

Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Clinica e Terapia d'Urgenza Veterinaria
Ciclo XXIII

Settore/i scientifico-disciplinare/i di afferenza: Area 07 – Scienze Agrarie e Veterinarie
VET/08 Clinica Medica Veterinaria

Immunohistochemical demonstration and distribution of Voltage-Gated
Calcium Channel alfa2 delta subunit in dorsal root ganglia
of neonates and adults dogs

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Esame finale anno 2011

Indice

Modes of Sensory Processing	3
Nociception and Neuropathic Pain.....	4
Pathophysiology of Neuropathic Pain	6
Clinical Presentation	9
Treatment of Neuropathic Pain.....	10
Voltage Gated Calcium Channel and Neuropathic Pain.....	11
Why investigate pain mechanisms in animals?	13
Objectives - Materials and Methods	15
Objectives	15
Material selection and general procedures.....	15
Histological investigations.....	16
Immunohistochemistry	17
Data collection	18
Statistic Analysis	19
Results	20
Technical Considerations.....	20
Obtained Values	21
Immunohistochemical analysis	22
Morphometric evaluation of the immunopositive cells	27
Statistical Analysis	29
Cervical Segment C4	29
Cervical segment C7	31
Lumbar segment L4	32
Lumbar Segment L7.....	35
Comparison of expression parameters in between the different spinal segments	38
Discussion	41
References	50

Modes of Sensory Processing

The constant exchange of information between animals and the environment has played a fundamental role in the design, evolution and optimization of the sensory system. Sophisticated neuronal mechanisms have been developed in order to detect and discriminate among various classes of environmental stimuli acting on the body surface and internal organs. Primary sensory neurons convey information from the periphery to the spinal cord or the brainstem. Their soma is located in the Dorsal Root Ganglia (DRG), for sensory information generated in the body, and trigeminal ganglia for sensory information originated in the head. This particular type of neurons are defined pseudounipolar because a single axon arise from the cell body and then bifurcates, sending one process toward the spinal cord through the dorsal root, while the other progress peripherally reaching skin, muscles and visceral organs. Each type of sensory stimulus thermal, mechanical, and chemical depolarise peripheral sensory receptors which turn this stimuli in to train of electric impulses. This neuronal activity is than conveyed from the periphery to the central nervous system, where it is interpreted by the brain as sensations corresponding to the type of stimulus applied. These receptors can be classified on the basis of the adequate stimulus which can elicit their activity, hence they are divided as chemoreceptors, thermoreceptors, mechanoreceptors and photoreceptors. Another classification take in to consideration their location in the body recognising two main type of receptors: exteroceptors, which are located near the body surface and detect changes in the external environment that affect the organism, and interoceptors located within the viscera which detect changes in the internal environment. Exteroceptors include primary sensory neurons for touch, temperature, pressure and noxious stimuli

which belongs to the General Somatic Afferent (GSA) System, neurons for light and sound belonging to the Special Somatic Afferent (SSA) System and neurons for General and Special Proprioception. Interoreceptors comprehend General Visceral Afferent (GVA) neurons responsible for detection of body temperature, blood pressure, gas concentration, pressure and movement of body viscera and the Special Visceral Afferent neurons responsible for chemical stimuli such as taste and smell . Nociceptors are a peculiar group of receptors giving the fact that they are not specific for the type of stimulus which is necessary to elicit their activity, hence mechanical, chemicals or thermal stimuli are able to generate electric impulses on this neurons. The specificity of this receptors is rather due to the intensity of the applied stimulus, thus they start firing when the degree of the stimulus is potentially destructive for the tissue, that is when the stimulus became noxious and the organism start experiencing pain .

Nociception and Neuropathic Pain

Pain has been defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage”. Pain is one of the most important function which help us to preserve the body and teach us to avoid potentially damaging situations, protect wounds while healing and prevent the recurrence of painful states in the future. Furthermore the experience of pain is not only physical but also emotional, thus it can heavily influence people and animals behaviours, especially when the acute painful state turns in to a chronic condition. .

