for analgesia by gabapentinoid drugs.

Thrombospondins as an Additional Target of Gabapentinoids

Thrombospondins are a family of extracellular matrix proteins (reviewed in: Risher and Eroglu, 2012) that in the brain are formed mostly by astrocytes. Astrocytes, both in spinal cord and neocortex, are thought to play a major role in the pathogenesis of chronic pain (Hansen and

Malcangio, 2013). Thrombospondins are released from astroctyes into the extracellular space in response to several stimuli including activation of glial P2X receptors (Tran and Neary, 2006; Kim et al., 2016) that respond to ATP from damaged cells or released from nearby glial calcium waves (Guthrie et al., 1999; Haydon and Carmignoto, 2006). The P2X receptors play a significant role in chronic pain (Chizh and Illes, 2001). The $\alpha_2\delta$-1 protein was shown to interact with thrombospondin (particularly subtypes 1 and 4) by immunoprecipitation (Eroglu et al., 2009) and by interaction between purified proteins (Park et al., 2016; Park et al., 2018), see Table 1. However, two separate studies have failed to show that gabapentin directly disrupts the molecular interaction between thrombospondin and $\alpha_2\delta$-1 proteins in vitro (Lana et al., 2016; El-Awaad et al., 2019), suggesting that the inhibition by gabapentin of thrombospondin actions could be indirect or require the presence of other proteins. Additional studies suggest that signaling proteins, including the scaffolding protein LRP1 (Kadurin et al., 2017) or activation of the small Rho GTPase, Ras-related C3 botulinum toxin substrate 1 (Rac1) are part of the biochemical pathway involved in the synaptogenic action that is inhibited by gabapentinoid drugs (Risher et al., 2018). Interestingly, LRP1 also was found to interact with β1-integrins at the cell surface to regulate their function (Theret et al., 2017).

Gabapentin and pregabalin inhibit several thrombospondin effects via $\alpha_2\delta$-1, and prevent the formation of new synapses in response to thrombospondin application or astroctye activation. It has been known for some time that astrocyte-conditioned media promotes the formation of new glutamate synapses in cultured neurons and it was found that this occurs because $\alpha_2\delta$-1 proteins act as neuronal thrombospondin receptors (Eroglu et al., 2009). Following neuropathic sensory nerve injury (Kim et al., 2012) or spinal cord injury (Zheng et al., 2005), thrombospondin induces chronic pain-like states and increase the number of excitatory synapses and the rate of glutamatergic mEPSCs in spinal dorsal horn in animal models (Nguyen et al., 2009; Kim et al., 2012).

The newly formed synapses in response to thrombospondin are initially silent glutamate synapses (with NMDA receptors but no functional AMPA receptors) (Eroglu et al., 2009; Risher et al., 2018; Yu et al., 2018; Wang et al., 2020). Recent findings indicate that pain related responses in mice from over-expression of either $\alpha_2\delta$-1 or thrombospondin-4 can be acutely blocked with gabapentin or pregabalin (Park et al., 2016), and also with a peptide fragment that prevents the interaction between $\alpha_2\delta$-1 and thrombospondin; this peptide also blocks analgesia with pregabalin in a spinal nerve injury model in vivo (Park et al., 2018). Although repeated treatment with gabapentinoids reduces the formation of new synapses induced by thrombospondin, once new synapses formed, gabapentin applied subsequently did not reduce the number or size of synapses but did effectively reduce pain-related behaviors. Therefore, there appear to be two distinct functions of drugs acting at $\alpha_2\delta$-1/thrombospondin, one long-lasting, involving synaptic size and the other more rapid and reversible, acutely reducing pain-related behaviors.

The effects of thrombospondin to promote chronic pain-like states are prevented by repeated doses of gabapentin acting at $\alpha_2\delta$-1 either in the spinal cord (Crosby et al., 2015; Pan et al., 2015; Park et al., 2016) or in the somatosensory cortex (Kim et al., 2016). A separate study showed that thrombospondin-4 application to isolated sensory (dorsal root ganglion) neurons for 4 hours alters voltage-gated calcium currents recorded at the cell body (Pan et al., 2016a). Although gabapentin itself did not acutely reduce voltage-gated calcium currents in these cells, it did prevent the long-term effects of thrombospondin-4 to decrease high-voltage activated currents and increase low-voltage activated (L-type) currents. These results strongly suggest that some actions of gabapentinoid drugs to reduce chronic pain may be independent of reducing calcium channel function.

The idea that a thrombospondin/$\alpha_2\delta$-1 interactions might underlie gabapentinoid drug actions other than analgesia in chronic pain also has been studied. Repeated prophylactic gabapentin

treatment in models of post-traumatic epilepsy (Li et al., 2012; Andresen et al., 2014; Lau et al., 2017; Takahashi et al., 2018) prevented the stabilization of abnormal new synapses in neocortex, presumably by blocking the action of astrocyte-derived thrombospondin.

