Differential upregulation in DRG neurons of an α2δ-1 splice variant with a lower affinity for gabapentin after peripheral sensory nerve injury

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1. Introduction

Voltage-gated Ca2+ channels of the Cav1 and Cav2 families contain 3 subunits: the pore-forming α1 subunit, together with 2 auxiliary subunits, β and αδ, both of which increase the functional expression of the channels [4,11,13,20,21]. The αδ subunits are each the product of a single gene (CACNA2D1-4), encoding an αδ preprotein, which is posttranslationally processed into αδ and δ [22]. We have recently shown that αδ subunits can form glycosylphosphatidylinositol-anchored proteins [19], which are constitutively endocytosed and reinserted into the plasma membrane via the recycling endosomes [5,48]. We have identified that the mechanism whereby αδ-1 and -2 subunits enhance plasma-membrane expression of calcium channels involves the metal ion-dependent adhesion site (MIDAS) motif in their von Willebrand factor A (VWA) domain [12,31], and it is also important for the enhancement of presynaptic vesicular release [31].

The αδ-1 protein is an auxiliary subunit of voltage-gated calcium channels, critical for neurotransmitter release. It is upregulated in dorsal root ganglion (DRG) neurons following sensory nerve injury, and is also the therapeutic target of the gabapentinoid drugs, which are efficacious in both experimental and human neuropathic pain conditions. The αδ-1 has 3 spliced regions: A, B, and C. A and C are cassette exons, whereas B is introduced via an alternative 3’ splice acceptor site. Here we have examined the presence of αδ-1 splice variants in DRG neurons, and have found that although the main αδ-1 splice variant in DRG is the same as that in brain (αδ-1 ΔA+B+C), there is also another αδ-1 splice variant (ΔA+B+C), which is expressed in DRG neurons and is differentially upregulated compared to the main DRG splice variant αδ-1 ΔA+B+C following spinal nerve ligation. Furthermore, this differential upregulation occurs preferentially in a small nonmyelinated DRG neuron fraction, obtained by density gradient separation. The αδ-1 ΔA+B+C splice variant supports Cav2 calcium currents with unaltered properties compared to αδ-1 ΔA+B+C, but shows a significantly reduced affinity for gabapentin. This variant could therefore play a role in determining the efficacy of gabapentin in neuropathic pain.

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extracellular matrix proteins, and this may influence its trafficking as well as the effect of gabapentinoid drugs [23].

Alternatively spliced isoforms of α2δ-1 in different tissues have been observed previously [10,22]. It was reported that the mouse cacna2d1 gene has 3 alternatively spliced regions (A, B, and C), and 5 splice variants were identified [3] (Fig. 1A). In the present study we have examined the hypothesis that there may be differential upregulation of specific splice variants of α2δ-1 following peripheral nerve damage, and that this might potentially contribute to either the state dependency [14,25] or to limiting the response to gabapentinoids, as these drugs are found to have variable efficacy in patients, with number-needed-to-treat values of about 4–5 [37,38].

2. Methods

2.1. Molecular biology and heterologous expression of cDNAs

Rat α2δ-1 (M86621.3) [47], which encodes the main brain splice variant ΔA+B+C, was used as the starting point for assembly of the α2δ-1 splice variants used in this study. All mutations were made by standard molecular biological techniques, and verified by DNA sequencing. Other calcium channel complementary DNAs (cDNAs) used were rat Caα2.1 (M64373), rabbit Caα2.2 (D14157), and rat β1b [47]. The cDNAs were cloned into the pMT2 vector for expression. For heterologous expression, tsA-201 cells were transfected with the cDNA combinations stated. The cDNA for green fluorescence (mut3 GFP) [17] was also included to identify transfected cells from which electrophysiological recordings were made. Transfection was performed as described previously [40]. In control experiments where α2δ was omitted, the ratio was made up with empty vector.

2.2. Isolation of detergent-resistant membranes (DRMs)

This procedure was performed as previously described [19,32]. Briefly, confluent cells from 6 175-cm² flasks (72 hours after transfection), or pelleted homogenate from rat whole brain, were resuspended in 1.5 mL 2-(N-morpholino)ethanesulfonic acid (MES)-buffered saline containing 1% (2% for brain tissue) Triton X-100 (Perbio, Tattenhall, Cheshire, UK) and left on ice for 1 hour. After the addition of an equal volume of 90% (w/v) sucrose, the sample was overlaid with a 10-mL discontinuous sucrose gradient and centrifuged at 140,000 g for 1 hour. After the addition of an equal volume of 90% (w/v) sucrose, the sample was overlaid with a 10-mL discontinuous sucrose gradient and centrifuged at 140,000 g for 1 hour.

Following the guidelines of the International Association for the Study of Pain [51], Selective spinal nerve ligation (SNL) surgery was conducted as previously described [34]. Briefly, the left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread under isoflurane anaesthesia (50% O2: 50% N2O). Haemostasis was confirmed and the wound was sutured. After surgery the animals were allowed to recover and housed at a maximum of 5 per cage. Food and water were available ad libitum. The foot posture and general behaviour of the operated rats were monitored throughout the postoperative period, and the development of mechanical hypersensitivity was confirmed at 7 days post surgery in the affected limb ipsilateral to the ligation, as previously described [5].

2.5. Spinal nerve ligation (SNL)

A total of 17 male Sprague-Dawley rats (Central Biological Services, University College London, London, UK) weighing 130–150 g at time of surgery were employed for this study. All experimental procedures were approved by the UK Home Office and followed the guidelines of the International Association for the Study of Pain [51]. Selective spinal nerve ligation (SNL) surgery was conducted as previously described [34]. Briefly, the left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread under isoflurane anaesthesia (50% O2: 50% N2O). Haemostasis was confirmed and the wound was sutured. After surgery the animals were allowed to recover and housed at a maximum of 5 per cage. Food and water were available ad libitum.

