Mechanism of Analgesia by Gabapentinoid Drugs: Involvement of Modulation of Synaptogenesis and Trafficking of Glutamate-Gated Ion Channels

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d) List of nonstandard abbreviations:

Adverse events: AEs

Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid receptor: AMPAR

AMPA receptor subunit A1: GluA1

AMPA receptor subunit A2: GluA2

Calcium channel alpha2/delta subunit genes 1-4: CACNA2/D1-4

Calcium impermeable AMPAR: CI-AMPAR

Calcium permeating AMPAR: CP-AMPAR
Ca\textsubscript{v} channels (Ca\textsubscript{v}.1.1, Ca\textsubscript{v}.1.2, Ca\textsubscript{v}.2.1, Ca\textsubscript{v}.2.2 and Ca\textsubscript{v}.3.2)

Voltage-gated calcium channel \(\alpha 2\delta 1\) subunit: Cacn\(\alpha 2\delta-1\) or \(\alpha 2\delta-1\)

Central nervous system: CNS

Cryo-electron microscopy: cryo-EM

Dorsal root ganglion: DRG

Endoplasmic reticulum: ER

Epidermal growth factor: EGF

Gamma-amino butyric acid: GABA

Gabapentin: GBP

Long-term potentiation: LTP

Miniature excitatory postsynaptic current: mESPC

N-methyl-D-aspartate receptor: NMDAR

Postsynaptic density protein-95: PSD95

Pregabalin: PGB

Theta-burst stimulation: TBS

Three times a day (ter in die): t.i.d.

Thrombospondins1-5: TSP1-5

Twice a day (bis in die): b.i.d.

Voltage-gated calcium channel: VGCC

Von Willebrandt factor A: VWF-A

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**Keywords:** Gabapentinoid drugs; calcium channel alpha2/delta-1 subunit; chronic pain, neuropathic pain; synapse development; glutamate-gated ion channel trafficking
ABSTRACT

Gabapentinoids have clinically been used for treating epilepsy, neuropathic pain, and several other neurological disorders for >30 years, however, the definitive molecular mechanism responsible for their therapeutic actions remained uncertain. The conventional pharmacological observation regarding their efficacy in chronic pain modulation is the weakening of glutamate release at presynaptic terminals in the spinal cord. While the \( \alpha_{2/\delta}-1 \) subunit of voltage-gated calcium channels (VGCCs) has been identified as the primary drug receptor for gabapentinoids, the lack of consistent effect of this drug class on VGCC function is indicative of a minor role in regulating this ion channel's activity. This current review targets the efficacy and mechanism of gabapentinoids in treating chronic pain. The discovery of interaction of \( \alpha_{2/\delta}-1 \) with thrombospondins established this protein as a major synaptogenic neuronal receptor for thrombospondins. Other findings identified \( \alpha_{2/\delta}-1 \) as a powerful regulator of NMDA and AMPA receptors by potentiating the synaptic expression, a putative pathophysiological mechanism of neuropathic pain. Further, the interdependent interactions between thrombospondin and \( \alpha_{2/\delta}-1 \) contribute to chronic pain states, while gabapentinoid ligands efficaciously reverse such pain conditions. Gabapentin normalizes and even blocks NMDAR and AMPAR synaptic targeting and activity elicited by nerve injury.

SIGNIFICANCE STATEMENT

Gabapentinoid drugs are used to treat various neurological conditions including chronic pain. In chronic pain states gene expression of \( \text{cacin}_{\alpha_{2/\delta}-1} \) and thrombospondins are upregulated and promote aberrant excitatory synaptogenesis. The complex trait of protein associations that involve interdependent interactions between \( \alpha_{2/\delta}-1 \) and thrombospondins, further, association
of NMDAR and AMPAR with the C-tail of α2/δ-1 constitutes a macromolecular signaling complex that forms the crucial elements for pharmacological mode of action of gabapentinoids.
INTRODUCTION

Strategies to design lipophilic GABA analogs that can pass through the blood-brain barrier led to the development of the clinical drugs gabapentin, 1-(aminomethyl) cyclohexane acetic acid, and pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid (Bryans and Wustrow, 1999) collectively termed: gabapentinoids. These compounds have been developed for clinical use as anticonvulsant drugs (Taylor, 1994) but several clinical case reports have also suggested that gabapentin is efficacious in treating neuropathic pain (Rosner et al., 1996) and in a number of other neurological conditions [for review see, (Stahl et al., 2013; Taylor and Harris, 2020)].

Gabapentin and pregabalin (Dworkin and Kirkpatrick, 2005) are approved for treatment of postherpetic neuralgia and epilepsy with partial onset seizures, and pregabalin is additionally approved in the United States for diabetic peripheral neuropathy, fibromyalgia and spinal cord injury (Wiffen et al., 2005; Wiffen et al., 2013; Moore et al., 2014; Calandre et al., 2016; Derry et al., 2019) (Table 1). Both drugs are prescribed off-label for treatment of various anxiety disorders (Greenblatt and Greenblatt, 2018). Pregabalin is also approved in the European Union to treat generalized anxiety disorder (Calandre et al., 2016; Chen et al., 2022). These drugs are now considered first-line treatments for various neuropathic pain conditions, also their anti-epileptic and anti-anxiolytic efficacies are well-recognized.

Meta-analyses of numerous clinical trials with gabapentin, at doses 1,200 mg or higher, have shown an outcome of about 32% of participants achieved at least 50% pain reduction in chronic neuropathic pain and fibromyalgia (Moore et al., 2014). On the other hand, 62% of the participants experienced at least one adverse event such as somnolence, dizziness, and gait balance or disturbance (Moore et al., 2014). The promising efficacy and such a high percentage of adverse events created an unmet need for improvement of this drug class. In recent years, the development of novel compounds contributed to approval of a newer
gabapentinoid, Mirogabalin, with improved safety characteristics as well as longer-lasting analgesic efficacy (Domon et al., 2018; Kato et al., 2021).

The observed drug efficacies by gabapentinoids in various clinical conditions, which all share neuronal hyperactivity, may all depend on interacting the drug with the α2/δ-1 protein receptor site. Further, gabapentinoid drugs are capable to reduce the release of excitatory neurotransmitters at synapses in the spinal cord, neocortex, and brainstem in hyperactive neuronal conditions (Alles and Smith, 2018). Since the key elements of drug action are very similar in different neurological conditions, we will focus in detailing the mechanism of action in chronic pain in this review.

GABAPENTINOID DRUGS

Gabapentin is a first-generation gabapentinoid. The drug has a moderate IC50 value for its receptor and has a short half-life and administered on a t.i.d regimen in a total dose ranging 900-3,600mg/day (Wiffen et al., 2005; Moore et al., 2014) (Table 1). Pregabalin is a second generation gabapentinoid, has a higher affinity to α2/δ-1 (IC50 80 nm vs 140 nm for gabapentin), better bioavailability and fewer side effects. The clinically relevant doses are also lower (100-600mg t.i.d). Efficacy outcomes of pregabalin in postherpetic neuralgia and painful diabetic neuropathy provided at least 30% pain reduction in 62% of the patient population, while 42% of the patients achieved 50% pain reduction. For each drug higher doses provided greater response. However, adverse event such as somnolence (25%) and dizziness (35%) were observed at lower percentage of the patient population for pregabalin (Derry et al., 2019). These data have shown that the affinity of the drug to its receptor correlated well with pharmacological efficacy and inversely correlated with the frequency of adverse events. Such a
challenge called for developing novel gabapentinoids and studying structure-function relationship.

