The anti-allodynic $\alpha_2\delta$ ligand pregabalin inhibits the trafficking of the calcium channel $\alpha_2\delta$-1 subunit to presynaptic terminals *in vivo*

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Abstract

Neuropathic pain is caused by lesion or dysfunction of the peripheral sensory nervous system. Up-regulation of the voltage-gated Ca^{2+} channel subunit $\alpha_2\delta$-1 in DRG (dorsal root ganglion) neurons and the spinal cord correlates with the onset of neuropathic pain symptoms such as allodynia in several animal models of neuropathic pain. The clinically important anti-allodynic drugs gabapentin and pregabalin are $\alpha_2\delta$-1 ligands, but how these drugs alleviate neuropathic pain is poorly understood. In the present paper, we review recent advances in our understanding of their molecular mechanisms.

Neuropathic pain

Unlike nociceptive or acute pain, neuropathic pain occurs without continuous noxious peripheral input. Patients with neuropathic pain experience spontaneous pain that is described as 'electric-shock-like, burning and tingling'. Further symptoms of neuropathy are the painful response to normally innocuous stimuli (allodynia) and the increased response to noxious stimuli (hyperalgesia). In addition, patients also suffer from depression, anxiety and insomnia as a result of their chronic pain condition. A recent survey showed that up to 8% of the population of the U.K. may endure chronic pain of predominantly neuropathic origin [1].

Neuropathic pain is caused by damage to primary sensory afferent neurons of the peripheral nervous system that relay nociceptive and non-nociceptive peripheral stimuli to the spinal cord and the brain [2] as a result of, e.g., trauma, diabetes, cancer and chemotherapy [3]. Diabetes is the most common cause of neuropathic pain in the U.K. The estimated annual NHS (National Health Service) expenditure on the treatment of peripheral diabetic neuropathy and its associated complications is in the range of £250 million and is expected to rise [4].

The axons of primary sensory afferents form the spinal nerves and dorsal roots, with their cell bodies residing within the DRGs (dorsal root ganglia) [2]. Damaged DRG neurons become hyperexcitable (increased probability to fire action potentials) and show ectopic activity (spontaneous firing of action potentials). Hyperexcitability and ectopic activity lead to increased transmitter release in the spinal cord and this causes central sensitization (increased excitability of neurons within the central nervous system). These changes in neuronal activity together with increased activation of descending pathways from the brain are the mechanistic components in the development and maintenance of neuropathic pain [3].

The role of the voltage-gated Ca^{2+} channel subunit $\alpha_2\delta$-1 in neuropathic pain

Voltage-gated Ca^{2+} channels are heteromultimeric complexes consisting of the pore-forming $Ca_v\alpha_1$ subunit that determines the main biophysical properties of the channel and the auxiliary subunits β and $\alpha_2\delta$ (for a review, see [5]). $\alpha_2\delta$ increases Ca^{2+} currents by increasing the number of functional channels at the plasma membrane through enhancement of $Ca_v\alpha_1$ trafficking to the plasma membrane, and by stabilization of the channels at the cell surface [5]. The $\alpha_2\delta$ subunit consists of two proteins that are derived from a single gene product by proteolytic cleavage, namely the extracellular α_2- and the δ-protein that is thought to be transmembrane. The proteins α_2 and δ remain linked via disulfide bonds. The α_2-protein is heavily glycosylated and harbours the vWF-A (von Willebrand factor-A) domain [5]. An intact vWF-A domain is a prerequisite for the positive effect of $\alpha_2\delta$ on Ca^{2+} channel forward trafficking [6]. So far, four genes encoding $\alpha_2\delta$ subunits ($\alpha_2\delta$-1–$\alpha_2\delta$-4) have been identified. $\alpha_2\delta$-1 was cloned from skeletal muscle, but was found to be relatively ubiquitously expressed, whereas $\alpha_2\delta$-2 and $\alpha_2\delta$-3 are more restricted to the brain and $\alpha_2\delta$-4 to certain endocrine tissues and retina [5].

Key words: $\alpha_2\delta$-1, neuropathic pain, pregabalin, spinal nerve ligation, trafficking, voltage-gated calcium channel.