A recent study in the striatum of mice showed that selective stimulation of astrocytes caused the formation of new and stronger glutamate synapses onto striatal medium spiny neurons. This synaptogenesis was mediated by glial-derived thrombospondin-1 acting at $\alpha_2\delta$-1, whose effects were blocked by gabapentin (Nagai et al., 2019). The formation of stronger synapses in striatum was associated with behavioral hyperactivity and disrupted attention and these abnormal behaviors also were prevented by repeated prophylactic gabapentin treatment. An additional study in mice shows that in the nucleus accumbens shell (an area implicated in drug addiction), cocaine administration triggers the formation of new silent glutamate synapses via thrombospondin-2, and this process is blocked by repeated gabapentin administration acting at $\alpha_2\delta$-1 (Wang et al., 2020). Finally, an important study (Risher et al., 2018) shows that in mouse neocortex, repeated gabapentin treatment reduces the normal formation of new cortico-cortical synapses by thrombospondin, and this occurs by a postsynaptic interaction between thrombospondin and $\alpha_2\delta$-1 that requires both NMDA receptors and the GTPase, Rac1. In this study, activation of the thrombospondin/ $\alpha_2\delta$-1 pathway had no effect on GABA synapses. Finally, a recent study (Brennan et al., 2020) shows that prophylactic treatment with gabapentin in an animal model of spinal cord injury prevents plasticity in spinal autonomic circuits that cause autonomic dysreflexia by preventing thrombospondin-induced changes.

In summary, $\alpha_2\delta$ drugs may prevent the stabilization of new or stronger glutamate synapses formed specifically in response to astrocyte activation and the subsequent release of thrombospondin. This astrocyte-activated process of synaptic strengthening may occur in response to neuronal damage in neocortex (from release of glutamate, potassium ions and ATP), sustained astrocyte activation via $GABA_B$ receptors in striatum, by neuronal activation by

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cocaine in nucleus accumbens and also by normal activation of glia during synaptogenesis. Each of these processes appear to activate astrocytes and strengthen nearby glutamate synapses in a process that is blocked by gabapentinoid drugs. It seems likely that the interaction between gabapentinoid drugs and thrombospondin function may be relevant for preventing long-lasting pain related anatomical changes. It is less clear that the interaction between $\alpha_2\delta$-1 and thrombospondin is necessary for short-lasting analgesia from acute treatment with gabapentinoid drugs in some animal models or clinical use.

Other $\alpha_2\delta$-1 Binding Proteins

BK type voltage-gated potassium channels compete with voltage-gated calcium channel α1 subunits for $\alpha_2\delta$-1 protein binding (Zhang et al., 2018). BK channels directly associate with $Ca_V2.1$ and $Ca_V2.2$ channels at presynaptic endings in brain (Berkefeld et al., 2006; Dai et al., 2009), putting them within a few nanometers of vesicle release machinery at synapses. This localization is required for BK channel function to rapidly hyperpolarize cells in response to presynaptic calcium influx and to moderate calcium-induced vesicle release. Although not studied by protein-protein interaction techniques, recent findings (Hoppa et al., 2014) indicate that changes in $\alpha_2\delta$-1 expression in cultured neurons alter the function of presynaptic K_V1 and $K_V3.1$ potassium channels. To date, no studies of gabapentin or pregabalin have been published on BK channel function or $K_V1/K_V3.1$ channel function at synaptic endings.

Brain $\alpha_2\delta$-1 also interacts directly with the membrane-bound protein trafficking molecule LRP1, a transmembrane protein that is involved in mediating $\alpha_2\delta$-1 effects on calcium channel traffic to and from the membrane (Kadurin et al., 2017). LRP1 has many protein-interacting domains and is known to interact with a variety of other proteins (Lillis et al., 2008) including the extracellular matrix proteins fibronectin and thrombospondin, apolipoprotein E and intracellular proteins such as RAP, Shc, protein kinase C, PSD-95 (which plays and important role with postsynaptic

NMDA receptors) and the endoplasmic reticulum protein calreticulin. Calreticulin is involved in protein processing in endosomes. It is possible that LRP1 participates in the interaction between $\alpha_2\delta$-1 and thrombospondin or other proteins like the cell signaling protein RAC1 (Risher et al., 2018).

There is evidence that $\alpha_2\delta$-1 interacts with several other proteins in brain (Table 2), although many of these interactions are very likely indirect, particularly those involving presynaptic vesicle release proteins that are known to directly interact with calcium channel α_1 subunits (reviewed in: He et al., 2018).

Summary and Conclusions

Since the discovery of the analgesic activity of gabapentin, identifying its mechanism of action at the cellular level has been challenging, with many promising, plausible, but apparently false leads. It is now clear that, although the analgesic effects of gabapentin-like drugs involve an interaction with $\alpha_2\delta$-1, those effects clearly are not limited to voltage-gated calcium channels. Instead, there is compelling new evidence that several gabapentinoid drug effects involve other proteins that interact with $\alpha_2\delta$-1, specifically a subset of NMDA receptors, neurexin-1α, and thrombospondins.