Development of newer gabapentinoid agents has been continued during the past decade (Table 1). Gabapentin enacarbyl, developed by XenoPort, is an acyloxyalkylcarbamate prodrug of gabapentin and has an improved bioavailability, otherwise the drug receptor affinity is the same as for gabapentin.

Mirogabalin, [(1R,5S,6S-6- (aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl] acetic acid, was recently developed by Daiichi Sankyo and approved as a novel gabapentinoid drug in Japan (Mirogabalin besylate, Tarlige) for peripheral neuropathic pain, including postherpetic neuralgia and diabetic peripheral neuropathy (Domon et al., 2018; Kitano et al., 2019; Kato et al., 2021; Chen et al., 2022; Domon et al., 2023). Mirogabalin exerts analgesic effects on thermal and mechanical hypersensitivity after partial sciatic nerve ligation (Oyama et al., 2021), acting at both spinal and supraspinal levels and has potent pain-modulating effect with a unique high affinity (IC$_{50}$ of 16 nm) and prolonged dissociation rate for the $\alpha_2/\delta-1$ subunit of VGCCs resulting in more sustained analgesia compared with traditional gabapentinoids. The significantly higher potency requires a very low dose in clinical applications (10-30 mg/day). Also, mirogabalin exhibits wider safety margin and superior adverse effect profile compared to gabapentin and pregabalin, which might be due to a rapid dissociation from the $\alpha_2/\delta-2$ subunit of VGCCs that is potentially implicated in central nervous system-specific AEs (Burgess et al., 2020). This greatly promising compound can be considered as a next generation of gabapentinoids (Chen et al., 2021).

HSK16149 is an investigative drug developed by Haisco, and currently tested in Phase III clinical trial in China. The drug is essentially a mirogabalin derivative as it has a tricyclo-nonan-2 moiety, [2-((1S,2S,3R,6S,8S)-2-(amino-methyl)tricyclo[4.2.1.0$^{3,8}$]nonan-2-yl)]acetic acid ben-
zenesulfonic acid (1:1)] attached to the 3-position of the GABA backbone. HSK16149 is a potent gabapentinoid, with its 3.96 nM IC_{50} is 23-fold more potent than pregabalin. The drug exhibited analgesic efficacy in various animal pain models, however, the oral exposure level in plasma was significantly lower than that of pregabalin (Gou et al., 2021). Another gabapentinoid in preclinical study is NVA1309, developed by Novassay. The IC_{50} at 2.1 nM is very low, and the compound is a derivative of mirogabalin as the carboxylic acid functionality is replaced by the tetrazole ring, a bioisostere (Chen et al., 2022).

A wide array of gabapentinoids, developed by Pfizer and many have been discontinued at preclinical or clinical stage (Table 1). Gababutins are bicyclic-substituted, cyclopentane-ring derivatives of gabapentin (Blakemore et al., 2010b). Gabutin has a week binding affinity to its receptor, therefore the development was terminated in preclinical stage. Gababutin A, a 3-methyl gababutin derivative has an IC_{50} value of 30 nM (Blakemore et al., 2010b), however, it cannot penetrate a blood-brain barrier. Atagabalin, 3,4-trans-dimethyl gababutin has a low IC_{50} value, 22 nM (Blakemore et al., 2010c). While the pharmacokinetic profile and absorption properties appeared beneficial in Phase II trial, frequent adverse event were noted, and the trial discontinued. PD-217014 is a bicyclic gababutin derivative with a IC_{50} value of 38 nM (Blakemore et al., 2010a). The study with this drug was terminated after the Phase II trial. Gababutin B is similar to PD-217014 except the GABA backbone is attached to a cyclobutane ring and the IC_{50} was at 46 nM. In phase II clinical trial AEs were identified and the trial discontinued. PD-0200347 is an oxodiazole bioisostere of gabapentin. The IC_{50} was at 210 nM and the drug exhibited poor brain uptake due to the weak affinity to System L amino acid transporter (Su et al., 1995).

Imagabalin, (3S,5R)-3-amino-5-methyloctanoic acid (PD-0332334) was developed by Pfizer (Quintero et al., 2011) and was tested due to its hypothesized anxiolytic, analgesic, hypnotic
and anti-convulsant activity in Phase III clinical trials, however, the trials were terminated and the drug is no longer under development.

Gabapentin analogues, alkyl and aryl substituted, mostly 3,4,5-substituted in the cyclohexane ring, have been examined for binding to the α2/δ-1 receptor and in vivo animal epilepsy model. Two methyl-substituted cyclohexane derivatives showed 2-3-fold greater binding affinity than gabapentin itself and have been identified as a potent novel anticonvulsant agent possessing a similar profile than gabapentin in an animal model (Table 1) (Bryans et al., 1998). On the other hand, heteroatom substitutions in the cyclohexane ring (O, N, S) drastically increased the IC50 of the compound.

A study with alkyl-substitutions on the GABA backbone of pregabalin analogs demonstrated structure-activity relationships for α2/δ-1 receptor interaction and System L substrate amino acid binding (Su et al., 1995). Changes to the size and stereochemical orientation of substituents on the pregabalin framework had a dramatic effect on in vitro affinities for α2/δ-1 and System L amino acid transporter. Essentially all substitutions decreased the affinity to the receptor site, in particular α- and β-substitutions on the GABA backbone lowered affinity. However, the potency to inhibit the System L amino acid transporter remained similar to pregabalin, except in case of α-isopropyl and isobutyl pregabalin derivatives where the System L substrate binding decreased 100-1000-fold. In vivo activity of alkyl-substituted pregabalin derivatives were mostly abolished or appeared substantially weaker than the parental compound (Belliotti et al., 2005).

Cyclopropyl derivatives of pregabalin efficiently bound to α2/δ-1 receptor sites, however, none of these compounds appeared to be substrates for the System L amino acid transporter. Consistent with inefficient brain penetration, efficacy was only achieved when the compounds were administered by injection into the cerebral ventricles (Schwarz et al., 2005).
The gabapentinoid compounds summarized in Table 1 have a characteristic of GABA backbone that represents the hydrophilic polar domain of the molecule. Lipophilic substituents, attached to the 3-position of the GABA backbone may represent mono-or disubstitution at this position and no other substitutions are tolerated (Chen et al., 2022), especially heteroatoms such as N, O, S. Substitution at 3-position with cyclohexane, cyclopentane or bicyclic and tricyclic moieties are tolerated in the structure-activity relationship, and in some cases resulting in favorable pharmacological profile (Domon et al., 2018). This observation correlates well with the mirogabalin cryo-EM structure that revealed the importance of contribution of hydrophobic components on the GABA core of the molecule (Figure 1B). Substitution of carboxylic moiety is generally unfavorable, however, in few cases the affinity to the receptor has improved (Chen et al., 2022).