Abbreviations used: DRG, dorsal root ganglion; GABA, γ-aminobutyric acid; SNL, spinal nerve ligation; vWF-A, von Willebrand factor-A.

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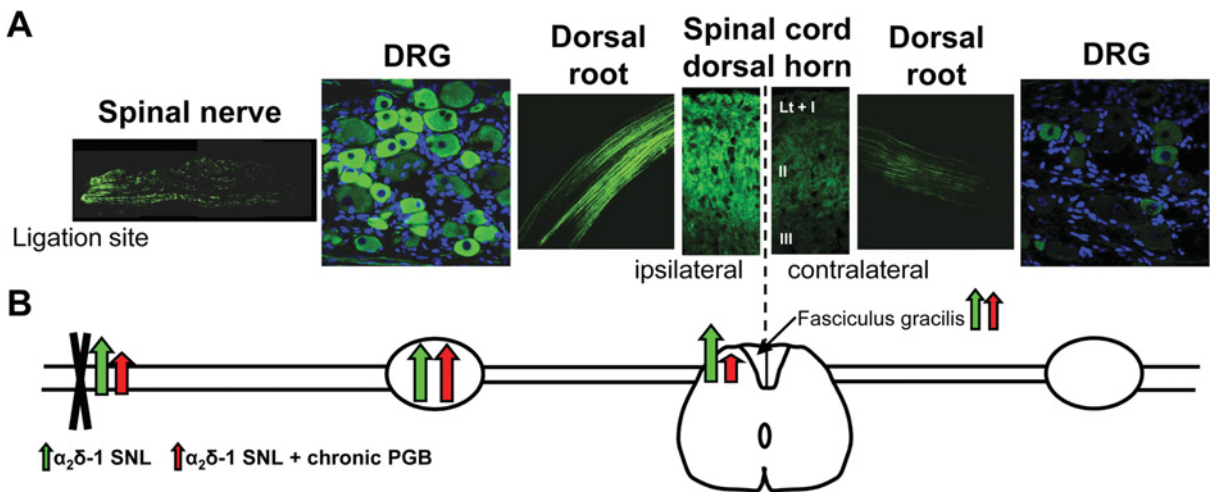
Figure 1 | Distribution of $\alpha_2\delta$-1 following SNL

(**A**) Immunofluorescence images showing the distribution of $\alpha_2\delta$-1 in (from left to right) ligated spinal nerve, DRG, dorsal root and the superficial layers of the spinal cord dorsal horn on the ipsilateral side of the lumbar region L5 compared with the spinal cord dorsal horn (Lt, Lissauer's tract; I, II and III, lamina 1, 2 and 3 respectively), the dorsal root and DRG on the contralateral side. $\alpha_2\delta$-1 is up-regulated in the ipsilateral DRG, dorsal root and spinal cord dorsal horn compared with the contralateral side. Moreover, $\alpha_2\delta$-1 accumulates at the ligation site. (**B**) Illustration of $\alpha_2\delta$-1 immunofluorescence staining and the effect of the chronic pregabalin (PGB) application on the distribution. $\alpha_2\delta$-1 protein is up-regulated following spinal nerve ligation (green arrows). This up-regulation is affected by chronic PGB (red arrow). The size of the red arrow relative to the green represents the effect of chronic PGB on $\alpha_2\delta$-1 distribution. The effect of chronic PGB on the $\alpha_2\delta$-1 level in the fasciculus gracilis was small, but significant [11]. However, there was no detectable effect in the DRGs. The ligation site is indicated by X.



Accumulating evidence from numerous groups points to an important role of $\alpha_2\delta$ in neuropathic pain. $\alpha_2\delta$-1 mRNA and protein levels are dramatically up-regulated in affected DRGs in several models of neuropathic pain, and this increase in $\alpha_2\delta$-1 correlates with the onset of allodynia [7–11]. In contrast with $\alpha_2\delta$-1, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 were found to be down-regulated in the unilateral lumbar SNL (spinal nerve ligation) model of neuropathic pain [12], demonstrating the dominant role played by $\alpha_2\delta$-1 in neuropathy [11]. Furthermore, transgenic mice overexpressing $\alpha_2\delta$-1 show allodynic symptoms even in the absence of nerve damage, indicating that increased levels of $\alpha_2\delta$-1 are sufficient to cause neuropathic pain [13].