In order to obtain sufficient material, both L5 and L6 DRG were extracted from the ipsilateral and contralateral sides of 2 rats, either naïve animals or 7 days after SNL. Nerve roots were trimmed and the isolated ganglia were incubated in 5 U/mL of collagenase (Sigma-Aldrich) in 2 mL of Hanks balanced saline solution in a shaking water bath at 37°C for 30 minutes, and then triturated. The cell suspension was sedimented at 4°C for 5 minutes at 200 x g. The pellet was resuspended in culture medium composed of Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (Gibco, Invitrogen, Life Technologies, Grand Island, NY, USA), penicillin (100 U/mL), streptomycin (100 μg/mL), l-glutamine (2 mM), nerve growth factor (100 ng/mL, Sigma-Aldrich), and 10% foetal calf serum. Four mL of DRG neurons in culture medium was filtered through a 100-μm cell strainer (BD Biosciences) and layered onto 5 mL of a solution of 40% Ficoll (Histopaque-1077, Sigma-Aldrich) and 60% phosphate-buffered saline containing 4.2 mM NaHCO₃. The final pH was adjusted to 7.3–7.4 with HCl. The preparation was centrifuged at 100 x g for 15 minutes at 4°C, and DRG neurons were separated according to their size into a low-density fraction (LDF) and a high-density fraction (HDF) [28]. The 2 fractions obtained were diluted to 10 mL with culture medium and then centrifuged at 200 x g for 5 minutes at 4°C. The LDF and HDF pellets, enriched in viable small and large neurons, respectively, were
Fig. 1. Distribution of different αδ-1 splice variants in rat tissue. (A) Diagram of possible splice variants of αδ-1, showing arrangement of exons within the region of alternative splicing, and position of cassette exon 18a encoding A (white), alternative spliced region B (black), and cassette exon 23 encoding C (dark grey), data obtained from Ensembl database, gene IDs ENSG00000153956 (human), ENSMUSG00000040118 (mouse), and ENSRNOG00000033531 (rat). (B) Identification of αδ-1 splice variants using outer primer pairs. Bands were recovered from the gel and sequenced, to verify identification. Lanes 1–4 were amplified from plasmids, lanes 5–8 were amplified from the tissues stated, lane 9 is the water control, and lane L is the size ladder. Samples were run on 2% MetaPhor Agarose Gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) in Tris Acetate Ethylenediaminetetraacetic acid, 100V. (C) ΔAΔBΔC is the most abundant αδ-1 splice variant in rat heart. Representative electropherogram of capillary electrophoresis/laser-induced fluorescence with polymerase chain reaction (PCR) products derived from rat heart. Reverse-transcription PCR was performed for 30 cycles on 100 ng of total RNA equivalent. αδ-1 transcript products were separated by size along the x-axis: ΔAΔBΔC (345 bp), ΔAΔBΔC (360 bp), ΔAΔBΔC (366 bp), ΔAΔBΔC (381 bp), +ΔAΔBΔC (402 bp), +ΔAΔBΔC (423 bp), +ΔAΔBΔC (438 bp). The y-axis indicates the fluorescence signal peak height, which corresponds to the expression level of the respective splice variant. (D) Diagram of domains in αδ-1 to show the approximate positions of the 2 domains with homology to bacterial chemosensory domains (CSDs), with respect to the von Willebrand factor A domain and to regions A, B, and C. (E) Structure prediction for the bacterial chemosensor-like domains of αδ-1. The sequence of αδ-1 including the 2 predicted bacterial chemosensory domains [starting at amino acid 491 [Asp-Val-Ser-Leu . . . -945 ( . . . Leu-Glu-Ala, which is the end of αδ-1] was submitted to Phyre2 [33] for structure prediction (http://www.sbg.bio.ic.ac.uk/phyre2). The amino acid numbering includes the N-terminal signal sequence of αδ-1. Region I (αδ-1 amino acids 491–607) was predicted with 99% confidence, and modelled on a number of bacterial CSDs, including the extracellular domain of the Bacillus subtilis CSD (mmhk1s-z2) and the putative sensory box/ggdef protein from Vibrio parahaemolyticus. Region II (αδ-1 amino acids 687–886) was modelled with a predicted 98% confidence on a separate subset of CSDs, including the C4-dicarboxylate transport sensor protein dcb, and the mec-p_n and cache domains of methyl-2 accepting chemotaxis protein from Vibrio cholerae. The regions between domains I and II and beyond II are not modelled with high confidence. The approximate positions of the two spliced regions B and C are indicated. They are both likely to be in exposed loops, B being within the first chemosensory domain, and C being between the 2 domains. A is not present in this model.
obtained and immediately used for RNA extraction, or plated on medium to large neurons [26,29] was employed to confirm the successful separation of neuronal fractions.

2.7. Quantification of splice variants and NF-200 by quantitative polymerase chain reaction (PCR) and capillary electrophoresis (CE)

RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR) was carried out as follows. Total RNA was extracted from individual L4 or L5 pulverized frozen DRG 7 days after SNL or sham surgery, and pelleted LDF and HDF after density gradient centrifugation. RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, GmbH, Hilden, Germany). RNA concentrations and purity were determined spectrophotometrically. RT was performed on 1 µg of total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), using random hexamer primers (Promega, Madison WI, USA) and RNaseOUT (Invitrogen).

