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Developmental Changes in Voltage-Gated Calcium Channel $\alpha_2\delta$-Subunit Expression in the Canine Dorsal Root Ganglion

Marco Rosati[a, b] Thomas Goedde[d] Frank Steffen[e] Gualtiero Gandini[a] Luisa De Risio[f] Sven Reese[c] Kaspar Matiasek[b]

[a]Section of Neurology, Veterinary Clinical Department, Alma Mater Studiorum, University of Bologna, Ozzano Emilia, Italy; [b]Section of Clinical and Comparative Neuropathology, Institute of Veterinary Pathology, Department of Veterinary Clinical Sciences, and [c]Institute of Anatomy, Histology and Embryology, Department of Veterinary Sciences, Ludwig Maximilians University of Munich, Munich, and [d]Neurology Referral Service, Tierarztpraxis Stauffeneck, Piding, Germany; [e]Neurology Unit, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; [f]Neurology Unit, The Animal Health Trust, Kentford, UK

Key Words

Canine DRG · VGCC · $\alpha_2\delta$ subunit · SP · CGRP · Primary sensory transmission

Abstract

The voltage-gated calcium channel subunit $\alpha_2\delta$ plays a fundamental role in propagation of excitatory signals associated with release of glutamate and neuropeptides substance P (SP) and calcitonin gene-related protein (CGRP). It can be selectively inhibited by gabapentinoids. Hence, investigation of the $\alpha_2\delta$ subunit may predict the efficacy of gabapentinoid therapy in neuropathic pain. Since sensory processing underlies significant age-related changes, this study was conducted in order to elucidate the role of the $\alpha_2\delta$ subunit in the sensory transmission during canine development. Dorsal root ganglia (DRG) were harvested from four spinal segments of 16 puppies and 10 adult dogs without a history of neurological signs, pain, spinal disease or orthopedic disorders. $\alpha_2\delta$-Subunit expression and coexpression with SP and CGRP was evaluated immunohistochemically regarding the number of immunopositive ganglion cells, staining intensity and subcellular distribution. All tested ganglia were immunopositive for $\alpha_2\delta$. Cell counts and expression levels were significantly lower in pups than in adult dogs ($p < 0.05$). In the cervical segments of both groups, the number and percentage of immunopositive neurons was significantly higher than in lumbar DRG ($p < 0.05$). Multilabeling studies in all tested animals confirmed the coexpression of $\alpha_2\delta$ and pain peptides SP and CGRP. This anatomical study for the first time documents the involvement of $\alpha_2\delta$ subunits in sensory signal processing in dogs. The proportion of positive neurons and the intracellular expression levels show a net increase from early postnatal life to adulthood. A significant portion of $\alpha_2\delta$-positive cells in the dogs exhibited C- and Aδ-phenotypes compatible with nociceptive neurons. The coexpression of $\alpha_2\delta$, SP and CGRP imply that these neurons are involved with peptidergic nociception. The cervicolumbar gradient of $\alpha_2\delta$ expression in adults reflects functional differences in between forelimbs and hind limbs. These data will facilitate translational studies on neuropathic pain states in this species such as common canine nerve entrapment syndromes.

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Dr. Marco Rosati, PhD
Section of Clinical and Comparative Neuropathology
Institute of Veterinary Pathology – LMU
Veterinaerstrasse 13, DE–80539 Munich (Germany)
E-Mail marco.rosati@neuropathologie.de

Introduction

Voltage-gated calcium channels (VGCC) are a class of cellular membrane receptors that represent the main current for calcium entry into excitable cells and that are responsible for the firing and propagation of action potentials in the nervous system through their influence on excitatory neurotransmitter release [1–3]. The receptors are heteromeric complexes with a pore-forming transmembrane α_1 subunit, an α_2 subunit, linked by disulfide bond to a δ subunit, which is anchored to the outer surface of the cellular membrane, a cytosolic β subunit and a transmembrane γ subunit [4]. The $\alpha_2\delta$ subunit plays a major role in the assembly of the VGCC, and its highly glycosylated constitution provides the crucial element for calcium channel activation [5]. To date, four different genes have been identified encoding for the $\alpha_2\delta$ isoforms: $\alpha_2\delta$-1, $\alpha_2\delta$-2, $\alpha_2\delta$-3 and $\alpha_2\delta$-4. These isoforms are expressed to a different extent in different tissues, suggesting that they play a role in tissue-specific tasks [6, 7]. The $\alpha_2\delta$ subunit is mainly expressed in skeletal muscle, myocardium, Purkinje cells, habenulae, septal nuclei and dorsal root ganglia (DRG) [2, 8]. The specific role of this subunit is to increase Ca^{2+} influx through the presynaptic membrane. Recently, it has been reported that this subunit also acts as a thrombospondin receptor in the regulation of excitatory synaptogenesis [5, 9]. In summary, the $\alpha_2\delta$ subunit is involved in propagation of excitatory signals mediated by glutamate and neuropeptide substance P (SP) and calcitonin gene-related protein (CGRP) [10]. Its upregulation under pathological conditions is associated with hyperexcitatory states such as seizures and neuropathic pain. The causal link between $\alpha_2\delta$-subunit expression and hyperalgesia has been documented in several experimental animal models of spinal nerve injury [4, 11]. Moreover, preliminary studies from our laboratory have demonstrated a strong immunoreactivity for $\alpha_2\delta$ subunits in DRG neurons from dogs affected by painful nerve root entrapment [12]. The conclusions to be drawn from these studies, however, are limited by a lack of data on the physiological $\alpha_2\delta$ expression pattern in dogs. Primary sensory transmission undergoes extensive structural and functional changes from birth to adulthood [13, 14]. New insight regarding the physiological expression of $\alpha_2\delta$ at various stages of synaptogenesis (i.e. ongoing vs. completed) between first- and second-order neurons is needed before using nonconventional analgesics such as gabapentinoids. Consequently, the present investigation was designed to shed light on the relative involvement of $\alpha_2\delta$ subunits in the sensory processing of domestic dogs *(Canis familiaris)* at two different stages of maturation.