The sensation of pain in the past was considered only related to events that could trigger the activity of peripheral receptors which subsequently transfer the painful information through the spinal cord up to the brain. In this simplistic view of the nociceptive pathway

central mechanisms were considered merely transmitters of impulses generated in the periphery . According to this theory, patients that reported painful experiences in the absence of evident peripheral noxious stimulus they were assigned to psychiatric care as neurotics, hypochondriacs or hallucinators . Nowadays it is well known that central mechanisms are of great importance in the experience of pain which has been initiated by peripheral stimulation of nociceptors . Recognising the importance of the somatosensory transmission mechanisms the International Association for the Study of Pain define also “pain initiated or caused by a primary lesion or dysfunction of the nervous system that, under normal conditions transmit noxious information” as **Neuropathic Pain (NP)**. This kind of pain is a pathologic condition which affect a sensory pathway and it does not bring any benefit for the organism for there is no apparent reason to trigger the nociceptive function. .

Neuropathic pain can develop following primary lesions or disfunctions in any part of the nervous system, from the peripheral receptor to the brain, thus degenerative spinal cord diseases, diabetes, herpes zoster infection, mechanical injuries of the nerves and spinal cord, AIDS, painful surgeries such as thoracotomies and mastectomies, amputation and stroke are just some of the condition that can cause this pathological state . Regardless of the initial primary site of pain, the condition evolve through time with a subsequent spread of pain generating mechanisms due to biochemical changes within the nervous system, therefore Neuropathic Pain should be considered a progressive nervous system disease . In addition at the unpleasant feelings of protracted pain, there are many psychological and social aspects because this condition affect also a person’s quality of life, in the meaning of interferences in sleep, work, recreation and emotional well-being .

Pathophysiology of Neuropathic Pain

So far, researches in vivo and in vitro indicate that a lesion of the afferent pathway is necessary for development of neuropathic pain, and it is now known that there is not a single but rather several mechanisms involved . Many of these mechanisms are independent on the cause of the primary disease, thus the same mechanisms can be found in several conditions, and different mechanisms can produce the same painful pattern. This highlight the importance of the understanding of the underlying pain mechanism for the individual patient in order to establish the correct management, because a mechanism-based treatment approach can lead to efficient analgesia . Physiologically pain is elicited through the application of an appropriate stimulus which, can be either mechanical, chemical or thermic in every part of the body. The cell bodies of peripheral sensory nerves are located in the dorsal root ganglia of the dorsal root of the spinal nerves and the trigeminal ganglia for cranial nerves. Under normal conditions A delta fibers and C fibers are those responsible for the transmission of noxious stimuli. The A delta fibers are rapidly conducting and thinly myelinated, they have small receptive fields and are activated by noxious thermal or mechanical input. Their transmission is rapid and therefore are responsible for the initial sensation of pain (first pain), which is sharp, pierce, localized and transient. This protective mechanism warns us immediately to withdraw a body part from a potential major damage. C fibers are slowly conducting and unmyelinated, they have larger receptive fields and are activated by thermal, mechanical or chemical stimuli of higher intensity compared to A delta fibers. They are responsible for the second pain which is burning and throbbing .

Once the information has reached the dorsal root of the spinal nerve it enters into the dorsal horn of the spinal cord, at the level of laminae I and II. The sensory input is than transferred via the ascending nociceptive pathway (spinothalamic tract) through the spinal cord and

brainstem up to the thalamus. From here the information reaches the cortex, where pain is perceived and a behavioural response, such as withdrawal from the noxious stimulation, is made. Furthermore projections to the hypothalamus initiate a neuroendocrine and autonomic response, while descending modulatory pathways, or inhibitory systems, function to reduce the degree of pain perceived, acting as a “gate” to further noxious inputs and activating endogenous analgesic systems .