Interactions between $\alpha_2\delta$-1 and neurexins could explain at least part of the gabapentinoid drugs ability to reduce neurotransmitter release from neocortical and hippocampal tissues, where there is not always a clear correlation between neurotransmitter release and decreased calcium channel function. In light of the findings reviewed here about interactions of $\alpha_2\delta$-1 with multiple synaptic proteins, and findings that overexpression of $\alpha_2\delta$-1 in cultured neocortical neurons actually *decrease* presynaptic calcium influx (Hoppa et al., 2012; Hoppa et al., 2014), it seems likely that synaptic proteins other than calcium channel $\alpha 1$ subunits may be required for gabapentinoid drugs to reduce mEPSC frequency and excitatory neurotransmitter release.

The interaction between $\alpha_2\delta$-1 and NMDA receptors is particularly intriguing because NMDA receptors have long been seen as promising targets for analgesic and antiseizure drugs, but have proven elusive in terms of drugs with favorable risk/benefit profiles. Although gabapentin and pregabalin clearly do not modulate all NMDA receptors, it also is clear that some subsets of NMDA receptors are modulated by drug binding to $\alpha_2\delta$-1. The interaction of thrombospondins with $\alpha_2\delta$-1 may also may be important for some pharmacological actions of gabapentin and pregabalin, particularly when pain is augmented by activation of glia that release thrombospondin to form new and enlarged glutamate synapses.

An important unanswered question with gabapentinoid drugs is whether different proteins can interact at $\alpha_2\delta$-1 at the same time or whether such interactions are mutually exclusive. In this regard, one study has shown that BK potassium channels compete with calcium channel α_1 subunits for binding $\alpha_2\delta$-1. However, it is not yet known if $\alpha_2\delta$-1 interactions with thrombospondins, NMDA receptors or neurexins are mutually exclusive with each other or with calcium channel α_1 proteins. An important step in this direction was provided by findings that a GPI anchor at the membrane portion of $\alpha_2\delta$-1 is required for increasing cellular calcium currents (Davies et al., 2011), but a similar modification *prevents* the $\alpha_2\delta$-1/thrombospondin action that enlarges synaptic spines (Risher et al., 2018). Furthermore, a full-length and apparently membrane-spanning $\alpha_2\delta$-1 is required for $\alpha_2\delta$-1/NMDA receptor interactions (Chen et al., 2018). Thus differently-processed $\alpha_2\delta$-1 proteins are required for some of the different gabapentinoid drug functions in neurons.

In conclusion, although gabapentinoids do appear to act by binding to $\alpha_2\delta$-1, analgesic effects appear to involve voltage-gated calcium channels, but appear also to involve other proteins including a subset of NMDA receptors, neurexins, thrombospondins, and possibly other proteins. In particular, existing evidence suggests the $\alpha_2\delta$-1/thrombospondin interaction

independent of calcium channels may be important for gabapentinoid drugs to reduce long-lasting pain with only acute treatment around the time of nerve injury.

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Footnotes:

Financial Disclosure: CPT previously was employed and owned stock in Pfizer, Inc. but no longer has financial conflicts. EWH claims no conflicts.

Legends for Figures:

Figure 1. Structures of the $\alpha_2\delta$-1 protein based on cryo-electron microscopy and molecular modeling (Wu et al., 2016) of calcium channels purified from skeletal muscle. A. The numbered $\alpha_2\delta$-1 sequence with major domains colored (as in B). The immature $\alpha_2\delta$-1 protein is cleaved (between residue 960 and 961) and the two resulting segments are joined by a disulfide bridge. The arginine at position 217 (Arg217) is critical for drug binding (see text) and is shown with a red arrow in each subpart of the figure. B. Atomic resolution map of the entire voltage-gated calcium channel protein, including the ion conducting α_1 subunit (white and shades of blue for the four transmembrane domains), the γ subunit (yellow, which is not found in brain calcium channels) and the β subunit (pale green, on the cytosolic side of the membrane). The $\alpha_2\delta$-1 protein includes the von Willebrand A (VWA) (green) with a metal ion dependent adhesion site (MIDAS), Cache 1 and Cache 2 (brown and purple), and the C-terminal domain (CTD, purplish-gray). Some of the polysaccharide (glycan) groups are shown as abbreviated stick-figure sugar molecules. The R217 residue (red arrow) is buried in the middle of the protein, between the VWA domain and the Cache1 domain. C. The presumed drug binding pocket (Kotev et al., 2018) consists in part of the Arg217 residue (red arrow) that coordinates with the acid moiety of gabapentin (GBP, purple). Other residues (numbered) coordinate with both the acid and the amine of gabapentin. It is presumed that drug binding to this pocket causes a large conformational change to $\alpha_2\delta$-1 that alters its interaction with other proteins. Figures are adapted from (Wu et al., 2016; parts A and B) with permission from AAAS and from (Kotev et

al., 2018; part C) with permission from Journal of Chemical Information and Modeling, copyright (2016) American Chemical Society.