MOLECULAR TARGET OF GABAPENTINOIDE DRUGS

Gabapentinoids are drugs that specifically bind to high affinity binding sites on the \( \alpha_2/\delta-1 \) (Gee et al., 1996) and \( \alpha_2/\delta-2 \) subunits (Klugbauer et al., 1999; Marais et al., 2001; Klugbauer et al., 2003) of VGCC (Figure 1). Even though gabapentinoids carry a significant structural resemblance to GABA, they lack any appreciable affinity to either \( \text{GABA}_A \), \( \text{GABA}_B \) and \( \text{GABA}_C \) receptors (Jensen et al., 2002; Taylor et al., 2007; Taylor, 2009; Li et al., 2011). Further, GBP has no binding affinity for NMDA, AMPA and glycine receptors (Taylor, 1994; Taylor, 2009; Li et al., 2011), does not interact with allosteric GABA receptor sites (Lanneau et al., 2001; Li et al., 2011), GABA transporter sites (Taylor et al., 2007; Li et al., 2011), and GABA synthetic or degradative enzymes (Taylor et al., 2007; Taylor, 2009; Li et al., 2011).

Gabapentin binds to members of Kv7 channels such as heteromeric KCNQ2/3, and to homomeric KCNQ3 and KCNQ5 and enhances K\(^+\) currents (Manville and Abbott, 2018b). While
the activation by GBP occurs at low nanomolar level (2-5nM), PGB does not activate KCNQ2/3, on the contrary, at higher concentration it exerts inhibitory function (Manville and Abbott, 2018b). Molecular docking and mutagenesis studies provided evidence that a conserved tryptophan (W265) in the S5 transmembrane segment of this class of K⁺ channels determine GBP binding. GABA also associates with this site in KCNQ2 and 3 channels and enhances channel currents (Manville and Abbott, 2018a; Manville et al., 2018). GABA modulation of these channel functions establish the correlates of neuronal M currents, which suppresses neuronal excitability, and such an enhancement of M current in neuronal cells may represent another possibility in the mode of action of gabapentinoids.

**Behavioral impact of ablation of the CACNA2D1 gene**

Ablation of the CACNA2D1 gene that encodes the α2/δ-1 subunit protein and studies of the transgenic (knock-in) mice expressing R217A mutant (Wang et al., 1999), a mutation that leads to significant reduction in the binding affinity of pregabalin (Figure 1 and legend to Figure 1), provided evidence for proof that gabapentin binding to α2/δ-1 is both necessary and sufficient for analgesic actions in neuropathic pain model (Field et al., 2006). Also, in the R217A mutant mice the anxiolytic and anticonvulsant actions of gabapentin were also abolished. In tissues from these mice, pregabalin binding was markedly reduced (Field et al., 2006; Fuller-Bicer et al., 2009). Antisense knockdown of α2/δ-1 subunit transiently reversed experimental neuropathic pain (Bauer et al., 2010). Further, it was also shown that anxiolytic activity of pregabalin was associated rather with the expression of α2/δ-1 gene product in mice than with the presence of α2/δ-2 (Lotarski et al., 2011).
Molecular structure of the gabapentinoid receptor

The overall domain structure of calcium channel α2/δ-1 subunits is depicted in Figure 1A. The protein contains four sequence regions that are characteristic for chemoreceptors in bacteria and archaea that possess amino acid and other organic ligand binding (Cache domains) (Figure 1A), (Anantharaman and Aravind, 2000; Gumerov et al., 2022). The ligand binding domain in bacterial chemoreceptors are termed dCache_1 that corresponds to a double Cache domain found in calcium channel α2/δ (Gumerov et al., 2022). Across the Tree of life, in eukaryotes, these domains, can only be found in calcium channel α2/δ subunits and in a structurally related protein CaChD1 (Whittaker and Hynes, 2002; Dahimene et al., 2018).

Based on the 3-D structure of α2/δ-1 (Wu et al., 2016) and using protein-ligand sampling algorithm, (Kotev et al., 2018; Gumerov et al., 2022) determined that the sequence arrangement in bacterial dCache_1 domain establishes two binding pockets, and ligands, depending on their chemical nature, bind either to membrane-distal (amino acid receptor) or membrane-proximal (ligands such as sugars, organic acids, or nucleotides) modules in the dCache_1 domain. By analyzing the amino acid sequence of a large group of bacteria that are known to bind amino acids and comparing them to another set of bacteria that bind ligands other than amino acids Gumerov et al., (Gumerov et al., 2022) found a consensus sequence in the distal module that is: YxxxxRxWY[x~1-17]Y[x~27-34]D. The amino acid binding property of this sequence was validated by utilizing extended data search throughout of the Tree of Life, and by the amino acid ligand binding to various recombinant dCache_1 domains by differential scanning fluorimetry-based thermal shift assays (Gumerov et al., 2022).

While identification of the consensus sequence made possible to deduct the gabapentinoid binding site (Gumerov et al., 2022; Page et al., 2023), a recent advance provided further
clarifications on the amino acid residue components that establish the binding pocket (Figure 1B). Kozai et al (Kozai et al., 2023) determined the 3-D structure of the recombinant human α2/δ-1 bound to mirogabalin. Cryo-EM studies revealed that the ligand binding site is in the first (dCache_1) domain’s distal pocket which is similar to the bacterial dCache_1 domain’s distal pocket that binds L-leucin. The combination of cryo-EM structure and comprehensive alanine scanning mutagenesis studies established that amino acids Y236, R241, W243 and Y450, D452, T461, D491 form the binding pocket for the carboxyl and amino groups, respectively. In addition, W205, V207, A215, Y217, W223 and L454 contribute to binding the hydrophobic skeleton of mirogabalin (Figure 1B). Mutations in the above amino acids resulted in substantially decreased mirogabalin binding affinity (Kozai et al., 2023) thereby complementing our knowledge on the precise structure of the binding domain for gabapentinoids in α2/δ-1 and -2 proteins. Further, these studies revealed the atomic contributors of mirogabalin recognition mechanisms by α2/δ-1.

The emphasis of particular importance on hydrophobic interactions in α2/δ-1 ligand recognition are novel observations and offer explanation for the tight binding of the ligand and long-lasting impact of the drug. This information coincides with observations based on medicinal chemistry studies (see Table 1) and it should provide more detail on current status of structure-activity relationships for gabapentinoids. In addition, the detailed knowledge of the molecular components of the drug receptor site may offer avenues to rational development of gabapentinoid drugs.
CORRELATION OF CaV α2/δ-1 EXPRESSION WITH THE DEVELOPMENT OF CHRONIC PAIN AND EPILEPTOGENESIS

Correlation of α2/δ-1 expression with the development of chronic pain

Using a rat neuropathic pain model Luo et al. (Luo et al., 2001) described that nerve injury resulted in enhanced α2/δ-1 subunit expression (>17-fold) in the spinal cord and dorsal root ganglia. This marked, time-dependent α2/δ-1 subunit upregulation appeared in rats with unilateral sciatic nerve crush, but not dorsal rhizotomy, indicating a peripheral origin of the expression regulation. In contrast, other calcium channel subunits that are extensively involved in neurochemical transmission (Hartung et al., 2021), such as N- or P/Q-type calcium channel CaVα2.1 or CaVα2.2 and β3 subunit expression were not co-upregulated with α2/δ-1 subunit after nerve injury.

Now it is widely accepted that α2/δ-1 upregulation in DRG plays a role in neuropathic pain processing (Luo et al., 2001; Luo et al., 2002; Valder et al., 2003; Boroujerdi et al., 2008; Bauer et al., 2009; Bauer et al., 2010; Boroujerdi et al., 2011). Data indicated that even though allodynia occurred in all types of nerve injury investigated, DRG and/or spinal cord α2/δ-1 subunit upregulation and gabapentin sensitivity only coexisted in the mechanical nerve injuries and diabetic neuropathies (Luo et al., 2002). In orofacial nerve constriction injury model, the resulting hypersensitivity also correlated with α2/δ-1 upregulation (Li et al., 2014), and blocking α2/δ-1 with gabapentin reversed the orofacial hypersensitivity.