Materials and Methods

Tissue Sampling

The investigation was conducted on tissues harvested from animals delivered to the necropsy service of the Ludwig Maximilians University, Munich, for diagnostic procedures unrelated to this study. The owners explicitly consented to the use of tissues for scientific purposes related to animal welfare. Collection of animals was random provided that dogs did not show evidence of neurological symptoms, spinal disease or orthopaedic disorders. Based on age distribution, the animals were divided into two groups with a clear cut-off: pups less than 8 weeks of age and adults older than 3 years of age. All dogs underwent a full post-mortem work-up including investigation of the central nervous system and peripheral nerves. The spinal cord and associated nerve roots, including the dorsal root ganglia, were removed in toto after dissection of the epaxial and paraxial musculature, laminectomy and removal of transverse processes and the crista iliaca.

After extracorporeal inspection of the spinal cord and nerve roots, the DRG were obtained from the following spinal segments: C4, C7, L4 and L7. The tissues were immersed in 10% neutral-buffered formalin. All the following procedures – apart from the incubation with the primary antibodies – were performed at room temperature. After 24 h of fixation, the DRG were trimmed transversely with a razor blade into slices of 1 mm thickness. The slices were postfixed in formalin for another 12 h and underwent routine processing by an automatic histoprocessor (TP 1020 Leica Instruments, Nussloch, Germany) after which they were embedded in Paraplast Plus® paraffin wax (Leica Biosystems, St. Louis, Mo., USA). Microtome sections were obtained from these blocks with a slice thickness of 5 μm. Slices were mounted on positively charged amino-propyl-ethoxy-silane-coated glass slides. Prior to histological and immunohistochemical staining, the sections underwent a standard dewaxing protocol employing xylene treatment followed by a decreasing alcohol series and washes with distilled water and phosphate-buffered saline (PBS; pH 7.4).

Histological Investigations

Before immunohistochemical investigations were launched, all DRG and the associated spinal cord segments underwent a neuropathological examination. This investigation employed multiple histological sections stained with haematoxylin and eosin (HE). The survey focused on histological evidence of trauma, infiltrative disease and degenerative features, such as those induced by foraminal stenosis. Moreover, samples that showed evidence of peripheral nerve damage with DRG involvement were eliminated from the subsequent investigation. The examination was carried out in accordance to general algorithms for prevertebral ganglia pathology [15]. All investigations were conducted on a Zeiss Axiophot® equipped with a digital camera at optic resolutions ranging from ×25 to ×1,000.

Immunohistochemistry

Pilot investigations were conducted in order to identify the optimal antigen retrieval and primary antibody concentrations as well as to establish a reliable double-staining protocol (see online suppl. material, www.karger.com/doi/10.1159/000343725). Antigen demasking included different types of heat pretreatment and enzymatic digestion. The following text summarizes the tech-

nique that proved to be most feasible in terms of specificity, reproducibility and paucity of nonspecific background signals.

The endogenous peroxidase activity was blocked using 3 ml of 30% hydrogen peroxide in 100 ml methanol. The incubation was carried out for 30 min, with subsequent washing steps in PBS for 15 min. Thereafter, the slides were mounted with caprine nonimmune serum (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:20 for 30 min.

The first marker applied was a rabbit polyclonal antibody directed at calcium channel L-type α_2 subunit (Abcam, Cambridge, UK). The sections were incubated in a humidified chamber at 4°C for 18 h using a primary antibody dilution of 1:200. Incubation was followed by repeated PBS rinses, each lasting 15 min.

Subsequent staining employed an avidin-biotin complex detection kit (Vectastain ABC kit®, Vector Laboratories, Burlingame, Calif., USA). Incubation with the biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories) was performed for 1 h in a humidified chamber. Unbound secondary antibodies were removed by PBS rinses. The sections were then incubated with the ABC complex for 1 h. After repeated washes in PBS, the slides were covered with 3,3′-diaminobenzidine (DAB) according to the manufacturer's protocol (Vector Laboratories) [2]. The sections were counterstained with hematoxylin and coverslipped routinely using a xylene-based mounting medium.