Figure 2. Proposed direct molecular interactions of $\alpha_2\delta$-1 protein (yellow) with other neuronal proteins. The outside (out) and inside (in) of the cell membrane are indicated and are the same in B through F. Evidence is based mostly on co-immunoprecipitation of solubilized proteins ex vivo (see text and Table 1 for more details). In each case, presumed sites of protein-protein interaction are shown with pink ovals. A. The extracellular loops of the voltage-gated calcium channel α_1 subunit (blue) bind to the VWA domain of $\alpha_2\delta$-1 (Gurnett et al., 1997; Wu et al., 2016). Note that this appears to require a GPI-linked $\alpha_2\delta$-1 protein (Davies et al., 2010). B. The EGF-like repeats of the thrombospondin trimer (thrombospondin 1 and 2, light green) or pentamer (thrombospondin-4, not shown) bind to VWA to signal across the membrane via the $\alpha_2\delta$-1 transmembrane domain (yellow) to intracellular proteins including Rac1 (red arrow), requiring a transmembrane variant of $\alpha_2\delta$-1 (Risher et al., 2018). C. An unknown extracellular part of $\alpha_2\delta$-1 interacts with the soluble ectodomain regions LG-1 and LG-5 of neurexin-1α (Tong et al., 2017). This interaction reduces radioligand binding of gabapentin to $\alpha_2\delta$-1 (San Segundo et al., 2019) and gabapentin applied to this complex signals to the presynaptic cytosol to reduces the effective size of the readily releasable pool of vesicles (San Segundo et al., 2019) D. The $\alpha_2\delta$-1 transmembrane domain interacts with an unknown sequence on NMDA receptor NR1/NR2A and NR1/NR2B proteins (red) (Chen et al., 2018) to alter NMDA receptor function. E. BK-type calcium dependent potassium channels (bright green) associate with $\alpha_2\delta$-1 in a mutually exclusive manner with calcium channel α_1 (Zhang et al., 2018) but effects of gabapentinoid drugs on BK channels (if any) are not known. F. LRP1 (lipoprotein related protein receptor 1, blue) binds to the $\alpha_2\delta$-1 VWA domain via two extracellular ligand binding domains (Kadurin et al., 2017).

Figure 3. Representative electron micrographs ipsilateral (***C***, ***D***) and contralateral (***E***, ***F***) to spinal nerve damage. Dots show immunogold labeling of $\alpha_2\delta$-1 in the dorsal horn (lamina I–III) of L4 and L5 spinal cord sections 14 days after sensory nerve ligation that causes allodynia. Scale bar, 0.5 μm. Arrowheads show presynaptic sites at the plasma membrane of excitatory axon terminal boutons ("b"), characterized by round synaptic vesicles and postsynaptic density). Arrows show postsynaptic sites at the extrasynaptic plasma membrane of dendritic shafts (Den); double arrows show intracellular dendritic sites. ***G***, Ratio of presynaptic to postsynaptic numbers of particles on the sides ipsilateral (filled bars) and contralateral (open bars) to injury. Figure reproduced from (Bauer et al., 2009), with permission (copyright 2009, Society for Neuroscience).

Figure 4. Proposed gabapentin-sensitive interactions of $\alpha_2\delta$-1 with synaptic proteins other than calcium channels. A. Gabapentin interferes with α-neurexin at synapses in model cells to reduce the size of the readily releasable pool of presynaptic vesicles (San Segundo et al., 2019). B. Gabapentin interferes with the action of presynaptic NMDA receptors in several systems (Chen et al., 2018; Luo et al., 2018; Ma et al., 2018; Zhou et al., 2018; Chen et al., 2019; Deng et al., 2019) to reduce the spontaneous release of synaptic vesicles and decrease NMDA receptor function. C. Gabapentin interferes with the action of thrombospondin from astrocytes and this reduces presynaptic vesicle release and also reduces postsynaptic spine enlargement (Risher et al., 2018; Wang et al., 2020). The effect of gabapentin to prevent spine enlargement is mediated by the small Rho GTPase protein, Rac-1, and requires activation of Rac-1 by guanine exchange factors (GEFs) and also requires NMDA receptors. This process then activates the actin cytoskeleton.

Table 1. Proteins Interacting Directly with $\alpha_2\delta$-1

Target Protein	Target Protein Region	$\alpha_2\delta$-1 Region	Experimental Method	Effect of $\alpha_2\delta$-1 on target	GBP or PGB Drug Effect	Reference
Ca channel α_1 subunit (Ca_V1, Ca_V2, but not Ca_V3)	Extracellular part of Domain III (?)	VWF-A domain MIDAS	Co-isolation with solubilized protein purification	↑ Ca channel membrane stability; alters Ca channel kinetics	↓ Ca channel number at membrane	(Felix et al., 1997; Canti et al., 2005)
Ca channel α_1 subunit ($Ca_V1.1$) (skeletal muscle)	Extracellular part of Domain III Loop 5; Domain I Loop 5 and Domain II Loop 5	VWF-A domain and Cache1 domain	Cryo-electron microscopy with molecular modeling of α_1 and $\alpha_2\delta$-1			(Briot et al., 2016; Wu et al., 2016)
NMDA-R NR1/2A and NR1/2B subtypes	NR1 and NR2A subunits (requires	Trans-membrane	Co-immunoprecipitation; FRET fluorescence	↑ NMDA current; ↑ presynaptic release	↓ NMDA current, ↓ $\alpha_2\delta$-1-NMDAR binding	(Chen et al., 2018; Luo et