In spinal nerve injury neuropathic allodynia model, dorsal rhizotomy diminished basal level expression and blocked injury-induced expression of the spinal dorsal horn α2/δ-1 subunit and reversed injury-induced tactile alldynia. Further, intrathecal injection of α2/δ-1 antisense oligonucleotides blocked injury-induced dorsal horn α2/δ-1 subunit upregulation and diminished
tactile allodynia (Li et al., 2004; Li et al., 2006). These findings indicate that α2/δ-1 subunit basal expression occurs presynaptically and postsynaptically in the spinal dorsal horn. Nerve injury induces mainly presynaptic α2/δ-1 subunit expression that derives from increased α2/δ-1 subunit in injured DRG neurons.

**Correlation of genomic aberrations and expression of CACNA2D1 with epileptogenesis**

In the neocortical partial isolation (undercut) model of posttraumatic epileptogenesis, gliosis, increases in thrombospondins (TSPs, TSP1/2) and α2/δ-1, and increased density of excitatory synapses occurred in the injured cortex, along with abnormal epileptiform burst discharges (Li et al., 2012). Chronic gabapentin or pregabalin treatment decreased the incidence of epileptiform discharges, decreased posttraumatic synaptogenesis and significantly reversed the gene expression of TSP1/2 and α2/δ-1 in this disease model (Li et al., 2012). Faria et al., have shown (Faria et al., 2017) that overexpression of α2/δ-1 alone in neocortex of uninjured transgenic mice might result in increased excitatory connectivity and consequent cortical hyperexcitability and epileptiform activity. Further, genomic aberrations of the CACNA2D1 gene were found in the total blood DNA of a limited number of patients with epilepsy (Vergult et al., 2015) and in peripheral blood of patients with early onset of epileptic encephalopathy (Hino-Fukuyo et al., 2015).

Copy number variations encompassing CACNA2D1 have been reported in patients with epilepsy and intellectual disabilities (Mefford et al., 2011). Interestingly, in search of genetic etiologies of West syndrome, Hino-Fukuyo identified deletions disrupting CACNA2D1 in a female patient with normal mental development, possibly due to the absence of other affected genes (Hino-Fukuyo et al., 2015). Biallelic variants of CACNA2D1 resulting in the absence, or severely diminished level of α2/δ-1 protein underlies the early onset developmental epileptic encephalopathy. Two patients with such genetic defects were described and interestingly both
patients were found insensitive to pain (Dahimene et al., 2022). Similarly, biallelic variants of CACNA2D2 are associated with encephalopathy and cerebellar atrophy (Punetha et al., 2019).

MOLECULAR MECHANISMS OF GABAPENTINOID ACTION

Although gabapentinoids have been used clinically for treating epilepsy and neuropathic pain for >30 years, the definitive molecular mechanism responsible for their therapeutic actions remained uncertain. However, the conventional view from the past twenty years of research was that synaptic expression and targeting of α2/δ-1 potentiates, whereas gabapentinoids attenuate neuropathic pain through modulation of VGCCs. While the predominant molecular mechanisms underlying the effects of gabapentinoids following their binding to the receptor site was considered to be a decrease in synaptic transmission in disease conditions, the involvement of calcium channel function in the above process has not been understood for some time.

The α2/δ-1 subunit modulates functions of the class of high voltage-gated calcium channels (Catterall, 2011; Dolphin, 2012). In addition, N-type and P/Q-type calcium channels trigger neurotransmitter release from presynaptic vesicles. Gabapentinoids through action at α2/δ-1 subunit might modulate the activity of VGCCs and consequently neurotransmitter release. Indeed, one study reported that chronic (48-hr) treatment with gabapentin reduced the VGCC activity and cell-surface expression in a cell line (Hendrich et al., 2008). However, other studies showed that treatment with gabapentin for >72 hr had no effect on VGCC-mediated neurotransmitter release or VGCC trafficking in cultured neurons (Hoppa et al., 2012), and that gabapentin had no effect on the α2/δ-1 interaction with VGCC α1 subunits (Cassidy et al., 2014). The lack of a consistent effect of gabapentin on VGCC function (Rock et al., 1993; Scott
et al., 2003) could be due to the weak interaction between \( \alpha2/\delta-1 \) and VGCC \( \alpha1 \) subunits (Muller et al., 2010) or a minor role of \( \alpha2/\delta-1 \) in regulating VGCC activity (Wu, 2009).

Unlike \( \alpha2/\delta-1 \), which is mainly expressed in excitatory neurons, \( \alpha2/\delta-2 \) is predominantly expressed in inhibitory neurons (Cole et al., 2005; Taylor and Garrido, 2008). The affinity of pregabalin and gabapentin binding to \( \alpha2/\delta-1 \) and 2 proteins (Kd) is nanomolar (Klugbauer et al., 2003; Dolphin, 2012), whereas the physiological actions of these drugs are usually in the low micromolar range. One likely reason for this discrepancy assumes that endogenous \( \alpha2/\delta-1 \) ligands from living tissues (e.g., L-leucine, L-isoleucine, L-valine) (Klugbauer et al., 2003; Dolphin, 2012) are present at micromolar concentrations and must be competed away from the receptor by drug molecules.

**Gabapentinoids interfere with synaptogenesis and maturation**

**Studies in cultured cells**

In the CNS, astrocytes are closely associated with synapses (Araque et al., 1999). Through this association, astrocytes actively control synaptic transmission, including synapse formation, maturation, function, and elimination (Chung et al., 2015). The synaptogenic role of astrocyte-secreted cell adhesion proteins was originally discovered by (Christopherson et al., 2005; Eroglu et al., 2009) and was found that addition of purified synaptogenic factors, such as TSP1-5, induces the formation of structurally mature but postsynaptically silent synapses (Allen and Eroglu, 2017; Stogsdill and Eroglu, 2017; Risher and Eroglu, 2020; Tan and Eroglu, 2021) in vitro and in vivo (Christopherson et al., 2005; Allen and Eroglu, 2017; Baldwin and Eroglu, 2017).

Further, the calcium channel subunit \( \alpha2/\delta-1 \) was identified as the major synaptogenic neuronal receptor for TSPs (Eroglu et al., 2009). All five mammalian TSPs share the ability to induce
synapse formation by binding via their type 2 EGF-like repeats to the von Willebrand factor A domain of \( \alpha 2/\delta-1 \) (Fig. 1). Besides \( \alpha 2/\delta-1 \), TSPs also interact with several other cell-surface receptors and mediate other functions in the CNS (Risher and Eroglu, 2012). TSP-1 was also found to interact with the postsynaptic adhesion protein neuroligin 1 and, in this way, accelerates excitatory synapse formation in cultured hippocampal neurons (Xu et al., 2010). Gabapentin prevented excitatory synapse formation by blocking the ability of TSP to bind its receptor \( \alpha 2/\delta-1 \), thereby inhibiting the synaptogenic signaling initiated by TSP–\( \alpha 2/\delta-1 \) interaction without affecting previously formed synapses (Eroglu et al., 2009) providing a line of evidence that TSP–\( \alpha 2/\delta-1 \) signaling and astrocyte-induced synapse formation, which latter is linked to secreted proteins by astrocytes (Stogsdill et al., 2017), might be involved in the pathophysiology of diseases, such as neuropathic pain and epilepsy, for which gabapentin is a common treatment.