In a recent study [11], we performed a detailed analysis of the distribution of $\alpha_2\delta$-1 in DRG neurons in the SNL model (Figure 1). DRG neurons are heterogeneous in morphology and their function correlates with the size of their somata. Small and medium-sized somata belong to mainly C- and Aδ-nociceptors, whereas non-nociceptive Aβ fibres have a large soma [7]. This study showed that $\alpha_2\delta$-1 protein levels were elevated in all three size groups of DRG neuron somata at the level of ligation, regardless of their function (Figure 1A) [11]. The augmentation occurred in the endoplasmic reticulum and at the plasma membrane.

$\alpha_2\delta$-1 protein levels were also found to be increased in the dorsal roots that are formed by the central axon branches of DRG neurons (Figure 1A) [11]. This increase intensified over days following ligation (experimental time points were 2, 4 and 7 days post-SNL). Within the dorsal roots, $\alpha_2\delta$-1 was localized to tubular-vesicular structures that are implicated in protein trafficking [11]. A subset of DRG neurons involved in touch and proprioception do not form synapses with spinal cord dorsal horn neurons, but their central axons directly project up to the brainstem. These axons form the fasciculus gracilis as part of the spinal cord dorsal column, and transection of the dorsal column prevents tactile allodynia symptoms in SNL rats [14]. $\alpha_2\delta$-1 was increased in the fasciculus gracilis starting at the level of ligation, and this augmentation continued up to the brain stem [11].

The majority of DRG neurons, however, form glutamatergic synapses on to second-order sensory neurons in the spinal cord dorsal horn. $\alpha_2\delta$-1 levels are increased in the spinal cord dorsal horn [10,11] and this up-regulation of $\alpha_2\delta$-1 is crucial for the aetiology of neuropathic pain [15]. The increase occurred in the superficial and to some extents also in the deeper layers of the dorsal horn (Figure 1A) [11]. This increase in $\alpha_2\delta$-1 protein levels was not accompanied by an increase in $\alpha_2\delta$-1 mRNA in the spinal cord dorsal horn neurons [11], but was due to an increase in $\alpha_2\delta$-1 in the presynaptic terminals of the DRG neurons [11]. Such an increase in $\alpha_2\delta$-1 in presynaptic terminals of affected DRG neurons is thought to enhance Ca^{2+} influx at the nerve

terminals and therefore increases synaptic transmitter release into the spinal cord which then causes central sensitization [16].

The $\alpha_2\delta$ ligands and anti-allodynic drugs pregabalin and gabapentin inhibit anterograde trafficking of $\alpha_2\delta$

The current first-line treatment of neuropathic pain comprises the anticonvulsant gabapentinoid drugs gabapentin and pregabalin. These drugs have an analgesic effect on neuropathic neuronal activity without affecting baseline nociception [17]. Gabapentin was originally designed as an analogue of GABA (γ-aminobutyric acid) with increased membrane permeability, but, like its more potent derivative pregabalin, showed little affinity for the relevant GABA-binding sites (for a review, see [18]). Various molecular targets have been proposed (for a review, see [19]), but, to date, just one high-affinity binding site has been found, namely $\alpha_2\delta$ [18].

Of the four known isoforms of $\alpha_2\delta$, only $\alpha_2\delta$-1 and $\alpha_2\delta$-2 interact with the gabapentinoid drugs, and $\alpha_2\delta$-1 has a higher affinity than does $\alpha_2\delta$-2 [5]. The arginine residue of $\alpha_2\delta$-1 at position 217 close to the vWF-A domain is critical for the binding [5]. Mutating this arginine residue to alanine (R217A) greatly reduces drug-binding affinity and R217A knockin mice develop neuropathic pain that is insensitive to pregabalin and gabapentin [20]. These observations indicate that the binding of the gabapentinoid drugs to $\alpha_2\delta$-1 is required for their analgesic effect.

