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## Calcium Channel $\alpha_2\text{-}\delta$ Subunit, A New Antiepileptic Drug Target

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Antiepileptic drugs (AED) protect against seizures through interactions with a variety of cellular targets. The actions on these targets are often categorized into three broad groups: (1) modulation of voltage-gated ion channels (mainly sodium and also calcium channels); (2) effects on GABA systems, including enhancement of synaptic inhibition mediated by GABA<sub>A</sub> receptors; and (3) inhibition of synaptic excitation mediated by ionotropic glutamate receptors (Rogawski and Löscher, 2004). The critical downstream action of AEDs that act on voltage-dependent sodium channels—such as phenytoin, carbamazepine and lamotrigine—may be to reduce action potential-dependent glutamate release, particularly that dependent upon prolonged high frequency firing as occurs during epileptic discharges (Lingamaneni and Hemmings, 1999; Rogawski, 2002). Voltage-dependent sodium channel block also may reduce the propagation of action potentials from the soma into dendrites (Jung et al., 1997; Colbert et al., 1997) and may reduce the dendritic amplification of synaptic potentials (Schwindt and Crill, 1995). Together, these actions inhibit the spread of epileptiform activity.

Electrophysiological recordings of synaptic responses demonstrate that sodium and calcium channel blocking anticonvulsants inhibit action potential-dependent synaptic events without affecting action potential-independent (‘miniature’) synaptic events. This is expected since these drugs do not block postsynaptic glutamate receptors and do not interfere directly with the synaptic release machinery. Interestingly, such drugs seem to have a preferential action on glutamate release and only weakly affect GABA release, possibly as a result of differences in excitation-contraction coupling in glutamatergic and GABAergic neurons (Prakriya and Mennerick, 2000; Westphalen and Hemmings, 2003).

In the past year, two entirely new AED targets have emerged. The targets were identified through similar pathways. The marketed AEDs gabapentin and levetiracetam—which were originally identified by virtue of their activity in empirical, mechanism-neutral screening models—were each found to bind with high affinity to unique recognition sites in brain. Two distinct proteins representing these recognition sites were identified and various lines of converging evidence supported the view that the binding proteins were the actual drug targets responsible for therapeutic activity. The two binding proteins have distinct subcellular localizations and undoubtedly have very different biological roles, although in both cases these roles are still largely obscure. Remarkably, both binding proteins may have roles in regulating vesicular neurotransmitter release. In the case of gabapentin (and the related AED pregabalin), drug binding has been shown to reduce neurotransmitter release. Because the levetiracetam target is localized to synaptic vesicles, it too may also influence neurotransmitter release. Recognizing that much remains to be learned, it is now appropriate to add a fourth category to

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the classification of AED mechanisms, as shown in Table 1 (see also illustration in Fig. 1). Here we describe evidence that one of these proteins—the calcium channel  $\alpha_2\text{-}\delta$  subunit—is an AED target; the other target, SV2A, is considered elsewhere (Rogawski, 2006).

The putative AED target  $\alpha_2\text{-}\delta$  actually represents a family of four related proteins (MW, ~125 kD) encoded by separate genes (Klugbauer et al., 2003; Arikath and Campbell, 2003; Qin et al., 2002). The first member to be identified,  $\alpha_2\text{-}\delta$  type 1, is expressed ubiquitously. The related proteins  $\alpha_2\text{-}\delta$  type 2,  $\alpha_2\text{-}\delta$  type 3 and  $\alpha_2\text{-}\delta$  type 4 are 54%, 26% and 25% identical, respectively, to  $\alpha_2\text{-}\delta$ -1 at the amino acid level and share common structural motifs.  $\alpha_2\text{-}\delta$ -2 is found in several tissues including brain and heart,  $\alpha_2\text{-}\delta$ -3 is brain-specific, and  $\alpha_2\text{-}\delta$ -4 is found in endocrine tissues and at very low levels in brain. The  $\alpha_2\text{-}\delta$  subunits are post-translationally cleaved into  $\alpha_2$ - and  $\delta$ -peptides, which are then covalently linked by a disulfide bridge.  $\alpha_2\text{-}\delta$ -1 and  $\alpha_2\text{-}\delta$ -2 are believed to form complexes with many calcium channel types (represented by different  $\alpha_1$  isoforms), allosterically enhancing current amplitude and also promoting channel trafficking to the membrane (Canti et al., 2003). The mouse mutant *ducky*, which is associated with mutations in the  $\alpha_2\text{-}\delta$  gene, exhibits spontaneous spike-wave seizures (Barclay et al., 2001). Similarly, targeted deletion of the  $\alpha_2\text{-}\delta$ -2 gene results in enhanced seizure susceptibility (Ivanov et al., 2004). These mouse models confirm a role for  $\alpha_2\text{-}\delta$ -2 in the regulation of seizure susceptibility.