With the use of hippocampal neuronal cell cultures from cross-bred various \( \alpha 2/\delta \) KO animals and combined with shRNA knock-down it was shown that upregulation of \( \alpha 2/\delta-1 \) and \( \alpha 2/\delta-3 \) triggered excitatory, glutamatergic synaptogenesis and presynaptic release of glutamate. Conversely, upregulation of \( \alpha 2/\delta-3 \), but not \( \alpha 2/\delta-1 \), increased the number of GABAergic synapses (Bikbaev et al., 2020) and enhanced presynaptic GABA release. Thus, \( \alpha 2/\delta \)s are essential for presynaptic differentiation, as well as the trans-synaptic alignment of postsynaptic receptors, however, the role \( \alpha 2/\delta-1 \) through 3 are highly redundant in regulating glutamatergic synapse formation and differentiation (Schopf et al., 2021).

In in vitro testing of biochemical interaction between recombinant, soluble TSPs and \( \alpha 2/\delta-1 \) has shown that only TSP-4 and \( \alpha 2/\delta-1 \) associate in saturation equilibrium (El-Awaad et al., 2019), and this binding cannot be disrupted by gabapentin. While the observation is somewhat disturbing, recombinant proteins, especially a soluble form of \( \alpha 2/\delta-1 \), might not represent
adequate native structure (Brown and Gee, 1998), this observation may also suggest the potential involvement of other factors in mediating gabapentin’s inhibitory effect in neuropathic pain.

**Studies in animal models and in genetically engineered mice**

In a rat nerve injury model, Kim et al (Kim et al., 2012) observed development of thermal hyperalgesia and mechanical allodynia, and these phenomena were associated with a twofold upregulation of TSP-4 protein in the injury site at the dorsal spinal cord (Pan et al., 2015). Blocking injury-induced spinal TSP-4 upregulation by intrathecal bolus injection of TSP-4 antibodies caused a complete reversion of thermal hyperalgesia and mechanical hyperalgesia but not tactile allodynia (Kim et al., 2012).

Park et al. (Park et al., 2016) directly immunoprecipitated TSP-4 and $\alpha$2/δ-1 from spinal cord, providing evidence for the direct interaction of these two proteins in the spinal cord. Intrathecal injection of TBS-4 induced tactile alldynia and this pain state was reversed by gabapentin injection. Preemptive knockdown of $\alpha$2/δ-1 by intrathecal injection of small hairpin RNAs prevented the behavioral hypersensitivity. Overexpression of $\alpha$2/δ-1 (TG mouse) increased mESPC frequency and pain states similar to SNL. However, TSP-4 antibodies reversed these effects.

CACNA2D1 overexpression/TSP-4 KO genetically modified mouse crosses resulted in behavioral hypersensitivities both to mechanical and thermal stimuli. This observation is consistent with the notion that TSP-4 basal level apparently is not critical in maintaining basal sensory/motor functions, however, the experimental animal strain was only TSP-4 KO and all the other TSPs were present, thereby fairly capable to provide TSPs for the behavioral hypersensitivities (Park et al., 2016).
Recently, it was shown that acute post-injury blockade of calcium channel α2/δ-1 subunit with human equivalent doses of gabapentin prevented pathological autonomic plasticity after spinal cord injury in a mouse model (Brennan et al., 2021). Gabapentin treatment prevented excitatory synaptogenesis, sprouting of sympathetic neurons that innervate lymphoid tissue and sprouting of nociceptive afferents starting at one day after post injury in a spinal cord injury mice model (Brennan et al., 2021). The beneficial impact of prophylactic GBP treatment persisted one month after stopping the treatment in this mouse model.

**Cacnα2/δ-1 subunit is a modulator of pre- and postsynaptic NMDAR and postsynaptic AMPA-type glutamate receptor activity**

*Regulation of NMDAR by calcium channel α2/δ-1*

The finding of Chen et al. revealed that α2/δ-1 is a powerful regulator of NMDARs, and that, it contributes to neuropathic pain by potentiating the synaptic expression and targeting of NMDARs (Chen et al., 2018; Deng et al., 2019b). Lentiviral overexpression of α2/δ-1 in spinal cord potentiated presynaptic and postsynaptic NMDAR activity of spinal dorsal horn neurons to cause pain hypersensitivity (Chen et al., 2018). Conversely, α2/δ-1 knockdown or ablation normalized synaptic NMDAR activity increased by nerve injury. They determined that α2/δ-1 forms a heteromeric complex with NMDARs in rodents and in human spinal cord, and this interaction predominantly occurs through the C-terminus of α2/δ-1 and it promotes surface trafficking and synaptic targeting of NMDARs (Chen et al., 2018) (Figure 2). Gabapentin or an α2/δ-1 C-terminus-interfering peptide (α2/δ-1 Tat) (Figure 1A) normalized NMDAR synaptic targeting and activity increased by nerve injury in experimental animals (Chen et al., 2018).

*The role of α2/δ-1 in regulating AMPA receptors in promoting postsynaptic dominance of calcium-permeating AMPARs*
Intrathecal injection of $\alpha_2/\delta-1$ in a lentiviral vector into spinal cord evoked thermal hypersensitivity and tactile allodynia that was reversed with IEM-1460, an AMPA channel blocker, selective for open channel CP-AMPAR (Li et al., 2021). Li et al. (Li et al., 2021) have also shown that $\alpha_2/\delta-1$ interacts via its C-terminal tail with GluA1 and GluA2 subunits of AMPARs and disrupts the assembly of GluA1 and GluA2 into heteromers (Sommer et al., 1991; Greger et al., 2002; Greger et al., 2003), thereby prevents the formation of calcium impermeable AMPARs.

AMPA-type glutamate receptors are the predominant postsynaptic receptors involved in fast excitatory neurotransmission in the CNS. Four subunits (GluA1-GluA4) may compose the functional AMPA receptor and GluA2 is a critical component in determining the biophysical properties of the channel. Most AMPA receptors in adult brain and spinal cord are composed of GluA1/GluA2 subunits that constitute CI-AMPAR channels. GluA2 is postranscriptionally edited (Sommer et al., 1991), a process that determines whether the channel is calcium impermeable (CI-AMPAR) or calcium permeable (CP-AMPAR). GluA1 can move to the synapse, but GluA2, in unedited form is entrapped in the ER (Greger et al., 2002; Greger et al., 2003; Greger et al., 2006; Greger et al., 2017). Only $\alpha_2/\delta-1$ interacted with GluA1 and GluA2, while other $\alpha_2/\delta$ subunits ($\alpha_2/\delta-2$ and -3) did not perform such an interaction and had no effect on surface expression of either GluA1 or GluA2 (Li et al., 2021).