After the immunostaining with the α_2-subunit antibody, a selected number of slides underwent a second antibody labeling using rabbit polyclonal antibodies against CGRP and SP (both Abcam Cambridge, UK). To remove the previously applied primary and secondary antibodies from the tissue, slides were treated with the LinBlock® kit in accordance to the manufacturer's protocol (Linaris Biologische Produkte, Dossenheim, Germany). Prevention of false labeling was demonstrated in a previous trial (see online supplementary material). After LinBlock® treatment, the slides were incubated with the respective second primary antibody (dilution of 1:500) in a humidified chamber overnight at 4°C. Nonbound antibodies were subsequently removed with PBS and the tissues were mounted with secondary antibodies (1:200) for 1 h. After additional PBS rinses and incubation with ABC complex, the slides were covered with the second chromagen Histogreen® (Linaris Biologische Produkte, Dossenheim, Germany). The staining reaction was blocked with buffer rinses, and the sections were counterstained with hematoxylin and coverslipped as described above.

Data Collection

Nonoverlapping photomicrographs were obtained from $\alpha_2\delta$ immunostained serial sections at either ×100 or ×200 optical resolution. DRG cells were scored according to the intensity of their cytoplasmic staining after background subtraction and adjustment of white balance. Scores were given as follows: 0 = immunonegative, 1 = mild cytoplasmic staining, 2 = moderate staining, 3 = marked staining. The different positive and negative ganglion cell fractions were counted using ImageJ® software after implementation of a cell counter plug-in. The plug-in also was used to establish the frequency of immunonegative versus single and double immunopositive cells after combined $\alpha_2\delta$ and neuropeptide staining.

For simple planimetric assessment, all nerve cell profiles of the sections in the ImageJ® window showing both nuclei and nucleoli were circumscribed manually with a high end pen tablet (Intuos3® Wacom, Vancouver, Wash., USA) after which the neuronal area was calculated from the pixels.

All data were fed into Microsoft Excel® and PAST® software for statistical analysis (see below). The investigators were fully blinded for the origin of the tissues. To achieve a standardized data collection, the subjective scoring was adjusted by two investigators until the intraobserver variability was less than 5% in a pilot procedure [16].

Statistical Analysis

The distribution characteristics of the values were checked for each linear parameter by Shapiro-Wilk test and normal probability plotting. Normal data were compared by Student's t test and one-way ANOVA with Tukey's posthoc correction. $p \leq 0.05$ was accepted as indicating a significant difference between the groups.

Results

Technical Considerations

All sections demonstrated the $\alpha_2\delta$-subunit antigen with high specificity and negligible background staining. The immunohistochemical signal was localised to perikarya and the contorted initial axon segments of DRG neurons. The previously tested dilution allowed for distinction of three different levels of protein expression (fig. 1). Double labeling for coexpression of $\alpha_2\delta$ with SP and CGRP, respectively, showed four neuron populations with either no staining, double staining or single staining (fig. 2).

Expression of $\alpha_2\delta$ Subunit amongst Canine DRG

DRG were harvested from a total of 26 dogs (10 adults, 16 pups). In pups, 56,907 DRG neurons were counted, with 22,521 cells (39.5%) expressing $\alpha_2\delta$. Amongst the different segments, the number of positive cells ranged from 32.1 ± 16.7 to 43.6 ± 7.7%. For both regions (cervical, lumbar), the percentage of positive cells increased toward the caudal parts of the intumescences. Only in the cervical area was this increase statistically significant ($p = 0.0019$).

In the adults, a total of 16,141 neurons were immunopositive, which represents 57.9% of all counted neurons (n = 27,840) throughout the different segments. Compared to the pups, all segments of adult animals showed a significantly higher proportion of $\alpha_2\delta$-positive cells ($p < 0.0001$). The counts ranged from 47.0 ± 11.9 to 63.0 ± 15.0% (fig. 3). Differences between the lower intumescence and more cranial segments were insignificant, although there was a mild gain of positive neurons from L4 to L7.

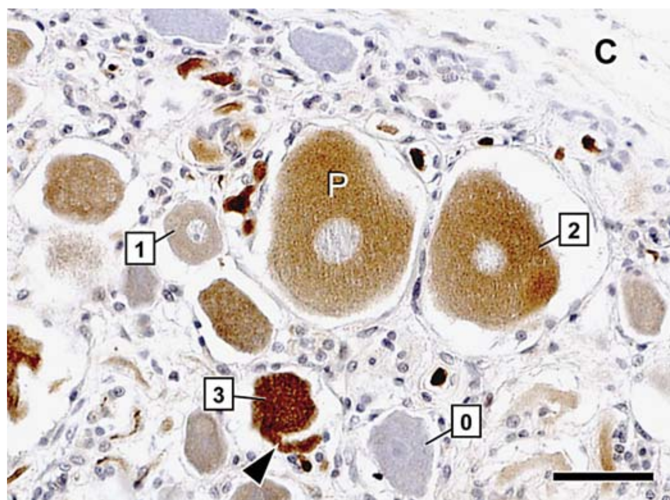

Fig. 1. Immunohistochemical staining for $\alpha_2\delta$-subunit expression in DRG slides. Immunopositive ganglion cells broadly exhibit three different grades of staining intensity: weak (1), moderate (2) and strong (3). Apart from the perikaryon (P), the antibody also labels the initial axon segments (arrowhead). 0 = Negative type B neuron. C = Capsule. Chromagen: DAB. Scale bar = 45 μm.