In order to quantify the α2δ-1 splice variant expression pattern in rat tissue (Fig. 1) and in DRG neurons following SNL, gene-specific primers for rat α2δ-1 (Eurofins MWG Operon, Ebersberg, Germany) were designed flanking all 3 splicing sites. For the analysis of the α2δ-1 splice variant expression pattern in LDF and HDF, gene-specific primers for NF-200 and α2δ-1, flanking only region C, were designed. TATA-box binding protein (TBP) was co-amplified in each reaction and served as an internal control in all experiments. The primer combinations resulted in the following target-specific PCR products: 145 bp (TBP), 360 bp (α2δ-1 splice variant ΔA+ΔB+C), 81 bp (α2δ-1 splice variant ΔA+ΔB+C), 101 bp (NF-200), 154 bp (α2δ-1 splice variant lacking region C), and 175 bp (α2δ-1 splice variant with region C). Either forward or reverse primers were labelled with 6-carboxyfluorescein. PCR was performed on 100 ng (whole DRG), 8 ng (for α2δ-1 in LDF and HDF), and 3 ng (for NF-200 in LDF and HDF) of total RNA equivalent, with Taq DNA Polymerase (Bioline Ltd., London, UK) in a total volume of 20 µL reaction volume. Thermal cycling was conducted with the following conditions: denaturation at 95°C for 2 minutes; 27, 28, or 29 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 40 seconds. The following PCR primers were used: rat α2δ-1 (NM_012919), 5'-CGATCTTATGTGTTGCTTATGCTG3'- (forward) and 5'-TCAAAATTGTGCCCTCAGGTTCTCTC3'- (reverse) flanking all 3 splicing sites; α2δ-1ΔC 5'-GCAATGCGGACAGATGACG3'- (forward); α2δ-1ΔC 5'-GTGCTATGAAAGTGTAGGC3'- (reverse) flanking only region C; rat NF-200 (NM_012607), 5'-AAAGTTGAAACGAGTCTAGTATGGC3'- (forward); NF-200 5'-GTCTTTTCTGAGCCTGCAAAC3'- (reverse); rat TBP (NM_001004198), 5'-GAATTGTGACACGCTCTAAAT-3'- (forward); 5'-GCCTGCTCTTATGCTGATG3'- (reverse). Data were analyzed either manually or using the open-source software Primer3. The techniques were optimized in a series of preliminary experiments for cycle number and starting quantity to ensure points of measurement were acquired during the linear phase of amplification (Supplementary Fig. 1, and data not shown).

Capillary electrophoresis (CE) was carried out following dilution (1:20 for α2δ-1 primers flanking all splice regions; 1:5 for NF-200) with deionized water of aliquots of RT-PCR products, and further analysed by laser-induced fluorescence (LIF). A detailed description and validation of the CE/LIF technique used for quantitative analysis of RT-PCR products was reported previously [43]. One µL of sample was diluted with 12 µL HiDi-Formamide (Applied Biosystems, Foster City CA, USA), and 0.5 µL of GeneScan 400HD ROX Size Standard (Applied Biosystems). Amplified PCR products were separated on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems) running a 50-cm capillary with 3100 POP-6 polymer (Applied Biosystems). Each sample was injected in triplicate from separate wells on the plate. The amplified products were sized and quantified in GeneMapper v3.5 software using the Local Southern method. This analysis method was chosen for its reciprocal relationship between fragment size and mobility.

The relative abundance of mRNA was determined as the ratio of integrated peak area for each PCR product relative to that of co-amplified TBP in order to allow a direct comparison between different preparations. Then, data from the ipsilateral side were normalized to their respective contralateral side. The percentage of the total transcript represented by the minor DRG isoform (ΔA+ΔB+C) was calculated as 100* pDpAΔBΔC/(pDpAΔBΔC+pDpAΔB+C) [44]. Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and SPSS statistical software (SPSS Inc, Chicago IL, USA) or GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined using the nonparametric Mann-Whitney U test and one-way analysis of variance with a suitable post hoc analysis. P values <0.05 were considered as statistically significant. All data are presented as mean ± SEM for the indicated number of experiments.

2.8. Electrophysiology

Calcium channel expression in tsA-201 cells was investigated by whole-cell patch clamp recording, essentially as described previously [7], using an Axopatch 1D amplifier (Axon Instruments, Burlingame, CA, USA). Recordings were made 2 days after transfection. Pipettes of resistance 2–4 MΩ were used, and the internal (pipette) and external solutions and recording techniques were similar to those previously described [30]. The patch pipette solution contained (in mM): Cs-aspartate, 140; ethylene glycol tetraacetic acid, 5; MgCl2, 2; CaCl2, 0.1; K2ATP, 2; HEPES, 20; pH 7.2, 310 mM osm with sucrose. The external solution for recording Ba2+ currents contained (in mM): tetraethylammonium Br, 160; KCl, 3; NaHCO3, 1.0; MgCl2, 1.0; HEPES, 10; glucose, 4; BaCl2, 1, pH 7.4, 320 mM osm with sucrose. Data were filtered at 1–2 kHz and digitized at 5–10 kHz. Current records were subjected to leak and residual capacitance current subtraction (P/8 protocol). Analysis was performed using pClamp 9 (Molecular Devices, Sunnyvale, CA, USA) and Origin 7 (Microcal Origin, Northampton, MA, USA).