Gabapentin has been known to bind  $\alpha_2\text{-}\delta$ -1 (encoded by the *CACNA2D1* gene) for nearly a decade (Gee et al., 1996). However, it is only recently that evidence has been developed that strongly links binding to the anticonvulsant activity of gabapentin and pregabalin [*S*-(+)-3-isobutyl GABA]. Indeed, several alternative theories have been proposed to explain the mechanism of action of these two drugs (Taylor, 2002). While gabapentin and pregabalin are GABA analogs and were originally synthesized with the intention of targeting GABA systems, they do not interact with GABA<sub>A</sub> or GABA<sub>B</sub> receptors or GABA transporters (Sills, 2006). Moreover, neither compound is active at other target sites commonly associated with anticonvulsant effects, including voltage-activated Na<sup>+</sup> channels or glutamate receptors (NMDA, strychnine-insensitive glycine, AMPA, KA or mGluR1, mGluR5). Both compounds, in contrast, are high-affinity ligands for the  $\alpha_2\text{-}\delta$ -1 and  $\alpha_2\text{-}\delta$ -2 proteins (but not  $\alpha_2\text{-}\delta$ -3 or  $\alpha_2\text{-}\delta$ -4) labeled with either [<sup>3</sup>H]gabapentin (Suman-Chauhan et al., 1993; Marais et al., 2001; Qin et al., 2002) or [<sup>3</sup>H]pregabalin (Piechan et al., 2004). Mutagenesis experiments have shown that the anticonvulsant binding site is probably confined to the  $\alpha_2$  protein and/or the external portion of the associated  $\delta$  component (Brown and Gee, 1998; Wang et al., 1999). Recent structure-activity studies with a variety of compounds related to gabapentin and pregabalin suggest that high affinity binding to  $\alpha_2\text{-}\delta$  is required for anticonvulsant activity with compounds structurally similar to these drugs (Bryans et al., 1998; Belliotti et al., 2005).

Additional strong evidence linking the pharmacological actions of gabapentin and pregabalin to  $\alpha_2\text{-}\delta$ -1 binding has come from studies with mice bearing a site-directed mutation in  $\alpha_2\text{-}\delta$ -1. With in vitro single amino acid mutagenesis experiments of the recombinant  $\alpha_2\text{-}\delta$  protein, substitution of arginine 217 with alanine was found to greatly reduce [<sup>3</sup>H]gabapentin-binding (Wang et al., 1999). Mice were then generated with this mutation (Bramwell et al., 2004; Taylor, 2004; Bian et al., 2004). Brains from these animals showed reduced [<sup>3</sup>H]pregabalin binding in the neocortex, amygdala, entorhinal cortex and hippocampus. The mutant mice exhibited pain responses and seizures that were indistinguishable from those of wild-type controls derived from crossing the same inbred lines. In addition, morphine was analgesic and phenytoin protected against seizures in the R217A animals. However, pregabalin (30 mg/kg) had no analgesic activity although it was analgesic in wild type controls. R217A mutant mice still show anticonvulsant responses to pregabalin in the maximal electroshock test, although the ED<sub>50</sub> value is shifted from 7.8 to 20 mg/kg (M. Vartanian and S. Baron, unpublished). These results indicate that  $\alpha_2\text{-}\delta$ -1 binding is required for the analgesic activity of pregabalin

and contributes to its anticonvulsant activity. It seems plausible that the additional anticonvulsant activity could result from binding to  $\alpha_2\text{-}\delta\text{-}2$ , but this will require experimental verification. In addition, further experiments with R217A mice that are genetically uniform (in contrast to the hybrid mice used for these early experiments) will clarify the present results.

How does binding to the  $\alpha_2\text{-}\delta$  proteins protect against seizures? Numerous studies have examined the effects of gabapentin or pregabalin on voltage-gated calcium channel function. There are several reports that the drugs reduce calcium current in neuronal cell body membranes (Stefani et al., 1998; Martin et al., 2003; McClelland et al., 2004). However, in other studies with neurons (van Hooft et al., 2002) or recombinant systems (Canti et al., 2003), gabapentin was inactive. Despite these conflicting data, studies of calcium influx measured with fluorescent probes in synaptosomes from rat neocortex (van Hooft et al., 2002) or human brain tissue obtained at epilepsy surgery (Fink et al., 2002) indicate that both gabapentin and pregabalin reduce calcium influx into presynaptic terminals. In addition, gabapentin and pregabalin have been shown to produce subtle but reproducible reductions in the calcium-dependent release of glutamate and other neurotransmitters, including norepinephrine, serotonin and dopamine, from neocortical tissues (Dooley et al., 2000a,b, 2002), and also to inhibit the release of glutamate stimulated by the peptide neurotransmitters substance P and calcitonin gene related peptide (CGRP) from sensory neurons (Maneuf et al., 2001). In some systems, pretreatment with agents that induce inflammation or with neuropeptides or protein kinase activators are required before clear drug-induced reductions in neurotransmitter release are seen (Fehrenbacher et al., 2003).