Target Protein	Target Protein Region	$\alpha_2\delta$-1 Region	Experimental Method	Effect of $\alpha_2\delta$-1 on target	GBP or PGB Drug Effect	Reference
(both pre- and post-synapse)	assembled subunits)	C-terminal region*				al., 2018; Ma et al., 2018)
Neurexin-1α	Neurexin-1α laminin-like globular (LG) domains 1 & 5	??	Co-immunoprecipitation		Not tested	(Tong et al., 2017)
Neurexin-1α ectodomain (applied to CHO cells with expressed Ca channel subunits)	Neurexin-1α ectodomain	??	[^{3}H]-gabapentin binding to CHO cell membranes	Added neurexin ectodomain reduces gabapentin-$\alpha_2\delta$-1 binding	↓ EPSC and size of readily releasable pool in cultured sympathetic neurons	(San Segundo et al., 2019)
Neurexin-1α	??	??	mobility of $\alpha_2\delta$-1 single particles reduced by added neurexin; reported nonspecific co-immunoprecipitation			(Brockhaus et al., 2018)

Target Protein	Target Protein Region	$\alpha_2\delta$-1 Region	Experimental Method	Effect of $\alpha_2\delta$-1 on target	GBP or PGB Drug Effect	Reference
Thrombospondin-1, -2 and -4**;** (source: astrocytes) neocortex	EGF-like repeats (central region)	VWF-A domain	Co-immunoprecipitation; change in $\alpha_2\delta$-1 drug binding when thrombospondin present	↑ new synapse formation	↓ New synapses; thrombospondin ↓ drug affinity to $\alpha_2\delta$-1	(Eroglu et al., 2009; Lana et al., 2016)
Thrombospondin-4	EGF-like repeats	??	Co-immunoprecipitation; Solid phase binding; Biacore instrument	Thrombospondin enhanced pain required $\alpha_2\delta$-1	↓ sensitivity to pain and ↓ glutamate release	(Park et al., 2016; Park et al., 2018)
BK-potassium channel	N-terminal region	C-terminal region	Co-immunoprecipitation	BK competes with $\alpha_2\delta$-1 actions at Ca channels	None	(Zhang et al., 2018)
LRP1 (cellular protein traffic)	Heavy chain LBD sequences	VWF-A domain	Co-immunoprecipitation; in vitro protein pull-down	Modulates $\alpha_2\delta$-1 traffic; increases $\alpha_2\delta$-1 at cell membrane	LRP1 expression ↓ drug binding B_{max} to $\alpha_2\delta$-1	(Kadurin et al., 2017)

Abbreviations: GBP – gabapentin; PGB – pregabalin; VWF-A domain – von Willebrand factor type-A homology domain; MIDAS - metal ion dependent adhesion site; NMDA-R – NMDA receptor; BK channel – Calcium-dependent potassium channel (rapidly opens

in response to elevated intracellular calcium ion concentration); CHO cells – Chinese hamster ovary cell line; LRP1 - Low density lipoprotein (LDL) receptor-related protein-1

* - $\alpha_2\delta$-2 and $\alpha_2\delta$-3 antibodies did not immunoprecipitate NMDA receptor subunits

<END TABLE 1>

Table 2. Evidence for Protein Interactions with $\alpha_2\delta$-1 in Whole Cells or Tissues

Target Protein	$\alpha_2\delta$-1 Region	Experimental Method	Effect of $\alpha_2\delta$-1 on target	GBP or PGB Effect	Reference
Collagen I	??	Fluorescent antibodies to $\alpha_2\delta$-1, siRNA to $\alpha_2\delta$-1 and measured cell motility	Increased adhesion and motility of muscle cells on collagen substrate	Not tested	(Garcia et al., 2008)
NMDA receptor NR1 subunit and thrombospondin	Membrane spanning C-terminal region	Thrombospondin effect on nascent dendritic spines (via $\alpha_2\delta$-1)	Increased size and stability of dendritic spines	Disrupts stability of new spines	(Risher et al., 2018)
NMDA receptors containing NR1 subunit	Membrane spanning C-terminal region	NMDA receptors and $\alpha_2\delta$-1 co-expressed in HEK-293 cells	Increased NMDA current at negative voltages	Blocks NMDA current only at negative voltages	(Chen et al., 2018)
Thrombospondin	VWA domain (requires membrane spanning protein)	New synapse formation in vitro and in vivo	Increased number of synapses and synaptic strength	Reduces new synapse formation	(Risher et al., 2018)
$K_V3.1$ and K_V1 potassium channels	??	Rapid membrane voltage fluorescent sensor	↑ Potassium channel function at release sites; ↓ presynaptic AP duration	Not tested	(Hoppa et al., 2014)