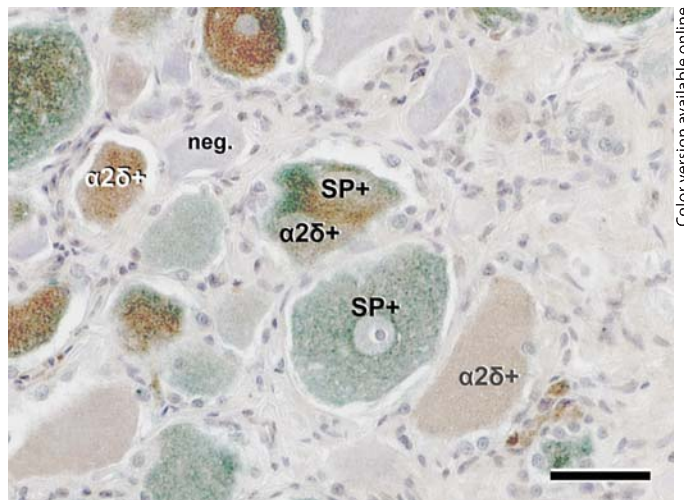

Fig. 2. Double staining for the $\alpha_2\delta$ subunit and neuropeptides. Four populations of cells are identified after double labeling: immunonegative (neg.) neurons, single positive cells for $\alpha_2\delta$ ($\alpha_2\delta$+) or the neuropeptide (in this case SP+) and neurons expressing both markers ($\alpha_2\delta$+/SP+). In this setting, there are also visible differences regarding the staining intensity for $\alpha_2\delta$, ranging from weak ($\alpha_2\delta$+ in dark grey) to strong ($\alpha_2\delta$+ in white). Chromagens: DAB and Histogreen®. Scale bar = 45 μm.

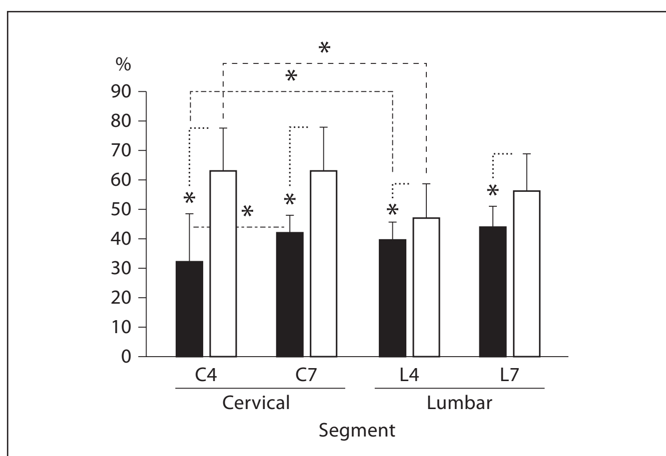

Fig. 3. Bar chart showing the percentage of $\alpha_2\delta$-positive cells for pups (black columns) and adults (white columns). Note the higher relative cell counts in adult dogs throughout all segments. Significant differences ($p \leq 0.05$) are highlighted by the asterisks.

The pups showed more weakly labeled neurons ($p < 0.0001$), whereas medium and strong immunolabeling were significantly more prevalent in the DRG of adult dogs (grade 2: $p < 0.0001$; grade 3: $p = 0.0215$). This difference remained constant throughout all investigated segments. The results of the distribution and degrees of expression for each segment are summarized in table 1.

Morphometric Analysis

Stereological assessment of immunopositive neurons in both groups uncovered an increase in the number of large cells in the caudal parts of the intumescences (fig. 4, 5). The asymmetric graph with a slightly elongated trough on the right side is compatible with a masked bimodal population profile, even though normal probability analysis revealed a normal distribution of neuronal areas in both groups. Direct comparison of all segments between adults and pups showed a substantially larger population of large-sized neurons in the adults ($p < 0.0001$ for all segments) (fig. 6). This feature held true for both $\alpha_2\delta$-positive and immunonegative neurons. The immunonegative population in each segment showed a smaller mean cell size than the immunopositive fraction in all segments ($p < 0.0001$) except L7 in pups ($p = 0.32$).