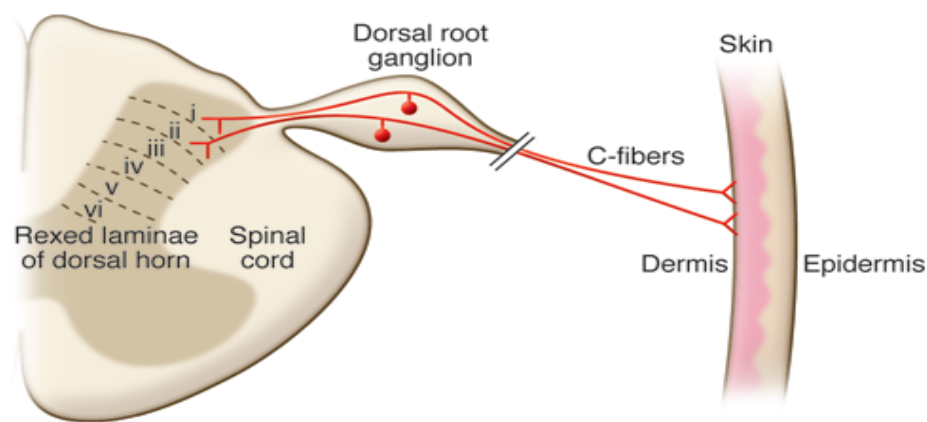


Fig 1. Most nociceptors are unmyelinated with small diameter axons (C-fibers, red). Their peripheral afferent innervates the skin (dermis and/or epidermis) and central process projects to superficial laminae I and II of the dorsal horn

In the absence of tissue injury the afferent discharges evoked by a painful stimulus terminate with cessation of the stimulation. Whether this stimulation is accompanied by an injury to a tissue, especially nervous tissue, an inflammatory states occurs and C-fibers produce an ongoing afferent discharge that persists after the original insult has ceased. The release of chemical mediators such as the Arachidonic Acid (AA) cascade and subsequent eicosanoid production facilitate neurons firing, through a recruitment and activation of more silent nociceptors which in turn release an increased amount of substance-P (S-P) a pronociceptive peptide neurotransmitter. Such

modifications of the peripheral afferent sensory pathway results in an increased input towards the spinal cord, which results in an increased pain response for a given stimulus that under normal conditions would have elicited less pain. This phenomenon is called **Peripheral Sensitization**

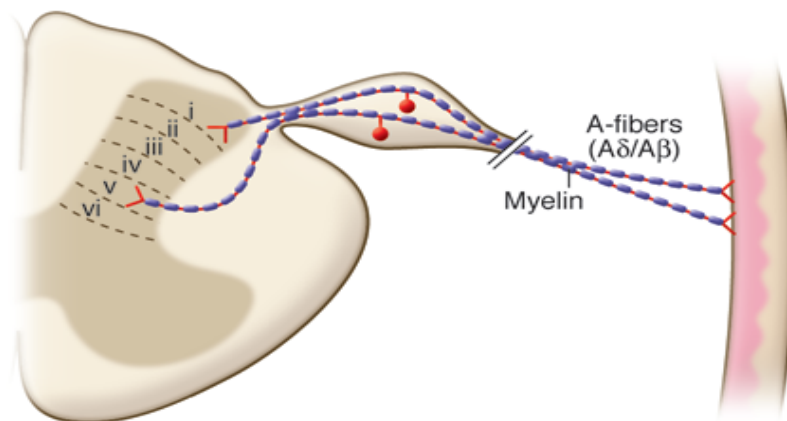


Fig. 2. A-fiber nociceptors are myelinated and usually have higher conduction velocities respect to C-fibers . A-fiber nociceptors project to superficial laminae I and V .