In neuropathic pain, induced by spinal nerve ligation injury, the $\alpha_2/\delta-1$ subunit is upregulated in the spinal dorsal horn and forms complexes with GluA1 and GluA2 via interaction with its C-terminal tail and prevents their heteromeric assembly in the ER. However, such an interaction does not prevent homotetrameric assembly and continued trafficking of GluA1 to the synapse. Changes in AMPAR composition is a characteristic phenomenon in neurological disorders, including chronic neuropathic pain (Chen et al., 2013; Henley and Wilkinson, 2016; Chen et al.,
2019a). In the development of neuropathic pain, in the spinal cord both potentiated NMDAR activity and the increased prevalence of CP-AMPAR are involved (Zhou et al., 2012; Chen et al., 2013; Chen et al., 2014). Post-synaptic prevalence of heteromeric AMPA receptors in the spinal cord is an important phenomenon to maintain low level cytoplasmic calcium in neural cells under physiological conditions (Isaac et al., 2007; Henley and Wilkinson, 2016). Most AMPA receptors in adult brain and spinal cord consist of heteromeric GluA1/GluA2 subunits; this composition renders the channel complex calcium impermeable (CI-AMPAR) (Isaac et al., 2007). These studies provided an elegant example showing that an auxiliary subunit of VGCCs, that is the α2/δ-1, in fact, is a critical interacting protein in regulating presynaptic and postsynaptic ionotropic glutamate receptors (NMDAR and AMPAR) (Figure 2).

While interaction of TSPs and α2/δ-1 modulates trafficking of NMDR and AMPAR, both of these ionotropic glutamate receptors at the synapse also have a fundamental role in synaptogenesis (Cline and Haas, 2008; Sobczyk et al., 2005). Most likely α2/δ-1 may act as a scaffold protein by interacting with both thrombospondins (via its VWF-A domain) (Canti et al., 2005; Eroglu et al., 2009) and NMDAR and AMPAR (via its C-terminal domain) (Chen et al., 2018; Li et al., 2021) either in concerted or consecutive manner. However, the spatial and temporal patterns of such interactions have not been elucidated yet (Carroll and Zukin, 2002; Lau and Zukin, 2007; Diering and Huganir, 2018; Groc and Choquet, 2020).

We should also mention that α2/δ-1 interacts with a number of other proteins, such as neurexin, LRP1 and prion protein but the analysis of the modulatory function of these interactions goes beyond the scope of this review and we refer for more comprehensive coverage of (Dolphin, 2016; Dolphin, 2018; Taylor and Harris, 2020).

Recently it was reported that sigma1 receptors (σ1R) promote, while the histidine triad nucleotide-binding protein 1 (HINT1) hinder the formation of α2/δ-1-NMDAR complexes in
periaqueductal gray. Thus, it appears that σ1R and HINT1 proteins coordinate the α2/δ-1-NMDAR association in such a manner that the development of mechanical allodynia requires the interplay of other proteins within the supraspinal periaqueductal gray (Rodríguez-Muñoz et al., 2021).

**Demonstration the impact of α2/δ-1 and NMDAR association in complex circuits**

To obtain broad scope proof for the novel phenomenon of α2/δ-1 and NMDAR interaction, the physiological existence and relevance of this association has been extensively demonstrated by utilizing the involvement of the molecular mechanisms in manifestation in complex circuits in numerous physiological aspects and systems (Table 2).

Chen et al., (Chen et al., 2019b) have shown that paclitaxel treatment in rats increased the α2/δ-1 expression level in the dorsal root ganglion and spinal cord, also potentiated the α2/δ-1–NMDAR interaction and synaptic trafficking in the spinal cord. Inhibiting α2/δ-1 trafficking with pregabalin, or disrupting the α2/δ-1–NMDAR interaction with an α2/δ-1 C-terminus–interfering peptide, or α2/δ-1 genetic ablation fully reversed paclitaxel treatment-induced presynaptic NMDAR-mediated glutamate release from primary afferent terminals to spinal dorsal horn neurons and markedly attenuated paclitaxel-induced pain hypersensitivity (Chen et al., 2019b).

Immunosuppressants, such as FK506 and cyclosporine, can cause pain that is termed as calcineurin inhibitor-induced pain syndrome. In an animal model, calcineurin treatment increased the amount α2/δ-1-NMDAR complexes in the spinal cord and evoked electrophysiological and pain characteristics that is typical for neuropathic pain. Similarly, α2/δ-1-NMDAR complexes are increased in chronic morphine treatment-induced hyperalgesia in an animal model. Systemic administration of gabapentin and α2/δ-1Tat peptide reversed the thermal and mechanical hypersensitivity (Huang et al., 2020) in both pain models, or
CACNA2D1 gene knockout abolished the increase in NMDAR activity and mechanical and thermal hyperalgesia (Deng et al., 2019a).

Brain injury-induced ischemia rapidly enhanced the $\alpha_2/\delta$-1-NMDAR physical interaction in the mouse brain tissue. Treatment with gabapentin, or $\alpha_2/\delta$-1 C-terminus-interfering peptide, or CACNA2D1 genetic knock-out, reduced middle cerebral artery occlusion-induced infarct volumes, neurological deficit scores, and calpain/caspase-3 activation in brain tissues (Luo et al., 2018).

Cac$\alpha_2/\delta$-1 and NMDARs were augmented in synaptosomes of spontaneously hypertensive rats compared to normotensive rats (Ma et al., 2018). Angiotensin II (Ang II) increased the prevalence of synaptic $\alpha_2/\delta$-1-NMDAR complexes in the hypothalamus. Furthermore, inhibiting $\alpha_2/\delta$-1, interrupting the $\alpha_2/\delta$-1-NMDAR interaction, or deleting $\alpha_2/\delta$-1 abolished the potentiating effects of Ang II on presynaptic and postsynaptic NMDAR activity in the hypothalamus (Zhou et al., 2021).

Theta-burst stimulation consistently induced corticosteroid long-term potentiation and increased the coincident presynaptic and postsynaptic NMDAR activity of medium spiny neurons. Inhibiting $\alpha_2/\delta$-1 trafficking with gabapentin or disrupting the $\alpha_2/\delta$-1-NMDAR interaction with an $\alpha_2/\delta$-1 C-terminus-interfering peptide abolished TBS-induced LTP (Zhou et al., 2018), (Huang et al., 2022).

Resinoferatoxin treatment induces small fiber sensory neuropathy and causes thermal sensory impairment and tactile allodynia, resembling to clinical features of postherpetic neuralgia. This neuropathy is also associated with $\alpha_2/\delta$-1 upregulation in the dorsal root ganglion, also enhanced presynaptic association between $\alpha_2/\delta$-1 and NMDAR was observed that was accompanied with an increased glutamatergic input to the spinal dorsal horn (Zhang 张广芬 et
al., 2021). Genetic or pharmacological manipulation of the interaction path between $\alpha_2/\delta-1$ and NMDAR largely normalized the electrophysiological and allodynia changes in resinoferatoxin treated rats.

**CONCLUSION**

Development of novel gabapentinoid drugs, such as mirogabalin resulted in higher potency, more efficacious and safer drug compared to first and second generation gabapentinoids. Mirogabalin-related compounds, still in development, potentially can demonstrate high efficacy and safety similar to the parent compound. The current breakthrough in determining the 3-dimensional structure of the mirogabalin drug receptor site on the $\alpha_2/\delta-1$ protein opened a new avenue for rational drug design of this drug class.

Gabapentinoid efficacy in chronic pain modulation manifests in weakening the glutamate release at presynaptic terminals in the spinal cord by interacting with calcium channel $\alpha_2/\delta-1$ and KCNQ2/3 and KCNQ5 potassium channels. This interaction has multiple consequences for synaptic transmission and network excitability. The discovery of interaction of $\alpha_2/\delta-1$ subunit with NMDAR and AMPAR broadened the knowledge on interacting domains of the $\alpha_2/\delta-1$ protein and extended the mechanistic implication of the role for this protein as a receptor for astrocyte-secreted thrombospondins that promote developmental synaptogenesis.