Target Protein	$\alpha_2\delta$-1 Region	Experimental Method	Effect of $\alpha_2\delta$-1 on target	GBP or PGB Effect	Reference
Unidentified vesicle release machinery	??	Synaptic vesicle fluorescence	↑ Vesicle release	↓ Vesicle release (non-Ca^{2+} stimulus)	(Micheva et al., 2006)
Unidentified vesicle release machinery	VWF-A domain MIDAS	Synaptic vesicle fluorescence	↑ Vesicle release despite smaller total Ca influx	No effect on probability of vesicle release	(Hoppa et al., 2012)
α-Neurexin (presynaptic cell adhesion molecule)	??	Presynaptic calcium influx (fluorescent dye)	$\alpha_2\delta$-1 Allows neurexin to ↑ calcium current; neurexin decreases $\alpha_2\delta$-1 mobility	Not tested	(Brockhaus et al., 2018)
Synaptotagmin (presynaptic release protein)	Secondary to effect at Cavα1 subunit?	Synapse fluorescence to antibodies	↑ synaptotagmin amount at active zone	Unknown	(Schneider et al., 2015)
Bassoon (presynaptic scaffold protein)	Secondary to effect at Cavα1 subunit?	Synapse fluorescence to antibodies	↑ Bassoon amount at active zone; ↑ size of bassoon puncta	Unknown	(Schneider et al., 2015)
RIM (presynaptic release protein)	Secondary to effect at Cavα1 subunit?	Synapse fluorescence to antibodies	↑ RIM amount at active zone; ↑ size of RIM puncta	Unknown	(Schneider et al., 2015)

Target Protein	**$\alpha_2\delta$-1 Region**	**Experimental Method**	**Effect of $\alpha_2\delta$-1 on target**	**GBP or PGB Effect**	**Reference**
RAP (receptor associated protein)	Secondary to LRP1 action?		RAP increases cell surface expression of $\alpha_2\delta$-1 (also requires LRP1)	Unknown	(Kadurin et al., 2017)

<END TABLE 2>

Table 3. Effect of Gabapentin and Pregabalin on NMDA Receptor-Dependent Responses

Preparation	Synapse	$\alpha_2\delta$-1 up-regulation?	Holding potential	Co-agonists	Drug Effect	Reference
Rat cultured striatal neurons	None (NMDA application)	No	-60 mV	Glycine (various)	GBP (10 to 200 μM) similar to glycine-site partial agonist; occluded by D-serine	(Sprosen, 1991)
Rat spinal dorsal horn neurons	Glutamate sensory afferents; NMDA application	No	+ 50 mV	None	GBP (100 μM) ↓ NMDA EPSCs (fraction of cells); GBP (100 μM) ↓ response to NMDA application	(Moore et al., 2002)
Rat dorsal spinal GABA neurons	None (NMDA application)	not measured	-60 mV	Glycine (various)	GBP (100 μM) ↑ NMDA response with PKC activation; occluded by glycine	(Gu and Huang, 2001; Gu and Huang, 2002)

Preparation	**Synapse**	**$\alpha_2\delta$-1 up-regulation?**	**Holding potential**	**Co-agonists**	**Drug Effect**	**Reference**
Rat hippocampus	Glutamate afferents to CA1	No	Extracellular record	None	GBP (50 to 200 μM) ↓ field EPSP by 25% (prevented by NMDA antagonist)	(Suarez et al., 2005)
Cultured rat hippocampal neurons	Recurrent glutamate synapses	No	Fluorescent signal	None	PGB (100 μM) ↓ vesicle release (prevented by NMDA antagonist)	(Micheva et al., 2006)
Mouse hippocampus	CA1 NMDA application	Yes	?	None	GBP (30 to 100 μM) NMDA response 40% only after ischemia	(Luo et al., 2018)
Rat hippocampus	Glutamate afferents to CA1 (after perinatal ethanol)	Yes?	-30 mV	None	GBP (30 μM) ↓ GluN2A-synaptic currents only after perinatal ethanol exposure	(Swartzwelder et al., 2017)

Preparation	**Synapse**	**$\alpha_2\delta$-1 up-regulation?**	**Holding potential**	**Co-agonists**	**Drug Effect**	**Reference**
Rat striatum	Medium spiny GABA neurons; cortex afferents	No	-80 mV	None	GBP (100 μM; 30 min) blocked NMDA-dependent LTP (theta-burst stimulation)	(Zhou et al., 2018)
Rat PVN hypothalamus (SHR hypertensive)	NMDA application; glutamate afferents	Yes	-60 mV; +30 mV	None	GBP (100 μM) ↓rate of glutamatergic mEPSCs; ↓ NMDA puff response 40%	(Ma et al., 2018)
Xenopus oocyte	NR1/NR2A human NMDA receptor	No	-70 mV	L-glutamate + 10 μM glycine	GBP (10 to 300 μM); glutamate response up to 50%; reversed by glycine	(Hara and Sata, 2007)

Abbreviations: GBP – gabapentin; GABA – gamma aminobutyric acid; LTP – long-term potentiation; NMDA receptor – N-methyl-D-aspartate type glutamate receptor; PGB – pregabalin; PKC – protein kinase C; PVN – paraventricular nucleus; SHR – spontaneously hypertensive rats < END TABLE 3>