Colocalisation Analysis

In all DRG, coexpression of neuropeptides SP and CGRP with $\alpha_2\delta$ subunit was seen. In pups, approximately half of all $\alpha_2\delta$-positive neurons (50.9 ± 4.3%) stained for SP, while about 40% (31.8 ± 5.4) coexpressed CGRP

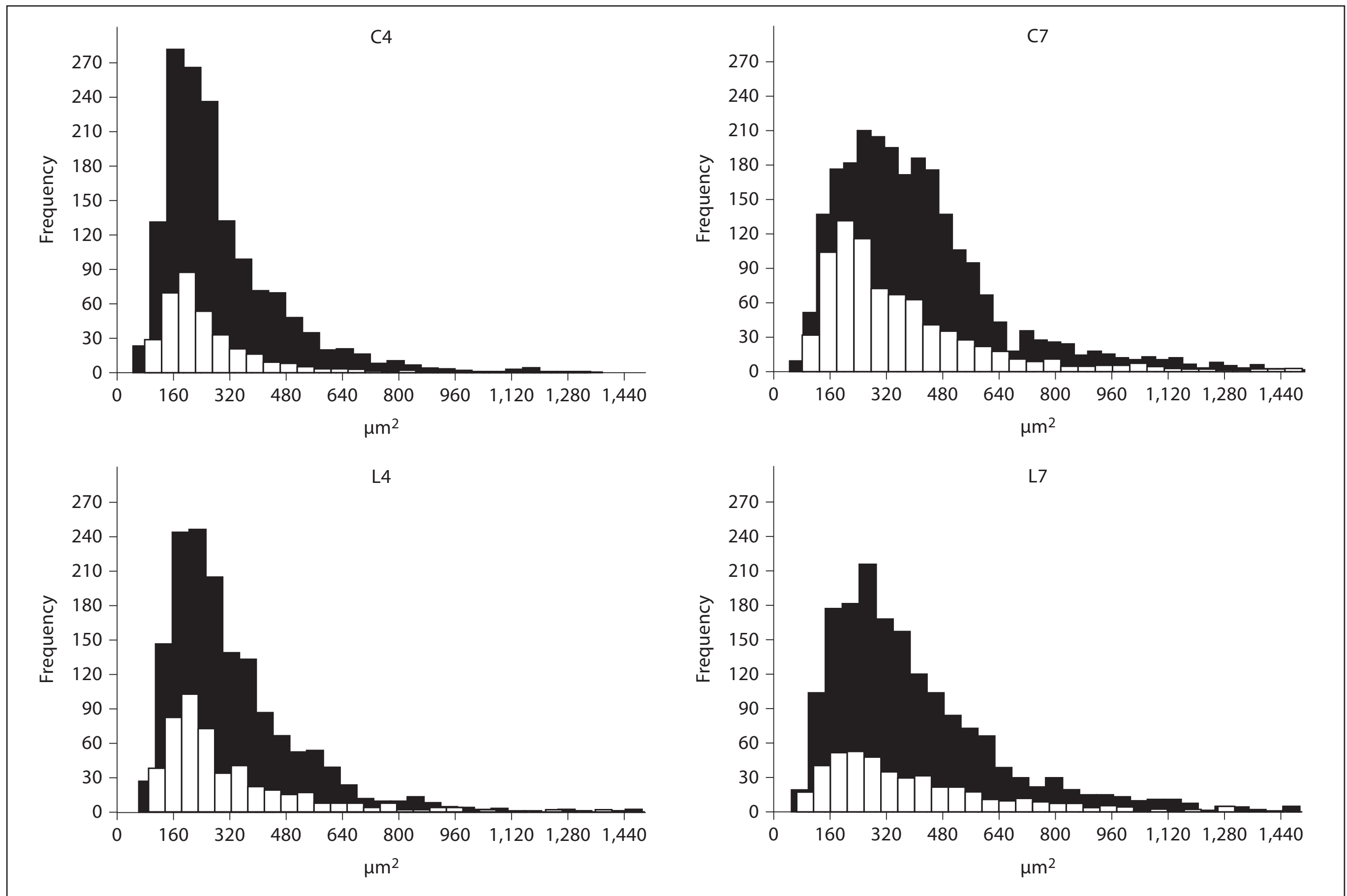

Fig. 4. Neuronal cell size distribution in the pups illustrated by histograms. Black bars = $\alpha_2\delta$-positive population;; white bars = $\alpha_2\delta$-negative population.

Table 1. Distribution and grade of staining intensity of $\alpha_2\delta$-positive neurons sorted for the different segments

%	C4		C7		L4		L7	
	pups	adults	pups	adults	pups	adults	pups	adults
$\alpha_2\delta$-Positive	32.1 ± 16.7	63.0 ± 15.0	42.1 ± 6.4	62.5 ± 15.4	39.3 ± 6.4	47.0 ± 11.9	43.6 ± 7.7	56.4 ± 12.8
Grade 1	60.9 ± 21.9	50.4 ± 9.9	66.2 ± 8.9	56.2 ± 12.9	69.0 ± 6.9	54.3 ± 12.5	63.3 ± 16.4	52.3 ± 14.6
Grade 2	24.7 ± 12.3	35.4 ± 7.8	22.1 ± 5.2	30.1 ± 9.2	20.6 ± 4.6	29.2 ± 7.6	20.3 ± 6.9	28.7 ± 9.6
Grade 3	11.1 ± 11.9	14.2 ± 5.2	11.6 ± 5.1	13.7 ± 5.5	10.4 ± 4.8	16.4 ± 7.2	16.4 ± 17.6	18.9 ± 7.0

The values are displayed as the means ± SD.