3. Results

3.1. Distribution of different splice variants of α2δ-1 in rat tissue

Splice variants of α2δ-1 containing the 3 alternatively spliced regions (A, B, and C) were identified previously in mouse tissue [3]. In that study it was reported that regions A and B were part of the same exon, with alternative 3’ splice acceptor sites, which spliced region A in or out [3]. However, the currently available human, mouse, and rat genomic sequences from Ensembl, ENSC00000135956 (human), ENSMUSG00000040118 (mouse), and ENSRNOC0000033531 (rat), indicate that regions A and B are in separate exons, with region A in rat being encoded by exon 18a, and B representing an alternative 3’ splice acceptor site (start site) of exon 19. A diagram of potential splice variants is given in Fig. 1A.

The following cDNAs were assembled by standard molecular biological techniques in the rat α2δ-1 backbone: +A+B+C,
Gabapentinoid drugs are used to treat neuropathic pain, so we wondered whether there were changes in splice variant expression in DRG following SNL, which could potentially influence the efficiency of Gabapentin binding affinity between the different splice variants used, the relative expression level in DRMs of each splice variant was analysed by Western blot. All splice variants partitioned similarly into DRM fractions, and their level of expression was also found to be similar (Fig. 2A, B). We found that both AAABAC (one of the splice variants found in heart) and AAAB+BC showed significantly lower affinity for 3H-gabapentin (K<sub>B</sub> values of 211 and 155 nM, respectively), compared to the major brain and DRG splice variant, AAAB+BC, and AAAB+BC. All constructs gave rise to products of the expected size with the PCR primers used in this study (Fig. 1B and data not shown). We then examined their distribution in adult rat tissues. AAABAC was found to be the main splice variant in rat heart, but a number of other splice variants were also identified by the much more sensitive CE/LIF, demonstrating that this method can be used for identifying and quantifying α<sub>2δ</sub>-1 splice variants (Fig. 1B, C). We found AAAB+BC in skeletal muscle and AAAB+BC in cerebral cortex (Fig. 1B), as shown previously for mouse tissues [3]. We also found the same brain splice variant, AAAB+BC, in DRG (Fig. 1B).

3.2. Location of the spliced regions within α<sub>2δ</sub>-1

The α<sub>2δ</sub>-2 subunits contain 2 domains with homology to bacterial extracellular chemosensory domains (CSDs) or Cache domains [2], which are downstream of the VWA domain. Bacterial extracellular CSDs bind a variety of nutrients and are generally associated with an intracellular histidine kinase signalling complex [15]. The plant ethylene receptor ETR1 is also a member of this family [15]. In α<sub>2δ</sub>-1, the first of these CSDs is situated between amino acids 491–607 [21]. Fig. 1D gives the approximate locations of the spliced regions, and Fig. 1E [33] illustrates a homology model of the 2 CSDs, showing that regions B and C are situated within a loop in the first CSD of rat α<sub>2δ</sub>-1 (I), and in the linker between CSD I and II, respectively. The model in Fig. 1E was generated using a sequence from which region A was absent, but A would be situated just upstream of region B. It is possible that within α<sub>2δ</sub> subunits, these domains might be important for their ability to bind small ligands, including gabapentin [21].

3.3. Determination of 3H-gabapentin binding affinity for α<sub>2δ</sub>-1 splice variants

We then examined whether there were differences in 3H-gabapentin binding affinity between the different α<sub>2δ</sub>-1 splice variants (Fig. 2). All determinations were performed on concentrated DRM fractions following expression in tsA-201 cells, since we have shown previously that there is a large increase in apparent affinity of both α<sub>2δ</sub>-1 and α<sub>2δ</sub>-2 for 3H-gabapentin in DRMs [18,30].

To ensure that the radioligand binding experiments were not influenced by differential expression of α<sub>2δ</sub>-1 protein between the different splice variants used, the relative expression level in DRMs of each splice variant was analysed by Western blot. All splice variants partitioned similarly into DRM fractions, and their level of expression was also found to be similar (Fig. 2A, B). We found that both AAABAC (one of the splice variants found in heart) and AAAB+BC showed significantly lower affinity for 3H-gabapentin (K<sub>B</sub> values of 211 and 155 nM, respectively), compared to the major brain and DRG splice variant, AAAB+BC (Fig. 2C, D). Both these splice variants with lower affinity lacked region C, whereas all the splice variants containing region C, including AAAB+BC, showed a higher affinity for 3H-gabapentin, with K<sub>B</sub> values between 80 and 102 nM (Fig. 2C, D). Thus, there was a nearly 3-fold difference in affinity between the splice variants with the highest and lowest 3H-gabapentin binding affinities. In contrast, all splice variants showed similar B<sub>max</sub> values, indicating that the number of binding sites was not affected (see legend to Fig. 2).