Several studies have shown that pregabalin and gabapentin reduce neurotransmitter release [18], but the molecular mechanism of this inhibition is unclear. Transmitter release by means of stimulus-coupled Ca^{2+}-dependent exocytosis depends on a tight spatial and temporal interplay of a plethora of processes and proteins [21]. Transmitters are stored in specialized vesicles and released in a highly controlled manner. Fusion of the vesicle with the plasma membrane is the final step of secretion when Ca^{2+} that enters the cell through Ca^{2+} channels triggers the fusion of the vesicle with the cell membrane. Therefore an obvious mechanism would be that the gabapentinoid drugs inhibit Ca^{2+} channels directly by binding to $\alpha_2\delta$-1, which would then reduce presynaptic Ca^{2+} influx and subsequent transmitter release. However, evidence for a direct inhibitory effect of gabapentinoid drugs on native Ca^{2+} currents and synaptic transmission is inconclusive [5]. Whereas some studies reported a small reduction of Ca^{2+} current when the drugs were applied acutely, others did not observe such an acute inhibitory effect on either heterologously expressed channels or endogenous Ca^{2+} currents in DRG neurons *in vitro* [22,23]. Only a prolonged and chronic application of pregabalin and gabapentin was able to reduce Ca^{2+} influx [22,23]. Thus it seems unlikely that gabapentinoid drugs inhibit Ca^{2+} channels directly. However, chronic application of gabapentin and pregabalin reduced the amount of the Ca^{2+} channel subunits $\alpha_2\delta$ and $Ca_v\alpha_1$ at the cell surface without affecting their rate of endocytosis [11,22]. This led to the conclusion that chronic application of gabapentinoid drugs *in vitro* reduced Ca^{2+} influx owing to a reduction of the forward trafficking of $\alpha_2\delta$ and $Ca_v\alpha_1$ to the plasma membrane. Interestingly, to reduce Ca^{2+} channels at the plasma membrane, gabapentin had to be taken up into the cell via the system L amino acid transporter and possibly displaced an endogenous ligand that is a positive modulator of $\alpha_2\delta$ forward trafficking [22].

A classical approach to study neuronal protein trafficking *in vivo* is to perturb trafficking by nerve ligation or nerve section. Proteins that are being trafficked accumulate at the site of the obstruction, and the level of accumulation is a measure of trafficking activity. In the SNL model of neuropathic pain, $\alpha_2\delta$-1 was found to accumulate proximal to the SNL site [11]. These results showed for the first time that endogenous $\alpha_2\delta$-1 is indeed subject to anterograde trafficking from the DRG somata to both peripheral and central terminals (Figure 1A). Chronic treatment of SNL animals with repetitive injections of pregabalin had a profound anti-allodynic effect on neuropathic pain symptoms and it inhibited the accumulation of $\alpha_2\delta$-1 at the SNL site [11]. Moreover, it also reduced $\alpha_2\delta$-1 in ascending DRG axons of the fasciculus gracilis and, most importantly, reduced the increase of $\alpha_2\delta$-1 in the presynaptic terminals of DRG neurons in the spinal cord dorsal horn. Because chronic pregabalin and gabapentin had no effect on the up-regulation of $\alpha_2\delta$-1 in DRG somata (Figure 1B) [11,24], these results strongly suggest that the anti-allodynic effect of chronic gabapentinoid drug treatment was due to an inhibition of anterograde trafficking of $\alpha_2\delta$-1 *in vivo*.

Conclusion and outlook

Recent advances in our understanding of the molecular mechanisms of gabapentinoid drugs indicate that pregabalin alleviates neuropathic pain by impairing the trafficking of $\alpha_2\delta$-1 to presynaptic terminals of DRG neurons which would reduce Ca^{2+} influx and transmitter release in the spinal cord and subsequently reduce spinal sensitization. This intracellular effectiveness of pregabalin is a novel molecular mechanism to explain the anti-allodynic effect of pregabalin and is in clear contrast with other analgesic drugs that influence their targets at the cell surface. Further studies are needed to unravel how and where in the cell gabapentinoid drugs affect the trafficking machinery of $\alpha_2\delta$-1.

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