(fig. 7). The values were quite similar in adults. SP coexpression was insignificantly (p = 0.07) more prevalent (58.1 ± 8.7%), and $\alpha_2\delta$-CGRP double-positive cells were within the same range as in pups (35.2 ± 5.0%; p = 0.10). A small subpopulation of SP- and CGRP-expressing cells did not exhibit $\alpha_2\delta$ immunopositivity. Resembling 10–15% of DRG neurons, these $\alpha_2\delta$-negative cells are more frequent amongst SP-expressing cells. Both $\alpha_2\delta$-negative populations are more prevalent in adult dogs (single positive SP: 11.5 ± 1.9% in pups vs. 16.0 ± 2.2% in adults; p = 0.055/single positive CGRP: 1.0 ± 0.4% in pups vs. 5.7 ± 2.0% in adults; p = 0.03).

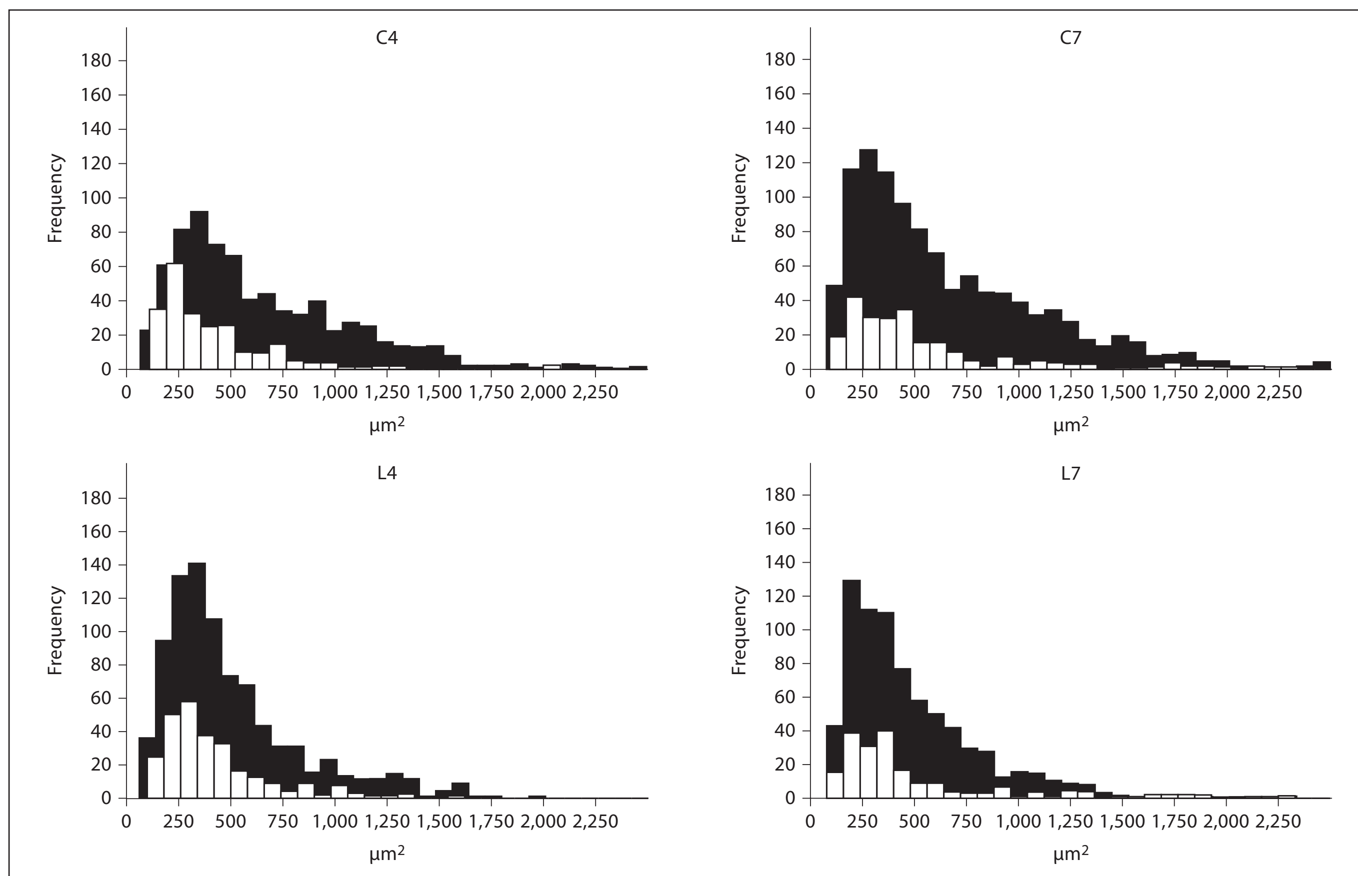

Fig. 5. Neuronal cell size distribution in adults illustrated by histograms. Black bars = $\alpha_2\delta$-positive population; white bars = $\alpha_2\delta$-negative population. Note the less steep decline of the curve to the right side compared to the pups. Note that a different scale is used in this graph than that used for the pups in figure 4.