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Figures

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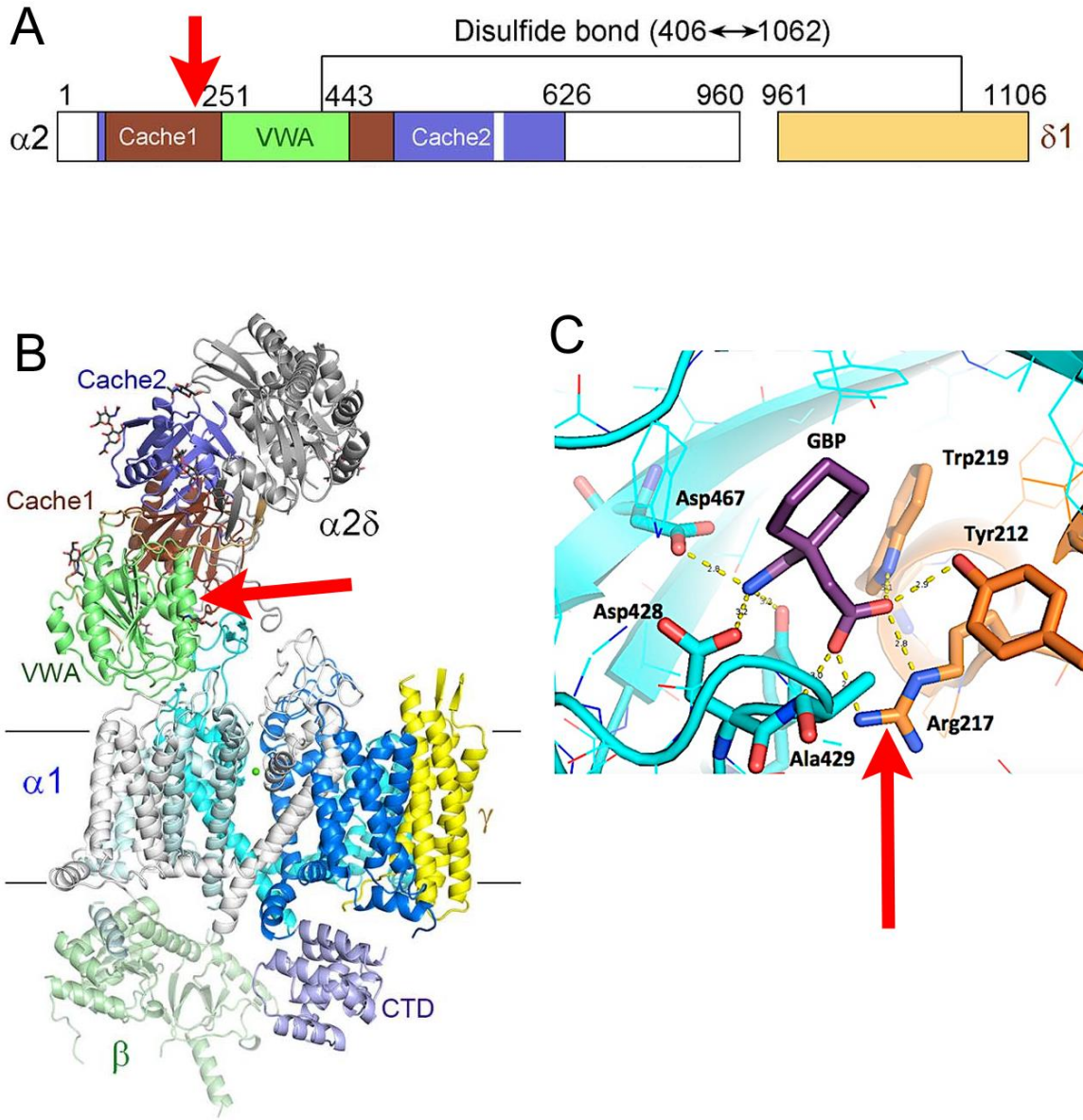