3.4. Do novel splice variants of α<sub>2δ</sub>-1 become expressed in DRG following SNL?

Gabapentinoid drugs are used to treat neuropathic pain, so we wondered whether there were changes in splice variant expression in DRG following SNL, which could potentially influence the effi-
Fig. 3. The effect of spinal nerve ligation (SNL) on mRNA levels of the α2δ-1 splice variants. (A, B) Representative electropherograms of capillary electrophoresis/laser-induced fluorescence with polymerase chain reaction products derived from L5 dorsal root ganglia (DRG) 7 days after SNL. Transcript products were separated by size along the x-axis. The y-axis indicates the fluorescence signal peak height, which corresponds to the expression level of the respective splice variant. mRNA levels of ΔA+B+C and ΔA+BΔC are higher in the side ipsilateral to SNL (B) than in the contralateral side (A). Note that the expression of the housekeeping gene (TATA-box binding protein) remains at a constant level on both sides. (C) SNL (7 days) leads to increased expression of the major α2δ-1 (ΔA+B+C) splice variant in L5 DRG. Data are expressed as percentage of relative peak areas of the ipsilateral side normalized to the respective contralateral side. The relative increase is shown in L4 DRG from sham-operated rats (black bar, n = 6), L4 DRG from SNL rats (white bar, n = 6), L5 DRG from sham-operated rats (grey bar, n = 6), and L5 DRG after SNL (cross-hatched bar, n = 6). There was no significant difference in L4 DRG between SNL and sham-operated animals. Error bars represent SEM. ANOVA, F = 9.012, P < 0.0001, and Gabriel post hoc analysis: **P < 0.01. (D) A pronounced upregulation of the α2δ-1 splice variant ΔA+BΔC was observed in ipsilateral L5 DRG 7 days after SNL. Data are expressed as percentage of relative peak areas of the ipsilateral side normalized to the respective contralateral side. The relative increase is shown in L4 DRG from sham-operated rats (black bar, n = 6), L4 DRG from SNL rats (white bar, n = 6), L5 DRG from sham-operated animals (grey bar, n = 6), and L5 DRG after SNL (cross-hatched bar, n = 6). Although ΔA+BΔC mRNA levels are 2.4-fold higher in SNL L4 compared to L4 sham-operated animals, this was not statistically significant (P = 0.814). Error bars represent SEM. ANOVA, F = 11.12, P < 0.0001, and Gabriel post hoc analysis: ***P < 0.001. (E) Comparison of the percentage of total transcript represented by ΔA+BΔC splice variant in DRG ipsilateral to SNL in L4 from sham-operated rats (black bar, n = 6), L4 DRG from SNL rats (white bar, n = 6), L5 DRG from sham-operated animals (grey bar, n = 6), and L5 DRG after SNL (cross-hatched bar, n = 6). The data show the pronounced shift in favour of the short splice variant in L5 after SNL. Data are expressed as percentage of relative peak areas for ΔA+BΔC mRNA transcripts normalized to the sum of ΔA+B+C and ΔA+BΔC peak areas. Error bars represent SEM. ANOVA, F = 9.39, P < 0.0001, and Gabriel post hoc analysis: **P < 0.01, ***P < 0.001.
nificantly higher in the HDF (13.9 ± 3.8 arbitrary units (AU)) compared to the LDF (1.8 ± 0.4 AU) (Fig. 4B), confirming that the HDF contains mainly larger neurons expressing NF-200.

We then used RT-PCR and CE/LIF to quantify the relative abundance of \( \alpha_2\delta-1 \) A\( \alpha \)+B+C and A\( \alpha \)+BAC in the small and large DRG neuron fractions after SNL. In agreement with the previous experiments, both A\( \alpha \)+B+C (Fig. 4C) and A\( \alpha \)+BAC (Fig. 4D) transcript levels were significantly increased on the ipsilateral side compared to the contralateral side after SNL. We further analysed whether the differential upregulation of A\( \alpha \)+BAC compared to A\( \alpha \)+B+C mRNA found in whole DRG (Fig. 3E) was present in both small and large neuron fractions. This was calculated as the percentage increase on the ipsilateral compared to the contralateral side, of the 2 transcripts in the small neuron fraction (white bars) compared to the large neuron fraction (grey bars). This analysis was performed to provide a direct comparison with the values found in whole DRG (Fig. 3C, D). We found that the increase of A\( \alpha \)+BAC mRNA following SNL was significantly greater compared to the corresponding A\( \alpha \)+B+C increase, only in the small neuron fraction, and not in the large neuron fraction (Fig. 4E).

Thus, although upregulation following SNL of the 2 DRG transcripts A\( \alpha \)+B+C and A\( \alpha \)+BAC occurs in both small and large DRG neurons (Fig. 4C, D), the increased upregulation of A\( \alpha \)+BAC compared to A\( \alpha \)+B+C following SNL occurs preferentially in the small DRG neuron fraction containing nonmyelinated nociceptors (Fig. 4E).

3.6. Electrophysiological properties of the 2 splice variants present in DRG

It was important to determine whether \( \alpha_2\delta-1 \) A\( \alpha \)+BAC was a functional splice variant, and we therefore compared the properties of Ca\( \alpha \)2.2 calcium channel currents co-expressed with \( \beta \)1b and either \( \alpha_2\delta-1 \) A\( \alpha \)+BAC or A\( \alpha \)+B+C. Ca\( \alpha \)2.2 was used because it is the main calcium channel in DRG neurons. We found that the properties of the currents formed from these combinations were very similar in terms of their ability to increase Ca\( \alpha \)2.2 current density compared to the absence of \( \alpha_2\delta \) (Fig. 5A–C), their ability to increase the inactivation rate of the currents (Fig. 5D, E), and their ability to hyperpolarize the steady-state inactivation of the currents (Fig. 5F). Since Ca\( \alpha \)2.1 is also present in DRG neurons, we also examined calcium currents formed by this channel. Similar results were obtained, in terms of the effect of the \( \alpha_2\delta-1 \) splice variants on current amplitude (Supplementary Fig. 2A–C), voltage-dependence of inactivation (Supplementary Fig. 2D), and kinetics of inactivation (Supplementary Fig. 2E).

4. Discussion

4.1. Genomic arrangement of \( \alpha_2\delta-1 \) giving rise to splice variants

The cacna2d1 gene encoding mouse \( \alpha_2\delta-1 \) was found to contain 3 alternatively spliced regions within the \( \alpha_2 \) moiety (A, B, and C) [3]. It was originally reported that regions A and B were part of the same exon, with alternative 3’ splice acceptor site, which split region A in or out [3]. In this scenario, splice variants of \( \alpha_2\delta-1 \) should not exist in which region A is expressed without B. However, our analysis of the mouse genomic sequence has found that A is encoded by exon 18a, and B is formed as a result of utilizing an alternative 3’ splice acceptor site for exon 19. In confirmation of this, we have found splice variants in rat heart in which A is expressed without B (Fig. 1C).