Discussion

Research on VGCC $\alpha_2\delta$ subunits has considerably increased due to their involvement in neuropathic pain following injury to the peripheral or central nervous system [9, 17]. In contrast to other pain markers, these subunits can be selectively targeted by nonconventional analgesics such as GBP and PGB [3, 18]. These observations laid the groundwork for the clinical use of gabapentinoids in the treatment of painful nerve root entrapment in humans. Rhizopathic pain also resembles a major welfare issue in dogs affected by foraminal stenosis (FS) [19]. Medical treatment is of particular relevance if surgery on the dog is not possible or if it has been declined by the owners. Failure to control pain via conventional analgesics in various neuropathic conditions has already prompted veterinarians to use gabapentinoids empirically [20]. Their efficacy as monotherapy of FS, however, varies from good to insufficient, one explanation of which may be an inconsistent involvement of $\alpha_2\delta$-1- and $\alpha_2\delta$-2-gated neurotransmission. First attempts to track the involvement of $\alpha_2\delta$ in affected dogs have just recently been undertaken by our laboratory [12]; however, no data are currently available on the expression of $\alpha_2\delta$ subunits in normal dogs and the developmental changes during maturation. This study aimed to close this gap by tracing $\alpha_2\delta$-subunit expression in DRG neurons in pups and adult dogs and by investigating their association with neuropeptides essential for propagation of painful signals.

The strong expression of $\alpha_2\delta$ molecules in both pups and adult dogs demonstrates their importance for sensory neurotransmission between first- and second-order neuron throughout the investigated ages. The percentage of $\alpha_2\delta$-expressing neurons and the level of expression show a net increase from early postnatal life to adulthood. Due to the rather broad age difference set in our animal

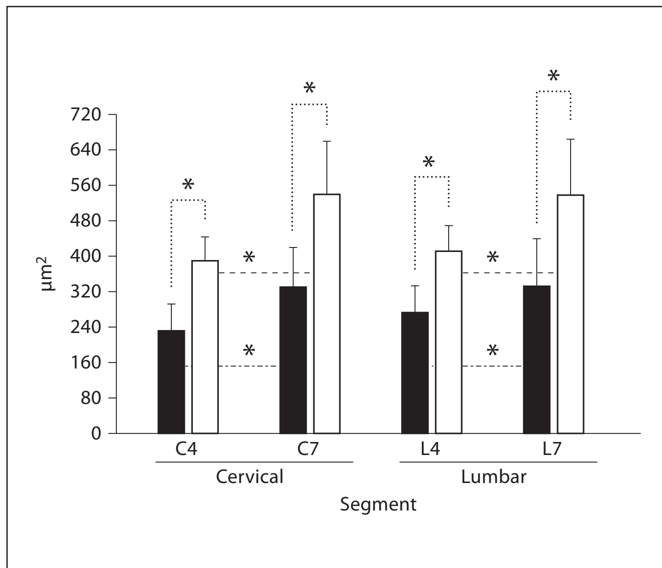

Fig. 6. Bar chart showing the mean neuronal areas of the $\alpha_2\delta$-positive population in pups (black bars) and adults (white bars). Note that the mean cell size is larger in adult dogs throughout all segments. Significant differences ($p \leq 0.05$) are highlighted by the asterisks.

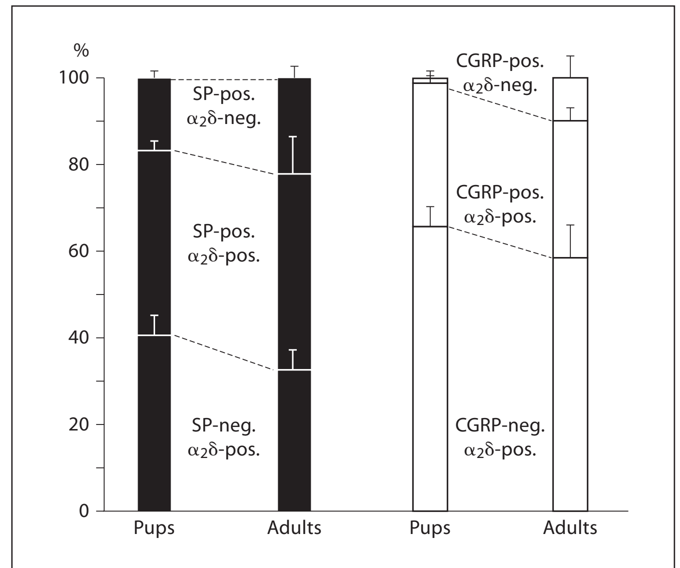

Fig. 7. Bar charts displaying the percentage of immunopositive neurons after double staining for $\alpha_2\delta$ and neuropeptides SP (black bars) and CGRP (white bars). The differences between the two age groups were not significant.

collective early postnatal variations in gene expression as noted in mice were not evaluated [21]. Considering a possible dissociation between the number of mRNA transcripts and the protein levels of $\alpha_2\delta$ [21], we chose the immunohistochemical investigation that also allows for topographic expression analysis if compared to quantitative PCR.