Recently, several studies have indicated that not only calcium-dependent release of neurotransmitters, but also asynchronous (spontaneous), calcium-independent release of individual transmitter vesicles from glutamate synapses in spinal cord slices (Shimoyama et al., 2000; Patel et al., 2000), entorhinal cortex slices (Cunningham et al., 2004) and cultured rat hippocampal neurons (K. Micheva and S. Smith, personal communication) is reduced by treatment with gabapentin or pregabalin. These results suggest that the actions of  $\alpha_2\text{-}\delta$  ligands to reduce neurotransmitter release may not require inhibition of calcium influx, and therefore may be mediated by an interaction of  $\alpha_2\text{-}\delta$  (or the calcium-channel complex containing  $\alpha_2\text{-}\delta$ ) with synaptic proteins that are involved in the release or trafficking of synaptic vesicles.

Could sites of action other than  $\alpha_2\text{-}\delta$  contribute to the pharmacological activity of gabapentin and pregabalin? It is, of course, impossible to rule out contributions from effects on other, as yet unidentified targets. However, the evidence accumulated to date suggests that other targets where the drugs are known to act are unlikely to contribute in a significant way to anticonvulsant activity. For example, 3-substituted GABA analogs like gabapentin and pregabalin have the ability to activate the GABA synthetase enzyme L-glutamic acid decarboxylase (GAD) (Andruszkiewicz and Silverman, 1989). However, the required drug concentrations are very high, and there is no correlation between enzyme activation and anticonvulsant activity. In structure-activity studies, pregabalin had the most potent anticonvulsant activity but was a weak activator of GAD, whereas other more potent activators of GAD had very weak anticonvulsant activity (Silverman et al., 1991). Gabapentin also inhibits GABA-transaminase (GABA-T), the enzyme responsible for degradation of GABA, although this effect is very weak and is not shared by pregabalin (Goldlust et al., 1995). If GABA-T inhibition were relevant to the anticonvulsant activity of gabapentin or pregabalin, the drugs should increase brain GABA levels, as is the case for the anticonvulsant drug vigabatrin. In the rat, neither gabapentin nor pregabalin increases GABA levels (Sills et al., 2003; Errante and Petroff, 2003), although small increases have been reported with gabapentin in human brain tissue slices (Errante et al., 2002). [Increases in GABA also were seen in human neocortex with topiramate (Petroff et al., 2001) and lamotrigine (Kuzniecky et al., 2002) which are not known to alter the synthesis or metabolism of GABA.] Since both gabapentin and

pregabalin are effective anticonvulsants in the rat, it is clear that GABA-T inhibition is not necessary for anticonvulsant activity in the model systems where it has been tested. Whether inhibition of GABA-T or elevated brain GABA concentrations contribute to the therapeutic activity in humans remains to be demonstrated.

Another target of gabapentin is the cytosolic isoform of branched-chain aminotransferase (Hutson et al., 1998). However, this potential target is not inhibited by pregabalin (T.-Z. Su, personal communication).

Finally, it is well recognized that gabapentin and pregabalin are substrates for the large neutral amino acid system L (leucine) transporter (Thurlow et al., 1993; Su et al., 2005). In fact, to be absorbed after oral administration and for active transport into the brain, 3-substituted GABA analogs also must be system L substrates (Fig. 2). The structural requirements for  $\alpha_2\text{-}\delta$  binding differ from those that confer system L substrate activity (Bellioti et al., 2005). Both *S*(+)- and *R*(-)-isobutyl GABA are system L substrates ( $\text{IC}_{50}$  values for inhibition of [ $^3\text{H}$ ]L-leucine uptake are 158 and 355  $\mu\text{M}$ , respectively), but the *S*-enantiomer (pregabalin) binds to  $\alpha_2\text{-}\delta$  with 10-fold greater affinity than the *R*-enantiomer. Consistent with the role of  $\alpha_2\text{-}\delta$  binding in mediating the anticonvulsant activity of pregabalin, only the *S*-enantiomer confers seizure protection in the maximal electroshock test (Taylor et al., 1993). Moreover, it is notable that analogs that bind to  $\alpha_2\text{-}\delta$  but are not system L substrates exhibit anticonvulsant activity but only when administered intracerebroventricularly (Schwarz et al., 2005). Clearly, the system L transporter is not a target for the pharmacodynamic activity of gabapentin and pregabalin, although substrate activity is essential for efficacy when the drugs are administered systemically. In sum, it does not appear that any of the previously described targets for 3-substituted GABA analogs other than  $\alpha_2\text{-}\delta$  are relevant to the pharmacodynamic actions of this class of drugs.