At the level of the spinal cord the increased amount of neurotransmitters act on a particular type of neurons called Widedynamic Range (WDR) neurons which tend to respond more vigorously to noxious stimulation, and are involved in the expression of spinal facilitation of pain, named also wind-up. The constant activation of WDR neurons which release glutamate into the synaptic cleft, lead to a major activation of N-methyl-D-aspartate (NMDA) receptors, thus reflecting a persistent membrane depolarization and a higher rate of firing for this neurons. Moreover glutamate, together with Substance P and Brain-derived Neurotrophic Factor (BDNF), participates in the activation of intracellular signalling cascade increasing the membrane's sensitivity toward subsequent stimulation. This modifications that occurs at the level of the central nervous system are

named **Central Sensitization**, which can last hours, weeks or even years after the primary triggering event. .

Clinical Presentation

Clinical signs reported by patients are areas of abnormal sensation or hypersensitivity in the affected zone, and classically this symptoms have been divided in to positive and negative sings. Paraesthesia, such as skin tingling or burning, spontaneous ongoing pain and burst of electric shock-like sensations are considered positive symptoms, while negative signs comprehend sensory deficit in response to touch, temperature or pinprink. . Positive symptoms are due either to peripheral or central sensitization. Changes in the peripheral nerves include ectopic activity related to injured sensory neurons and increased excitability of the adjacent uninjured neurons, while central sensitization is related to the loss of inhibitory mechanisms and an increased sensitivity of neurons in the spinal cord and higher centers, has previously mentioned. On the other hand, negative symptoms are due to neuronal cell loss .

Patients can experience also mechanical or thermal hypersensitivity, and regarding hypersensitivity two distinct type are described: allodynia and hyperalgesia. **Allodynia** is defined as pain in response to a nociceptive stimulus, thus even gentle mechanical stimuli such as a slight bending of hairs, which under normal conditions wouldn't cause pain, it is capable of evoke severe discomfort. The term **Hyperalgesia** is used to describe an increased pain sensitivity to a nociceptive stimulus. For thermally evoked pain, the terms cold and heat hyperalgesia have been widely accepted instead of allodynia which refers mainly to mechanical stimulation .

In human medicine, from the clinical point of view, neuropathic pain is a well characterized condition, mainly because patients can verbalize and describe what they're experiencing, what it feels like, which body

parts are affected and whether or not the treatments are appropriate in relieving pain. Animals can experience Neuropathic Pain as well as human beings do, but because veterinary patients cannot tell us whether they are painful or not things are more difficult for clinicians and owners. Disease conditions that can produce neuropathic pain in animals include trauma, surgical procedures, pelvic fractures, limb nerve entrapment, amputation, lumbosacral lesions, spinal cord injury, intervertebral disc herniation, fibrocartilaginous embolic myelopathy, syringomyelia, discospondylitis, polyradiculoneuritis, tumors of the central nervous system, inflammatory bowel disease and pancreatitis . Clinical manifestations are consistent with a painful condition which can start in an acute manner and then evolve in to a chronic state, or be more insidious and begin to manifest after weeks or months. Behavioral changes are frequently reported. Animals can prefer to hide or withdraw them to contacts with the owner or other pets, while some other can even become aggressive all of a sudden with dangerous consequences for people . When the pain is limited to a particular part of the body, such as a limb, the animal can manifest lameness, obsessive licking, scratching or even biting of the affected area with development of severe self-injuries.

Treatment of Neuropathic Pain

The management of Neuropathic pain is challenging because despite the efforts to develop more rationale therapeutic approach it still remains unpredictable the response to most drugs . Many patients do not respond or minimally respond to standard analgesic drugs like non steroidal anti-inflammatory drugs (NSAIDs) or opioids. This lack of pain relief through medications might be a result of the complexity with which neuropathic pain develop and the coexistence of psychological and emotional aspects of chronic pain . Furthermore, the variety of conditions that can lead to neuropathic pain development decrease the

likelihood that a single therapeutic intervention can be effective on every patient. In the medical literature there are only a moderate number of clinical studies on the efficacy of treatment for neuropathic pain in humans, while in veterinary medicine there are not even published clinical studies, thus the amount of knowledge in this field is rather sparse .