Independent of the specific sensory modality, the rising acquisition of stimuli from the environment and growth-related increase of the receptive fields are likely to explain the higher level of $\alpha_2\delta$ expression in the adult dog population. With regards to pain, a faster, sharper and more detailed set of nociceptive information, carried by DRG neurons, can certainly contribute to the minimization of tissue injuries and ultimately to the preservation of an organism's life. Hence, from a neurobiological point of view, this maturational accumulation could be related to the refinement in the generation and conduction of action potentials. Large-fiber receptive areas of the dorsal horn, which predominate when electrical activity develops in postfetal stages, make way for nociceptive C-fibers to insert at laminae I and II after several weeks [22]. From this stage of neurogenesis, nociceptive and thermal signaling become more prominent and result in an increased need for $\alpha_2\delta$ subunits in excitatory synapses projecting onto the central pain pathways. Indeed, a significant portion of $\alpha_2\delta$-positive cells in the dogs exhibited cell phenotypes compatible with nociceptive neurons and also co-expressed neuropeptides, known to be involved in the procession of painful signals.

Because nociception is processed by small fibers, nociceptive neurons are classically sought amongst small DRG neurons. The observation that all immunopositive and immunonegative cells in the pups were significantly smaller than in adult dogs is attributable to the fact that the volumes of the nerve cell perikarya increase during maturation in relation to the size of the receptive fields and thus correlate with body size and surface [23]. In comparing the histograms, both groups show a similar size distribution of $\alpha_2\delta$-positive cells with a peak amongst small thermoceptive and nociceptive neurons. The less steep curve in the adults indicates a larger population of fast-conducting Aδ-type neurons. Immunohistochemistry also highlights positive Aα-type sensory neurons (including Ia/b afferents) and Aβ-type neurons which are responsible for proprioception, pressure/vibration and tactile senses [24, 25]. Because these sensory modalities are more influential on the appendicular musculature and palmar/plantar dermatomas, a shift towards larger immunopositive neurons was noted in the lower intumescences of all dogs. In situ hybridization studies in rats have shown that in type A DRG neurons, the $\alpha_2\delta$-3 iso-

type, which is not apparently associated with nociception, predominates, while subtypes $\alpha_2\delta$-1 and $\alpha_2\delta$-2 are abundantly transcribed in C-type and Aδ-type neurons [26]. It is therefore likely that the anti-dihydropyridine antibody used in our experiments binds an epitope shared by 'noxious' and 'non-noxious' $\alpha_2\delta$ isoforms. A similar cross-reactivity has been observed with other antibodies used in an avian $\alpha_2\delta$ study [2]. Currently, there is a lack of data on the dependency of non-nociceptive sensory neurotransmission on $\alpha_2\delta$ subunits. However, spinal nerve ligation in rats appears to trigger the expression of pain-associated $\alpha_2\delta$-1 subunits in all neuronal populations, regardless of their initial function [27, 28]. De novo expression of $\alpha_2\delta$-1 may be involved in neuropathic pain.

To further investigate the possibility that $\alpha_2\delta$-expressing cells indeed include nociceptive neurons, we stained the neurons for coexpression of pain-related neuropeptides SP and CGRP [29, 30]. About half of the $\alpha_2\delta$-expressing cells in both groups stained positive for SP, and about one third coexpressed CGRP. Neuropeptide-containing DRG neurons are considered to represent polymodal nociceptors activated by chemical, thermal and high-threshold mechanical stimuli [31]. Because the majority of them appear to be dependent on $\alpha_2\delta$, their recruitment in neuropathic pain can predictively be modified by gabapentinoids. Hence, these drugs may suppress peptidergic pain from various sources of stimuli.

Type C neurons with $\alpha_2\delta$ expression that lack a neuropeptide signal, on the other hand, may reflect thermoceptive cells or contribute to the nonpeptidergic pain reception by upstream glutamatergic signaling [32]. The small but consistent percentage of double-negative and SP or CGRP single-positive neurons implies that not all peptidergic neurons rely on $\alpha_2\delta$-mediated neurotransmission. Because presynaptic calcium channels are constitutively expressed, it is likely that the synaptic terminals of these cells are equipped with different VGCC subtypes.

The differences between the evaluated segments may mirror the functional diversity of the spinal cord regions. DRG at the level of the lower intumescence receive sensory information from the entire limb, including the sensitive palmar/plantar aspects, and therefore show more type A neurons, reflecting the importance of touch and proprioception in these territories. Moreover, the craniocaudal decrement of $\alpha_2\delta$ expression along the spinal cord suggests functional differences between forelimbs and hind limbs in dogs. Even in the dog, the front limbs are not used merely for locomotion but are also able to perform specialized tasks at an intermediate level of dexterity, which therefore requires more sensory information than the hind limbs. How these differences also result in different pain thresholds remains to be elucidated.

In summary, the present study provides new insights into the anatomical distribution of the VGCC $\alpha_2\delta$ subunit in canine primary sensory neurons and highlights some developmental changes. The base data may be used for the design and interpretation of future investigations in dogs experiencing neuropathic pain in clinical and experimental settings.

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