In skeletal muscle, \( \alpha_2\delta-1 \) A\( \alpha \)+BAC was the only splice variant detected [3]. We did not identify any minor splice variants in skeletal muscle in our study. We also found that the A\( \alpha \)+BAC splice
cacy of these drugs. We chose 7 days after SNL as the time point for our analyses because we found previously that the increase in \( \alpha_2\delta-1 \) mRNA was not significantly different between 7 and 14 days after SNL, and we also noted less variability in the increase in \( \alpha_2\delta-1 \) mRNA at 7 than at 14 days [5].

In order to detect and quantify any minor \( \alpha_2\delta-1 \) splice variants in DRG, we used CE/LIF, and confirmed the presence of the major splice variant A\( \alpha \)+B+C both in unaffected contralateral DRG (Fig. 3A), and in ligated DRG after SNL (Fig. 3B). The relative abundance of A\( \alpha \)+B+C mRNA was quantified by CE/LIF after 27 cycles of RT-PCR (Fig. 3C). The A\( \alpha \)+B+C transcript level in ligated L5 showed a 3.6-fold increase, compared to the contralateral side. It was also significantly higher in L5 DRG following SNL, compared to L5 sham-operated animals, and compared to L4 SNL DRG and L4 DRG from sham-operated rats. There was no significant difference in \( \alpha_2\delta-1 \) levels in L4 DRG between small and sham-operated animals (Fig. 3C).

Surprisingly, we also observed a novel splice variant in DRG neurons, A\( \alpha \)+BAC \( \alpha_2\delta-1 \), which was particularly evident following the upregulation of \( \alpha_2\delta-1 \) mRNA that occurs after L5/L6 SNL (Fig. 3B). A\( \alpha \)+BAC was identified in DRG both by DNA sequencing following agarose gel separation (unpublished results), and by the size of the product on CE/LIF after RT-PCR (Fig. 3A, B). The mRNA level of the A\( \alpha \)+BAC splice variant was quantified by CE/LIF after 29 cycles of RT-PCR (Fig. 3D). The expression level of A\( \alpha \)+BAC was found to be ~10 times higher (1083%) on the ipsilateral side, compared to the respective contralateral side in L5 DRG. Moreover, A\( \alpha \)+BAC mRNA levels were significantly higher compared to L5 sham-operated animals, and L4 SNL and sham-operated rats. Although A\( \alpha \)+BAC mRNA levels were 2.4-fold higher in SNL L4 compared to L4 sham-operated animals, this was not statistically significant (P = 0.14).

Thus, the proportion of A\( \alpha \)+B+C was significantly increased in L5, ipsilateral to SNL compared to sham-operated L5, and this was not the case for L4, which was not ligated. The A\( \alpha \)+BAC splice variant represented 5.1 ± 0.5% of the total \( \alpha_2\delta-1 \) mRNA in sham-operated L5, and 8.4 ± 0.4% in SNL L5 DRG (Fig. 3E).

3.5. The relative abundance of A\( \alpha \)+B+C and A\( \alpha \)+BAC in different DRG classes

DRG neurons are heterogeneous in morphology and the size of their somata relates to different functional subtypes. DRG with small and medium-sized somata form mainly nonmyelinated C fibres and myelinated A\( \beta \) fibres, which conduct pain sensation, whereas nociceptive A\( \beta \) fibres have larger somata [39]. \( \alpha_2\delta-1 \) is expressed in every sub-type of DRG neuron [5], but to date, the distribution of \( \alpha_2\delta-1 \) splice variants in DRG subpopulations is unknown. For the most prevalent DRG calcium channel \( \alpha_1 \) subunit, Ca\( \alpha \)2.2, it has been found that there is a differential distribution of a particular splice variant in small DRG neurons [1,6], and this splice variant is downregulated following SNL in rats [1]. Therefore, we wished to determine whether there was differential distribution in small and large DRG neurons of the \( \alpha_2\delta-1 \) A\( \alpha \)+BAC splice variant, which showed reduced gabapentin binding affinity and a more pronounced upregulation following SNL, compared to the predominant \( \alpha_2\delta-1 \) splice variant A\( \alpha \)+B+C.

To obtain populations enriched with small or large neurons, DRG neurons extracted from L5 and L6 of 2 SNL rats were separated according to their size, as described in Materials and Methods. The LDF was enriched in smaller neurons, while the HDF was enriched in larger neurons (Fig. 4A). The mean diameter for neurons in the LDF was 10.0 ± 0.2 \( \mu \)m (n = 418), with a pronounced peak at 5–10 \( \mu \)m. In the HDF, the smallest neurons (<15 \( \mu \)m in diameter) were almost absent, and the neuron diameter was up to 50 \( \mu \)m, with a peak at 21–25 \( \mu \)m. The mean diameter was 24.5 ± 0.7 \( \mu \)m (n = 260). As expected, NF-200 mRNA, a marker for large myelinated DRG neurons [42], measured by CE/LIF, was sig-
variant bound $^3$H-gabapentin with high affinity, in agreement with previous findings of high-affinity binding of $^3$H-gabapentin to native rat skeletal muscle $\alpha_2\delta-1$ [27]. Our results confirm that the lack of effect of gabapentin on skeletal muscle function is not a result of its inability to bind to the skeletal muscle $\alpha_2\delta-1$ isoform.