In 2009 the European Federation of Neurological Societies published guidelines for neuropathic pain management and Gabapentin (GBP) and Pregabalin (PGB) have resulted as first drugs of choice for this condition . Gabapentin is an antiepileptic agent and it was designed at first as a GABA analog, but as a matter of fact, it has no effect on the GABA systems . Both drugs, GBP and PGB bind to Voltage Gated Calcium Channels (VGCC) on central terminals of primary afferent nociceptors but the exact mechanism of action is controversial. Acute inhibition of calcium channel currents, chronic inhibition of calcium channel trafficking and interference with binding to thrombospondin are some of the suggested mechanisms .

Published studies on Gabapentin and Pregabalin in the treatment of neuropathic pain in veterinary medicine are related to few reports, and the need of further studies and randomized clinical trial is fundamental for an evidence based approach to this debilitating condition. Clinical observations support the hypothesis that, as well as in rodents models, dogs can present analogous receptors for gabapentinoids, given the fact that gabapentin can produce pain relief .

Voltage Gated Calcium Channel and Neuropathic Pain

Voltage-Gated Calcium Channels (VGCC) are a class of cellular membrane receptor, which represent the main gate for Calcium entry inside excitable cells, and are thus responsible for firing and propagation of action potentials in the nervous system through influence over neurotransmitters release and activation of intracellular

responses . These class of receptors are heteromeric complexes constituted by a pore-forming $\alpha 1$ subunit with a transmembrane domain, an alpha 2 subunit linked by disulfide bond to a delta subunit, which is anchored to the outer surface of the cellular membrane, a cytosolic beta subunit and a transmembrane gamma subunit . The alpha 2 delta subunit play a fundamental role in the assembly of the VGCC and his highly glycosylated constitution provides the crucial element for calcium channel stimulation. A common gene encode for both peptide $\alpha 2\delta$ subunit, and so far four genes have been identified with a further subdivision in alpha 2 delta 1, alpha 2 delta 2, alpha 2 delta 3 and alpha 2 delta 4 subunits respectively. These latter subunits are present in different tissues, suggesting that they can play a role in tissue-specific functions . The presence of the alpha 2 delta subunit has been reported in skeletal muscles, heart, Purkinje cells of the cerebellum, habenulae, septal nuclei and dorsal root ganglion neurons (DRG) . An up regulation of alpha 2 delta subunit in dorsal root ganglion cells has been observed in sperimental animal models of spinal nerve injury with consequent development of neuropathic pain, thus suggesting a correlation of the expression of this VGCC subunits and the development of pathologic pain transmission .

The alpha 2 delta subunits are target receptors for gabapentinoid drugs, such as gabapentin (GBP) and pregabalin (PGB), which are, as already sad, currently used as antiepileptic drugs, but also for the treatment of neuropathic pain conditions . Many researches in sperimental animal models have been conducted over the interaction among gabapentinoids and these receptors in normal and pathologic conditions, but as before mentioned, the mechanisms of action still remain unknown .

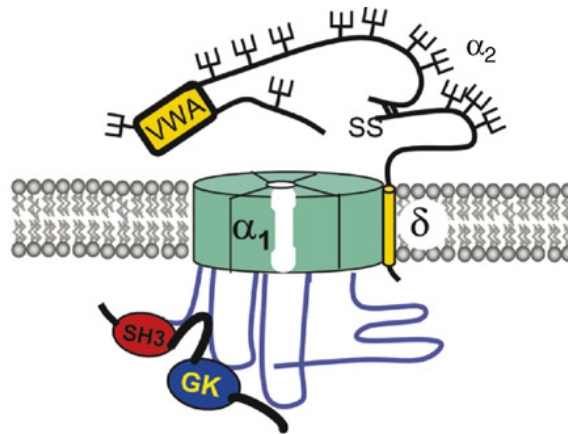


Fig. 3. Transmembrane topology of Voltage Gated Calcium Channel subunits. Modified from .