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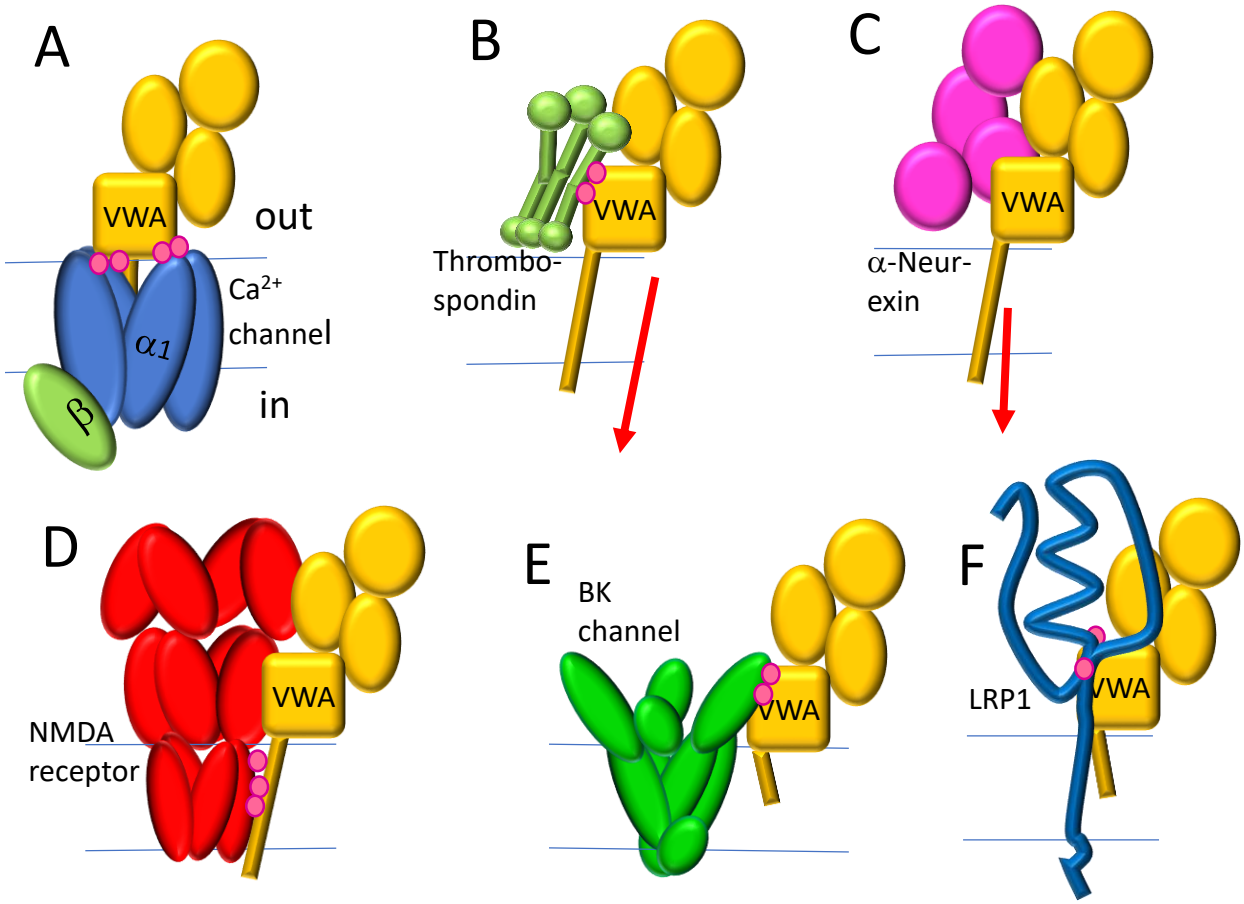

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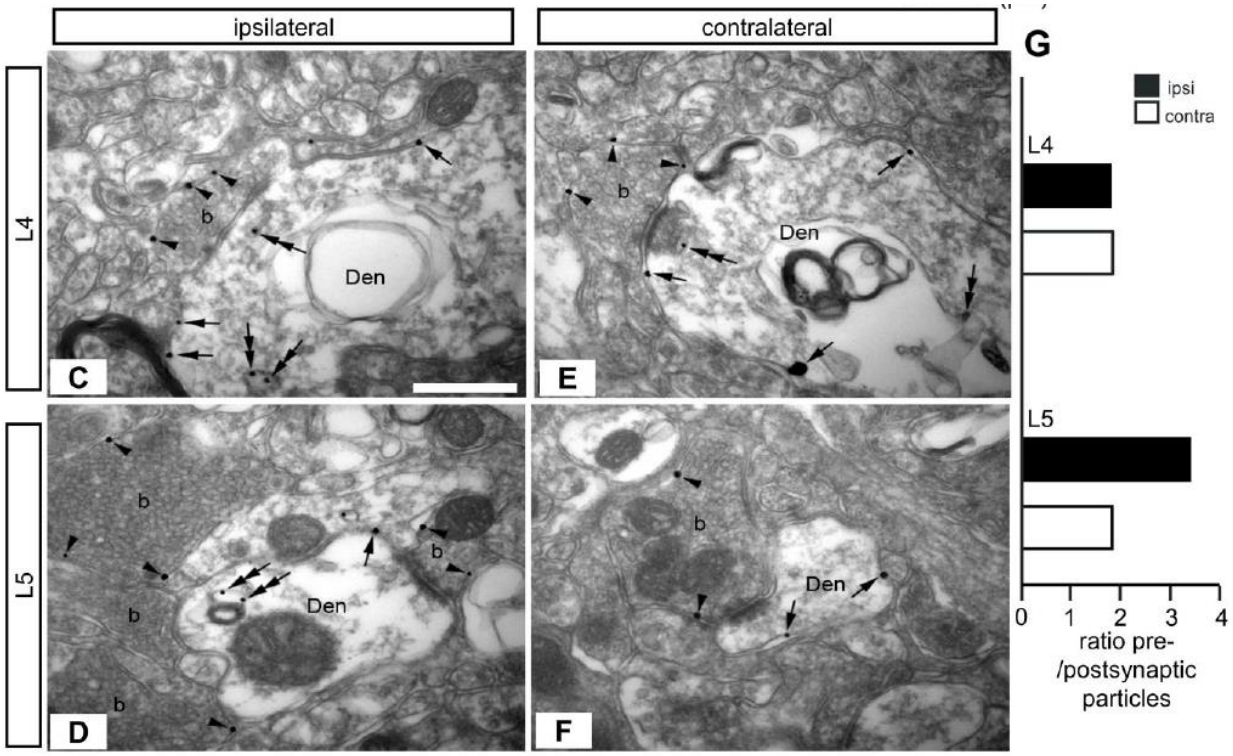

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