In mouse heart, $\alpha_2\delta-1$ $\Delta\DeltaB+C$ was previously found to be the most prevalent splice variant, although other splice variants were also observed, specifically $\DeltaAA\DeltaBAC$, and $\DeltaAA+BAC$ [3]. In this study we found that $\Delta\DeltaBAC$ was the predominant isoform in rat heart tissue, but we also found 6 other splice variants [43x79]
Fig. 5. Comparison of the effect of the 2 main dorsal root ganglion $\alpha_2\delta$-1 splice variants on CaV2.2 calcium channel current properties. (A) Representative current traces elicited between −30 and +40 mV in 10-mV voltage steps from a holding potential of −90 mV for CaV2.2/β1b, co-expressed in tsA-201 cells either without $\alpha_2\delta$ (left, ■), or with $\alpha_2\delta$-1 ΔA+ΔB+C (middle, ●) or $\alpha_2\delta$-1 ΔA+ΔBΔC (right, △). 1 mM Ba$^{2+}$ was used as the charge carrier. (B) Current-voltage (I-V) relationships for the 3 experimental conditions: CaV2.2/β1b/ $\alpha_2\delta$-1 ΔA+ΔB+C (●, n = 16), CaV2.2/β1b/ $\alpha_2\delta$-1 ΔA+ΔBΔC (△, n = 20), or CaV2.2/β1b (■, n = 12). Current amplitude was normalized to whole-cell capacitance and plotted against membrane potential. Data are fitted using a modified Boltzmann function, as previously described[12]. (C) Peak current density was $103.56 \pm 13.9$ pA/pF in presence of ΔA+ΔB+C (black bar) and $116.63 \pm 13.9$ pA/pF in presence of ΔA+ΔBΔC (grey bar). These values were both significantly higher than the current density measured in the absence of $\alpha_2\delta$, which was $9.6 \pm 2$ pA/pF (white bar). Error bars represent SEM. Statistical analysis was performed using 1-way analysis of variance (ANOVA) and Bonferroni post hoc analysis, $^\ddagger^\ddagger$ denotes $P < 0.001$. (D) Representative current traces in response to a long depolarizing voltage step (0.9 second) to 0 mV for CaV 2.2/β1b, co-expressed in tsA-201 cells either without $\alpha_2\delta$ (left), or with $\alpha_2\delta$-1 ΔA+ΔB+C (middle) or $\alpha_2\delta$-1 ΔA+ΔBΔC (right). 1 mM Ba$^{2+}$ was used as a charge carrier. Holding potential was −90 mV. Traces are normalized to their peak. (E) $\alpha_2\delta$-1 ΔA+ΔB+C and ΔA+ΔBΔC significantly accelerated the inactivation of currents compared to no $\alpha_2\delta$. The decay phase of individual current traces at 0 mV was fitted with a single exponential function, and the mean time constant (τ) of inactivation was 204.3 ± 10.3 ms for ΔA+ΔB+C (black bar, n = 10), 186.4 ± 15.8 ms for ΔA+ΔBΔC (grey bar, n = 14), and 248.0 ± 23.6 ms for CaV2.2/β1b without $\alpha_2\delta$ (white bar, n = 10). Statistical analyses were performed using 1-way ANOVA, Bonferroni post hoc analysis, *P < 0.05. (F) Steady-state inactivation curves for IBa evoked by a test pulse to +20 mV after a 10-second conditioning prepulse of between −100 and 0 mV, CaV2.2/β1b/$\alpha_2\delta$-1 ΔA+ΔB+C (●, n = 15), CaV2.2/β1b/$\alpha_2\delta$-1 ΔA+ΔBΔC (△, n = 13), CaV2.2/β1b (■, n = 10). Error bars represent SEM. Data were fitted with a single Boltzmann equation, and the mean voltages at which the channel is 50% inactivated were −61.3 ± 1.2 mV, −60.0 ± 1.9 mV, and −50.7 ± 1.9 mV, respectively.
3H-gabapentin, was differentially upregulated in SNL. In the calcium currents when applied chronically [30]. One of the other of region B, and another is just upstream of region C.

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In brain, the only splice variant found previously was ΔA+B+C [3], and its presence was confirmed in our study. We found that this was also the main splice variant in DRG neurons.

4.2. Differential upregulation of the minor α2δ-1 splice variant in DRG after SNL

Following SNL in rats, we found previously that the level of α2δ-1 mRNA was increased >500% in L5/L6 DRG neurons, ipsilateral compared to contralateral to the ligation, and this was accompanied by a similar increase in α2δ-1 protein [5]. We confirmed that result here, for the main splice variant ΔA+B+C, where a 3.6-fold increase was observed. However, by CE/LIF we were also able to observe the presence in DRG of a second, minor splice variant ΔA+ΔB+C, whose level was increased over 10-fold following SNL. Therefore, this splice variant, which has a lower binding affinity for 3H-gabapentin, was differentially upregulated in SNL. In the experiments described here, ΔA+ΔB+C increased from 5.1% of the total transcript in sham-operated L5 DRG to 8.4% in L5 ipsilateral to SNL.

4.3. Involvement of spliced regions of α2δ-1 in 3H-gabapentin binding

To date, several sites in α2δ-1 have been established to be important for its ability to bind gabapentin [9,50]. The triple arginine motif, situated just upstream of the VWA domain in α2δ-1, is essential for high-affinity 3H-gabapentin binding, and also for the ability of gabapentin to alleviate hyperalgesia [24], and to inhibit calcium currents when applied chronically [30]. One of the other regions identified by Wang et al. [50] is located just downstream of region B, and another is just upstream of region C.