Why investigate pain mechanisms in animals?

All vertebrate possess a sensory system that detect and process noxious stimuli, hence it is reasonable to assume that conditions which can cause pain in humans would evoke a similar experience in animals. What can largely differ among different species is the behaviour pattern in response to pain, which for certain animals may be barely detectable and thus more difficult to recognise, mainly because they can not express themselves verbally like humans do . As individuals we understand the negative impact of pain on our lives and we expect and require to treat it efficiently, while as clinicians we need to treat painful animals under our care because we have a moral, ethical and medical obligation toward them . The increased attention for pain and pain management has lead to the establishment of organisation such as the International Academy of Veterinary Pain Management, but also to the birth of a special interest group of the International Association for the Study of Pain focused on pain in non-human species .

Neuropathic pain conditions can be easily encountered in veterinary medicine, diseases like laminitis, cauda equina syndrome and

lameness in large animals, and intervertebral disc disease, syringomyelia and discospondylitis in small animals, are just few examples where this painful state develops. Despite the number of conditions in which neuropathic pain is registered, its treatment still represents a challenge for the clinician. In many countries in small animal and equine practice, non-steroidal anti-inflammatory drugs (NSAIDs) and opioids form the most frequently used means of managing postoperative pain. Hence it is not surprising that the previously mentioned class of drugs as well as local anaesthetics, α 2-adrenergic agonists and ketamine are all licensed for their specific use as analgesics in companion animals. Unfortunately neuropathic pain often does not respond or partially responds to these standard analgesic approaches thus the veterinary surgeon is forced to use unlicensed drugs which have been proven to be effective for similar conditions in human medicine, reaching variable degrees of success in animals. Scientific investigations aimed at the discovery and at the understanding of the multiple mechanisms underlying the development, establishment and perpetration of neuropathic pain are needed in order to create new and more effective therapeutic strategies with a target drug based approach. Furthermore companion animals, dogs in particular, experience nowadays a lifestyle which is much closer to the one of human rather than experimental animal models in laboratories, and they can develop disease conditions which are quite similar to those of people thus giving them a new role as research subjects. Animal pain management for several years has benefited from the use of analgesic drugs developed for man, but in the next future a better understanding of neuropathic pain mechanisms might lead to translation of therapies in the opposite direction.

Objectives - Materials and Methods

Objectives

The primary sensory transmission between DRG neurons and the secondary neurons at the dorsal horn of the spinal cord are susceptible of modifications under different circumstances thanks to neuronal plasticity, as the alterations in neuropathic pain testify during pathological states. In the present literature there is a lack of information regarding possible changes in the sensory pathway that can develop physiologically, but knowledge of the normal situation is the cornerstone of a better understanding of pathological changes. To prove if modification in the expression of alpha 2 delta subunits in DRG cells can occur under physiologic circumstances, we examined two distinct populations of dogs, neonates and adults using an immunohistochemical method.

Material selection and general procedures

The present investigation was conducted on tissues harvested from animals that were delivered to the pathology unit of the Ludwig-Maximilians Universität, Munich, Germany, for unrelated diagnostic procedures. Admission of animals was accompanied by an owner's consent regarding the use of tissues for scientific purposes related to animal welfare. Collection of animals was non-selective apart from exclusion of dogs that exhibited neurological signs or any evidence of spinal disease and also orthopaedic disorder.

For the purpose of our study, the dogs were divided into two groups based on age: neonates and puppies (less than 8 weeks of age; in following text summarised under "neonates") and adult dogs (more

than 3 years of age).

All dogs underwent a full post-mortem examination including investigation of the CNS and peripheral nerves. The spinal cord and associated nerve roots, including the dorsal root ganglia, were removed in toto after removal of the epaxial and paraxial musculature, complete laminectomy and removal of transverse processes and crista liliaca, as needed.