Since the discovery of gabapentin in the mid 1970s and pregabalin in 1990, various theories have been proposed to explain the anticonvulsant properties of these molecules (Taylor, 2002; Sills, 2006). Gabapentin and pregabalin show a similar spectrum of activity in animal models that is distinct from that of other AEDs, suggesting that they share a common mechanism of action (Vartanian et al., 2006). Recently, converging lines of evidence have pointed to the  $\alpha_2\text{-}\delta$  proteins as critical targets for the anticonvulsant effects of both drugs. However, since pregabalin protects against seizures in  $\alpha_2\text{-}\delta\text{-1}$  R217A mutant mice (albeit with reduced potency), an important question is whether binding to  $\alpha_2\text{-}\delta\text{-2}$  is responsible for the residual anticonvulsant activity or whether there are additional targets.

While it is now clear that  $\alpha_2\text{-}\delta\text{-1}$  represents a site of action for gabapentin and pregabalin, it is still not fully defined how binding results in therapeutic effects. There is evidence that presynaptic inhibition of neurotransmitter release (presumably of glutamate) is the critical effect that emerges from drug binding. However, to what extent this occurs through modulation of nerve terminal calcium channel activity or alternatively by direct effects on the release of synaptic vesicles remains to be determined. Finally, much needs to be learned about the functional roles of  $\alpha_2\text{-}\delta$  proteins: how they modulate calcium channel behavior or whether they have other roles in the cell. Only then will it be possible to have a complete picture of the pharmacology of anticonvulsant drugs that act at this novel target.