In the present study, we found that both the ΔA+ΔB+C and ΔAΔBΔC splice variants possessed significantly lower affinity for 3H-gabapentin, compared to the other splice variants tested. However, there was no significant difference in the gabapentin binding affinity to α2δ-1ΔA+B+C, containing region A, compared to its absence in ΔA+B+C. Similarly, for the presence or absence of region B, the 3H-gabapentin binding affinity was similar for α2δ-1ΔA+ΔB+C and ΔAΔBΔC. These data indicate that A and B are not directly involved in the binding of gabapentin. This suggests that it is primarily the absence of region C in α2δ-1ΔA+ΔB+C and ΔAΔBΔC that is critical for the reduced 3H-gabapentin binding affinity of these 2 splice variants. The sequence of region C in rat α2δ-1 is SKKGKMK, which is positively charged, as is the triple arginine motif that is essential for 3H-gabapentin binding.

It is notable that the affinity of gabapentin for α2δ-1 is much greater (ie, a lower Kd) than the clinically relevant concentrations. One potential factor contributing to this discrepancy is that an endogenous ligand may occupy the gabapentin-binding site on α2δ-1 in vivo, and compete with gabapentin. This also accounts for the finding that purification of the α2δ-1 proteins results in a marked increase in apparent affinity for gabapentin, as the endogenous ligand is removed during purification [8,18].

4.4. Efficacy of gabapentin following SNL

It has been demonstrated that the effect of gabapentin is state dependent, in that in most circumstances it is able to inhibit neuropathic pain responses in experimental animals, while having no effect on physiological nociception [14,24,25,41]. Alterations in gene expression occur following neuropathic nerve damage, which lead to changes in primary afferent inputs into the spinal cord, including the upregulation of α2δ-1 in these terminals [5]. It has been demonstrated that gabapentin blocks calcium currents acutely in DRG from mice overexpressing α2δ-1, but not in control animals [36], and this model may mimic the neuropathic condition. Our results indicate that the upregulation of specific splice variants of α2δ-1 in SNL does not contribute to determining the state dependence of the efficacy of gabapentin, since no splice variants with an increased affinity for gabapentin were identified in DRG neurons following SNL. Thus, the state dependence of gabapentin is likely to depend on the overall elevation of all α2δ-1 splice variants.

4.5. Biophysical properties of α2δ-1 splice variants found in DRG

It is thought that increased presynaptic calcium currents, resulting from the upregulation of α2δ-1, contribute to the hyperexcitability of DRG that underlies hyperalgesia and allodynia [36]. If the alternative splicing of α2δ-1 were to modify the electrophysiological properties of α2δ-1-containing channels, the consequences of altered function would be augmented following differential upregulation of the α2δ-1 ΔA+ΔB+C splice variant, as a consequence of nerve damage. However, we have demonstrated here that the ability of α2δ-1ΔA+ΔB+C to elicit calcium currents is not affected by deletion of region C. α2δ-1ΔA+ΔB+C enhances calcium currents resulting from the co-expression with either CaV2.2 or CaV2.1 to the same extent as α2δ-1ΔA+ΔB+C. This result indicates that the differential upregulation of α2δ-1ΔA+ΔB+C in DRG neurons does not contribute to triggering hyperexcitability to a greater extent than the main DRG splice variant.

4.6. Changes in calcium channel splicing following sensory nerve damage

It is of interest to compare our results with those for the exon 37a variant of CaV2.2, which is selectively expressed in DRG neurons, and conducts larger calcium currents than the exon 37b variant [1,6]. Despite being present overall at ~7% of the main splice variant 37b of CaV2.2, it was found selectively in small DRG neurons also expressing VR1 [1,6]. Interestingly, the 37a splice variant was downregulated to ~2% in DRG following SNL [1], whereas there was no change in the main CaV2.2 splice variant containing exon 37b.

In contrast, in our study there was a greater increase of α2δ-1ΔA+ΔB+C compared to the main splice variant α2δ-1ΔA+B+C following SNL, and this differential increase occurred particularly in the nonmyelinated small DRG neuron fraction. This, together with our finding that ΔA+ΔB+C had a significantly lower affinity for gabapentin, and an equivalent ability to enhance neuronal calcium currents, could be highly relevant to the response to gabapentin in patients with chronic neuropathic pain. The rat α2δ-1ΔA+ΔB+C mRNA sequence used in this study has 95% amino acid homology with the human sequence, and C region is identical in both species. Therefore, it is reasonable to speculate that human α2δ-1ΔA+ΔB+C (accession number P54289 isoform 5) is expressed in DRG neurons and undergoes similar upregulation in conditions leading to the development of chronic neuropathic pain. It is, further, tempting to speculate that differences in the extent of upregulation of α2δ-1ΔA+ΔB+C in humans could potentially contribute to the variable efficacy of gabapentinoid drugs [37,38].

4.7. Conclusion

We have identified a novel α2δ-1 splice variant ΔA+ΔB+C, which is expressed in DRG neurons and is differentially upregulated com-
pared to the main DRG splice variant 2βδ-1 AA+B+C, particularly in small nonmyelinated DRG neurons following SNI. This splice variant supports Ca2+ calcium currents with unaltered properties, but shows a significantly reduced affinity for gabapentin. It could therefore play a role in determining the efficacy of gabapentin in different forms of neuropathic pain. Therefore, targeting this splice variant for drug discovery could increase the therapeutic efficacy of gabapentinoid drugs in the future.

Conflict of interest statement

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.12.001.

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