After careful inspection of the spinal cord and nerve roots, the DRG taken from segmental levels: C4, C7, L4 and L7. The tissues were immediately transferred into 10% neutral buffered formalin. After 24 hours of immersion fixation at room temperature on a shaking plate, the DRGs were trimmed transversely with a razor blade into slices of about 1 mm diameter each. These slices were post fixed in formalin and thereafter underwent an automatic processing including an ascending alcohol series and embedding in paraffin wax. Of these paraffin blocks, microtome sections were performed with a slice thickness of 5 μ m, and then mounted on positively charged, amino-propyl-ethoxy-silane coated slides.

For both the histological and immunohistochemical staining procedures, the sections underwent a standard dewaxing protocol employing xylene treatment followed by a decreasing alcohol series and washing in distilled water and phosphate-buffered saline PBS (pH 7.4).

Histological investigations

Before immunohistochemical investigations were launched, all DRGs and the associated spinal cord segments underwent a neuropathological examination after staining with Haematoxylin-Eosin (HE). The investigation focused in particular on histological evidence of trauma, infiltrative diseases and degenerative features,

as induced by foraminal stenosis. Moreover, DRGs anyhow indicated or not specifically excluded peripheral nerve damage with retrograde DRG involvement were excluded from the group. The examination employed general algorithms for pre-vertebral ganglia pathology. All investigations were conducted on a Zeiss Axophot® equipped with a digital camera.

Immunohistochemistry

Pilot investigations were conducted in order to identify the optimal antigen retrieval scheme and primary antibody concentration for the planned setting. These procedures include different modes of heat pre-treatment and enzymatic digestion. The following text only includes the most suitable and reproducible method.

Once dewaxed, the slides were treated for Endogenous peroxidase activity blocking using a solution with methanol and hydrogen peroxide (100ml of Methanol plus 3 ml of Hydrogen Peroxide 30%) for 30 minutes, with a subsequent washing in PBS for 15 minutes. Non-immune goat serum at a dilution of 1:200 was added to reduce unwanted binding between antibodies and proteins of no interest for the investigation, for a 30 minutes period. All these steps were conducted at room temperature.

The primary antibody used for this study was a rabbit polyclonal antibody (Calcium channel L type DHPR alpha 2 subunit antibody, (ab65266) from Abcam®, Cambridge, UK) produced by immunization of rabbit with a purified 1,4-dihydropyridine (DHP) from synthetic peptide human Calcium channel L type DHPR alpha 2 subunit. The sections were incubated with the primary antibody at a dilution of 1:200 at 4°C overnight in a humidified chamber, followed by a PBS bath for 15 minutes to remove the excess of antibodies. An avidin-biotin complex method (Vecstatin ABC kit, Vector

Laboratories) was used as staining procedure. Secondary Ab Goat anti-mouse were incubated at room temperature in a humidified chamber for 1 hour. Once removed the excess of secondary antibodies through PBS washing for 15 minutes, the sections were incubated with the ABC complex for 1 hour at room temperature. After a further passage in PBS the peroxidase activity was detected with 3,3'-diaminobenzidine (DAB) according to the manufacturer's protocol (Vector Laboratories). The sections were then counterstained with haematoxylin and cover slipped for microscopic analysis.

Data collection

Photomicrographs were obtained from immuno-stained serial sections at x100 and x200 magnification resembling nonoverlapping tessels of the full section. Immunopositive cells were identified after adjustment of white balance and normalisation for possible background activity in areas not occupied by ganglion cells and the negative controls, in which the primary antibody was replaced by dilution medium only. Counting of positive and negative cells as well as large and small cell subsets was carried out by ImageJ® software after implementation of the cell counter plug-in. All data were immediately transferred into Microsoft Excel® and later into PAST® for further statistic analysis (see below). The investigator was fully blinded for the origin of the tissues. In order to achieve a standardised data collection, in a pilot procedure the cell counts were repeated randomly until the intraobserver variability was less than 5%.