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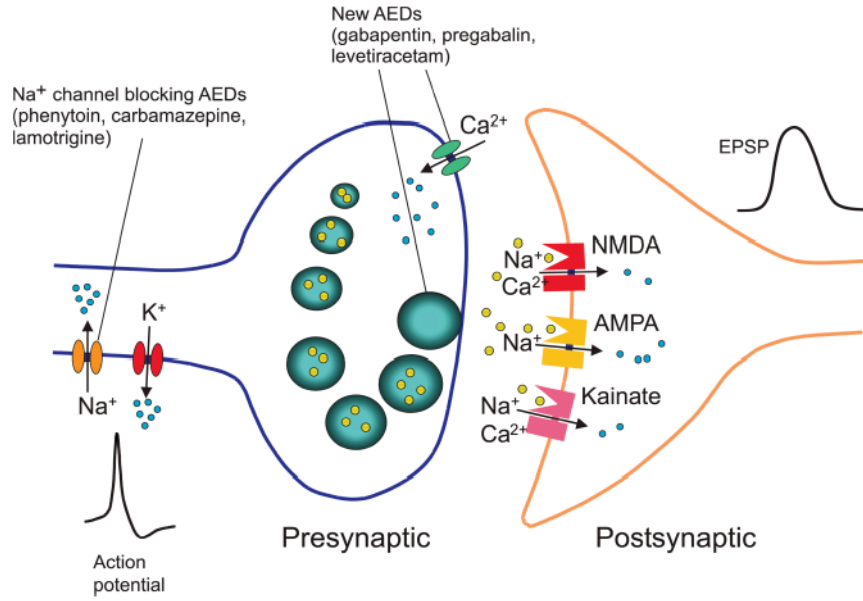
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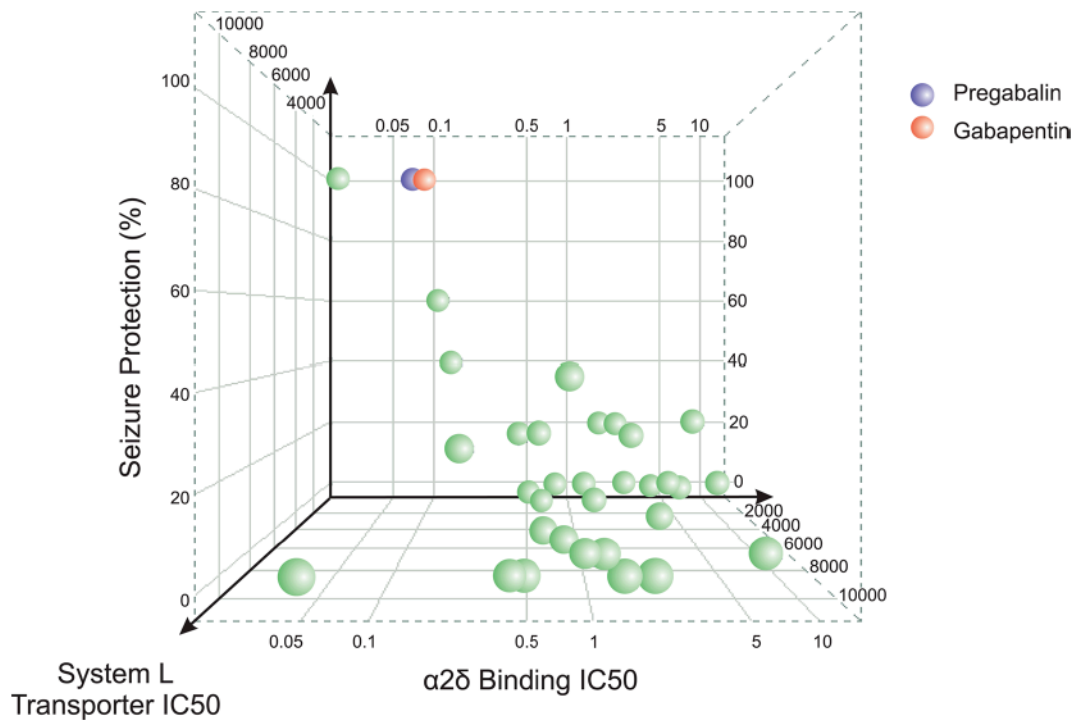
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**Figure 1.** Model synapse illustrating interaction of Na<sup>+</sup> channel blocking AEDs with voltage-activated Na<sup>+</sup> channels and putative sites of action of newer AEDs that may more directly interact with release machinery. Gabapentin and pregabalin bind to α<sub>2</sub>-δ, which may inhibit voltage-activated Ca<sup>2+</sup> entry through high voltage-activated Ca<sup>2+</sup> channels or affect the way in which Ca<sup>2+</sup> channels interact with vesicular release. Levetiracetam may also affect release by binding to synaptic vesicles protein SV2A. Action potentials are mediated by voltage-activated Na<sup>+</sup> and K<sup>+</sup> channels; Na<sup>+</sup> channel blocking AEDs suppress epileptiform action potential firing, which leads to inhibited release. Small blue circles indicate ions; larger yellow circles represent glutamate within synaptic vesicles and free in the synaptic cleft. Glutamate acts on ionotropic receptors (of the NMDA, AMPA and kainate types) to generate an excitatory postsynaptic potential (EPSP) in the postsynaptic neuron.





**Figure 2.**

Relationship between  $\alpha_2$ - $\delta$  binding affinity, system L transporter affinity, and potency for protection against audiogenic seizures in DBA/2 mice for gabapentin, pregabalin and structural analogs. The Y-axis represents the percent protection (out of 5 animals) in the seizure model at a dose of 30 mg/kg, p.o. The Z-axis represents the concentration ( $\mu$ M) producing half-maximal inhibition of [ $^3$ H]L-leucine uptake by the system L transporter in CHO-K1 cells. The X-axis represents the concentration ( $\mu$ M) producing half-maximal inhibition of specific [ $^3$ H] gabapentin binding to pig brain membranes. Seizure protection correlates with  $\alpha_2$ - $\delta$  binding only for those compounds that are substrates for the system L transporter, which appears to be required for absorption by the gut and delivery to the brain (D.J. Wustrow and C.P. Taylor, unpublished).

**Table 1**

## Revised classification of AED mechanisms

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1	Modulation of voltage-dependent Na <sup>+</sup> or Ca <sup>2+</sup> channels (leading to a secondary inhibition of synaptic release, particularly of glutamate; e.g., phenytoin, carbamazepine, lamotrigine) or to inhibition of intrinsic bursting (e.g., ethosuximide)
2	Enhancement of GABA-mediated inhibition or other effects on GABA systems (e.g., benzodiazepines, vigabatrin, tiagabine)
3	Inhibition of synaptic excitation mediated by ionotropic glutamate receptors
4	Modulation of synaptic release, particularly of glutamate, through direct actions on release machinery (e.g., gabapentin, pregabalin, levetiracetam)

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