While gabapentinoids do not inhibit calcium channel currents, thrombospondins exert their synaptogenic effects via binding to their primary neuronal receptor site that is the calcium channel $\alpha_2/\delta-1$ subunit. Thrombospondins promote the establishment of synaptic connectivity, which is linked with excitatory synaptogenesis, and gabapentinoids efficaciously tone down this process (Figure 2).
Animal chronic pain models and analysis of limited number of human tissue samples established that overexpression of calcium channel $\alpha2/\delta$-1 and thrombospondins is correlated with the development of chronic pain. Gabapentinoid drugs potently block the $\alpha2/\delta$-1/TSP association, also block the association of $\alpha2/\delta$-1 with NMDARs and AMPARs and tone down the forward mechanisms of excitatory synaptogenesis, thereby efficaciously modulate the pain condition (Figure 2).

**AUTHOR CONTRIBUTION**

Wrote the manuscript: Varadi, G.

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**DATA AVAILABILITY**

This article contains no datasets generated or analyzed during the current study.
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Table 1. Clinically Approved Gabapentenoid Drugs and Gabapentenoids Used in Research

<table>
<thead>
<tr>
<th>GABAPENTINOID DRUG</th>
<th>DRUG NAME</th>
<th>BRAND NAME</th>
<th>APPROVAL/ TERMINATION</th>
<th>DEVELOPER</th>
<th>DOSES (mg/day)</th>
<th>IC\textsubscript{50} (nM)</th>
<th>REFERENCE</th>
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</thead>
<tbody>
<tr>
<td><strong>Drugs on market</strong></td>
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<tr>
<td>Gabapentin</td>
<td>Neurontin,</td>
<td>Gralise,</td>
<td>1993 (USA)</td>
<td>Parke-</td>
<td>900-3,600 t.i.d.</td>
<td>140</td>
<td>Brown et al., 1998,</td>
</tr>
<tr>
<td>Enacarbil (Gabapentin prodrug)</td>
<td>Horizant,</td>
<td>Regnant,</td>
<td>2011 (USA, Japan)</td>
<td>GlaxoSmith</td>
<td>600-1200</td>
<td>59.0\textsuperscript{b} (mice)</td>
<td>Cundy et al., 2004, 2008</td>
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<tr>
<td>Pregabalin</td>
<td>Lyrica,</td>
<td>(USA, EU)</td>
<td>2004</td>
<td>Pfizer</td>
<td>150-600 b.i.d.</td>
<td>80</td>
<td>Domon et al., 2018</td>
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<td></td>
<td>Lyrica CR</td>
<td></td>
<td></td>
<td></td>
<td>t.i.d</td>
<td>62.5\textsuperscript{b} (hum)</td>
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<td>Daiichi-</td>
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<td>Company</td>
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<tr>
<td>Atagabalin PD-0200390</td>
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<td>22</td>
<td>Blakemore et al., 2009c</td>
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<td>Pfizer</td>
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\*aC_{50} defined as concentration (nM) of test drug that produces half-maximal inhibition of ^3[H] gabapentin binding either to pig, rabbit brain membranes, or COS-7 or HEK293A or 293ts cell membranes expressing species-specific recombinant α2/δ-1 subunit.

\*bK_D of ^3[H]gabapentin, ^3[H]pregabalin, ^3[H]mirogabalin
Table 2. Demonstration of NMDAR-α2/δ-1 Association in DRG and Dorsal Spinal Cord in the Development of Synaptic Trafficking and NMDAR Hyperactivity in Several Pain and other Physiological Function Animal Models

<table>
<thead>
<tr>
<th>Physiological condition or disease model</th>
<th>Measured parameters</th>
<th>Detection of</th>
<th>Inhibition of process by</th>
<th>Reference</th>
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<td>ESPSs/ NMDAR Other</td>
<td>NMDAR-α2/δ-1</td>
<td>GBP α2/δ-1 Cacna2d1 KO C-tail peptide</td>
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<td>Pain hypersensitivity</td>
<td>mEPSCs activity association</td>
<td>Immunoprecipitation, luminescence resonance</td>
<td>Chen, 2018</td>
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<td>Chemotherapy-induced pain (Paclitaxel)</td>
<td>Immunoprecipitation, mRNA expression</td>
<td>Chen, 2019</td>
<td></td>
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<td>Calcineurin inhibitor-induced pain syndrome</td>
<td>Immunoprecipitation</td>
<td>Huang, 2020</td>
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</table>

*a* indicates significant difference.
(CIPS)

Opioid-induced hyperalgesia, and analgesic tolerance ↑ ↑ ↑\(^a\) Immunoprecipitation ↓ ↓ ↓ Deng, 2019

Resinoferatoxin-induced Neuropathy (postherpetic ↑ ↑ ↑\(^{a,g}\) Immunoprecipitation ↓ ↓ ↓ Zhang, 2021

Neuralgia model)

Focal cerebral ischemia Cerebral artery occlusion, ↑ ↑ ↑\(^{a,b}\) Immunoprecipitation ↓ ↓ ↓ Luo, 2018

and reperfusion

Long-term potentiation Learning and memory ↑ ↑ ↑\(^{c,d,e}\) Immunoprecipitation ↓ ↓ ↓ Zhou, 2018
### Theta-burst stimulation
LPT in spinal cord $\uparrow$ $\uparrow^{a,c,g}$ Immunoprecipitation $\downarrow$ $\downarrow$ $\downarrow$ Huang, 2022

### Development of hypertension
$\uparrow$ $\uparrow$ $\uparrow^{f}$ Immunoprecipitation $\downarrow$ $\downarrow$ $\downarrow$ Ma, 2018

Zhou, 2021

---

*a* pain hypersensitivity; *b* neurological deficit, brain infarct volume, spectrin breakdown, caspase-3 activation, long-term potentiation; *c* T-maize test; *d* rotarod test; *e* sympatoexcitatory response; *f* pain hypersensitivity was diminished both in *Cacna2d1* and *Grin1* cKO animals
LEGENDS TO FIGURES

Figure 1.
Structure of the $\alpha_2/\delta$-1 Protein and the 3D Structure of the Mirogabalin Binding Pocket.

A. Domain Structure of the $\alpha_2/\delta$-1 Protein.

The three-dimensional structure of the rabbit Cav1.1 channel was determined by cryo-electron microscopy (Wu et al., 2015; Wu et al., 2016), and the domain structure of the $\alpha_2/\delta$-1 protein is taken from these studies. Calcium channel $\alpha_2/\delta$ subunits are encoded by four genes (CACNA2D1 through CACNA2D4) (Ellis et al., 1988; Williams et al., 1992; Klugbauer et al., 1999; Qin et al., 2002; Hofmann et al., 2015) and the corresponding protein subunits ($\alpha_2/\delta$-1-4) are widely expressed in excitable tissues. The $\alpha_2/\delta$-1 and 2 exert specific binding affinity to GBP (Marais et al., 2001). The $\alpha_2/\delta$-1 protein is encoded by one gene (CACNA2D1) and the $\alpha_2$ and $\delta$ subunits are generated by posttranslational processing (De Jongh et al., 1990). The $\alpha_2$ and $\delta$ subunits are held together by four disulfide bonds, and the $\delta$ subunit intramolecularly also carries two disulfide bonds. Four Cache domains (amino acid and small nutrient molecule recognition domain typical in calcium channels and in a wide range of chemotaxis receptors) were identified in $\alpha_2/\delta$ proteins (Anantharaman and Aravind, 2000), and these domains are typical structural motifs in chemoreceptors of bacteria and archaea. Despite the distinct domain organization in the three-dimensional space, the domains are intertwined in the primary sequence, therefore, for clarity, they are schematically depicted as contiguous units in this figure. The ligand binding domain in bacterial chemoreceptors are termed dCache_1 that corresponds to double cache domains found in $\alpha_2/\delta$-1 (Gumerov et al., 2022). The von Willebrand factor A (VWF-A) domain contains a MIDAS (metal ion-dependent adherence site)
sequence (Wu et al., 2016) and this domain is also the major interacting site with the α1 subunit (Wu et al., 2016). The VWF-A domain is inserted in the first dCache-1 and splits the GBP binding site. Gabapentin and TSP-4 and other thrombospondins strongly compete in characteristic biological impacts, such as the manifestation of chronic pain in rat and mouse models (Park et al., 2016) and in synaptic growth (Eroglu et al., 2009). The interaction site between the δ subunit and the NMDAR (Chen et al., 2018) and AMPAR (Li et al., 2021) subunits, respectively, are located on the very C-terminal portion of the δ subunit. This region is between amino acids 1062-1092 of the rat sequence (Gibbs et al., 2004). It was debated whether the α2/δ-1 is glycosylphosphatidylinositol (GPI) -anchored to the outer leaflet the lipid raft of the cell membrane (Davies et al., 2007; Davies et al., 2010), or δ is a type I single transmembrane spanning protein and inserted in the membrane (Jay et al., 1991; Robinson et al., 2011). Unfortunately, the 3-D structure of the rabbit VGCC (Wu et al., 2015) did not provide proof for either anchor possibilities as the C-terminal region of the δ subunit showed very poor resolution for this region. Similarly, in the cryo-EM structure of (Kozai et al., 2023) the proteolytic cleavage site and the C-terminal domain appeared disordered and could not shed light on details of this region.

B. Three-dimensional Structure of the Recombinant Human α2/δ-1 and Representation of the Mirogabalin Ligand in the Binding Pocket

Left, electron density map of the recombinant human α2/δ-1; Middle, ribbon diagram of portions of the α2/δ-1 structure; colors in pink and brown, first dCache_1 domain; green, VWA-F domain; grey, second dCache_1 domain. The first double Cache domain 1 (dCache_1) in the α2/δ-1 protein is split by the VWF-A, and this latter also splits a consensus sequence

(YxxxRxWY[x=13-17]Y[x=27-34]D) that is essential for amino acid and nutrient binding in
chemoreceptors (Gumerov et al., 2022). Also, the R residue in this domain is the third arginine in the triple-arginine sequence that is essential for GBP binding (Wang et al., 1999). Right, the mirogabalin binding site structure in the of the distal binding pocket of dCache_1 of α2/δ-1 with mirogabalin. Mirogabalin is in turquoise, and the carboxyl and amino groups are in red and blue, respectively. Amino acid side-chains important in establishing the binding pocket are depicted. Selected residues Y236, R241, W243, Y450, D452, T461, D491, W205, V207, Y217, W223, L454, H167, and A215 are shown as sticks. The cryo-EM data and alanine-scanning mutagenesis data of (Kozai et al., 2023) further established that amino acids Y236, R241, W243 and Y450, D452, T461, D491 form the binding pocket for the carboxyl and amino groups, respectively. While amino acid residues W205, V207, Y217, W223 and L454 contribute to binding of the hydrophobic skeleton of mirogabalin, H167, V207 and A215 participate more in the binding the hydrophobic moiety of L-leucin (Kozai et al., 2023), in particular, substitution of A215 and Y217 in the α2/δ-3 and 4 sequence might explain their insensitivity to gabapentin. Residues (W205, A215, Y217) critical for binding the hydrophobic skeleton of mirogabalin are boxed. Also, the cryo-EM structure revealed a slight structural change in a region of VWF-A induced by mirogabalin binding (Kozai et al., 2023). Figure 1B is reproduced from (Kozai et al., 2023), (© 2023 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)).

Figure 2. Generalized Mechanism of Gabapentinoid Action in Hyperactive Neuronal Conditions.

Chronic pain, nociception, and numerous other behavioral phenomena involve the interaction of α2/δ-1 subunit protein and thrombospondins. The α2/δ-1 and TSPs are upregulated in chronic pain condition via mechanisms not known in detail yet. The interaction of TSP to α2/δ-1
contributes promoting synaptic growth and development and maintenance of chronic pain mechanisms and ultimately might contribute to central mechanisms. Postsynaptic $\alpha_2/\delta-1$ subunits are key organizers of glutamatergic synapses (Schopf et al., 2021). Postsynaptic, not presynaptic $\alpha_2/\delta-1$ subunit is required and sufficient for TSP-induced synaptogenesis in vivo and in vitro (Eroglu et al., 2009; Risher et al., 2018; Risher and Eroglu, 2020). The binding of TSP to $\alpha_2/\delta-1$ may serve as an initiating event to recruit and stabilize NMDA receptors to the postsynaptic site which may then control other downstream events of synaptogenesis. The $\alpha_2/\delta-1$ may recruit NMDAR by interaction with its C-terminal tail segment. Or alternatively TSP-$\alpha_2/\delta-1$ interaction could recruit NMDARs through intermediaries such as neurexins and neuroligins to the postsynaptic sites. TSP induces the formation of silent synapses containing NMDAR but no AMPAR on the postsynaptic surface. AMPARs are also recruited to the postsynaptic site by interacting with the C-terminal peptide of $\alpha_2/\delta-1$. Collectively these evidences show that binding of TSP to the extracellular domain of postsynaptic $\alpha_2/\delta-1$ facilitates clustering of pre-and postsynaptic proteins to nascent synaptic sites. The transmembrane region and the C-terminal tail of $\alpha_2/\delta-1$ trigger signaling events such as recruitment of factors including activation of Rho family of small GTPases member Rac1 for activities and reorganizing the cytoskeleton (Risher and Eroglu, 2020). Gabapentinoids can block the $\alpha_2/\delta-1$ and TSP interaction and consecutively synaptic growth. The $\alpha_2/\delta-1$ may also act as a scaffold protein to establish link to NMDARs and AMPARs. This latter interaction might contribute to synaptogenesis and promotes a massive glutamate impact in the spinal cord and central nervous system. Gabapentinoid drugs break interactions in the initiating event of synapse formation and reduce synaptic targeting and expression of NMDARs and AMPARs. These processes can function simultaneously and gabapentinoids have marked pharmacological efficacy in the development and modulation of chronic behavioral phenomena.
Figure 1

A

- S-S
- S-S
- S-S
- S-S

First dCache_1

GBP binding domain

Metal ion

Second dCache_1

MIDAS domain

VWA domain

C-tail peptide

B

Cryo-EM structure of human α,δ1 with mirogabalin and mutagenesis binding assays
Figure 2

Chronic pain
Glutamate release in
the spine

GBP

TSP

NMDAR

S-S

AMPAR

Rac1

Noxious, chronic stimulus
Facilitated processing