Statistic Analysis

The distribution characteristics of all parameters was checked by Shapiro-Wilk test and normal probability plotting. Regarding the former a p-value ≤ 0.05 was considered indicating loss of normality. Grouping of data into the semi-quantitative scores (0-3) was evaluated via Chi-Square test with a significance threshold of $p \leq 0.05$. Normal data were compared by Student's T-Test. A p-value ≤ 0.05 was accepted as indicating a significant difference between the groups.

Results

Technical Considerations

The immunohistochemical protocol realized for this investigation has given successful results in terms of detection of the Voltage Gated Calcium Channel alpha 2 delta subunit in all sections tested of the Dorsal Root Ganglia of dog. The method described has furthermore proven to be reproducible giving a uniform pattern of immunoreactivity in terms of both distribution and intensity of staining that we have reached. Background staining associated to DAB detection technique, which can represent an issue in immunohistochemical prepared sections, was minimal and specific precipitates of the chromagen were easily identified without significant influence over the outcome of the microscopic analysis.

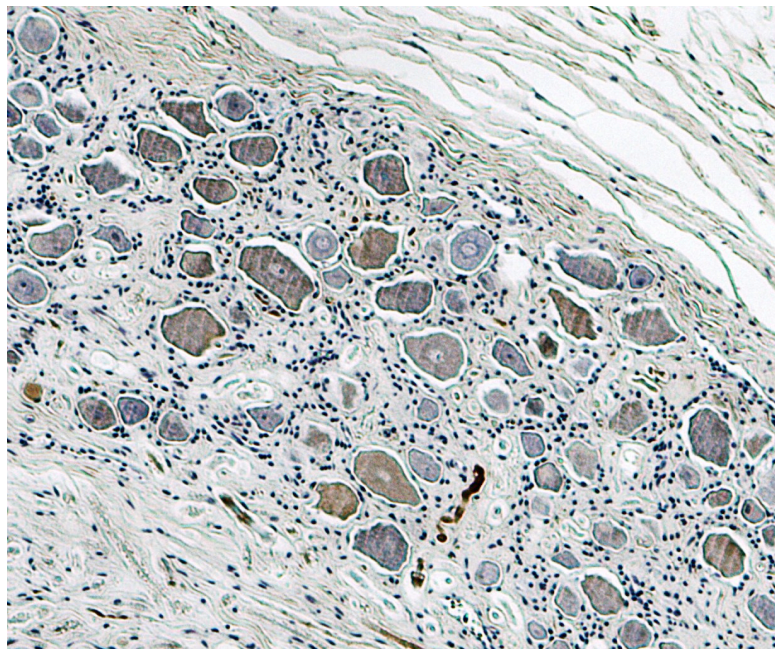


Fig. 4. Histological section of Dorsal Root Ganglia showing neurons with various degrees of immunopositivity toward VGCC alpha 2 delta subunit (brown cells) and immunonegative neurons (blue cells).

Obtained Values

Dorsal root ganglia were harvested from a total of 24 dogs. The two groups were constituted of 16 animals for the neonates group and 8 animals for the adults group.

The quantitative analysis in the neonates group showed a total of 56.907 cells with an immunopositivity for the VGCC alpha 2 subunit expressed in 22.521 cells (40%). The morphometric evaluation of the cells has been performed on a total of 4.458 stained cells, with 2.849 small sized cells (64%) and 1.609 large sized cells (36%).

In the adults group a total of 19.448 cells were counted with 10.962 (56%) cells exhibiting VGCC alpha 2 subunit immunopositivity within the cytoplasm. A total of 3.516 immunoreactive cells with a visible nucleus and nucleolus were examined for cell sizes, subsequently divided into 2.041 small sized cells (58%) and 1.475 large sized cells (42%).

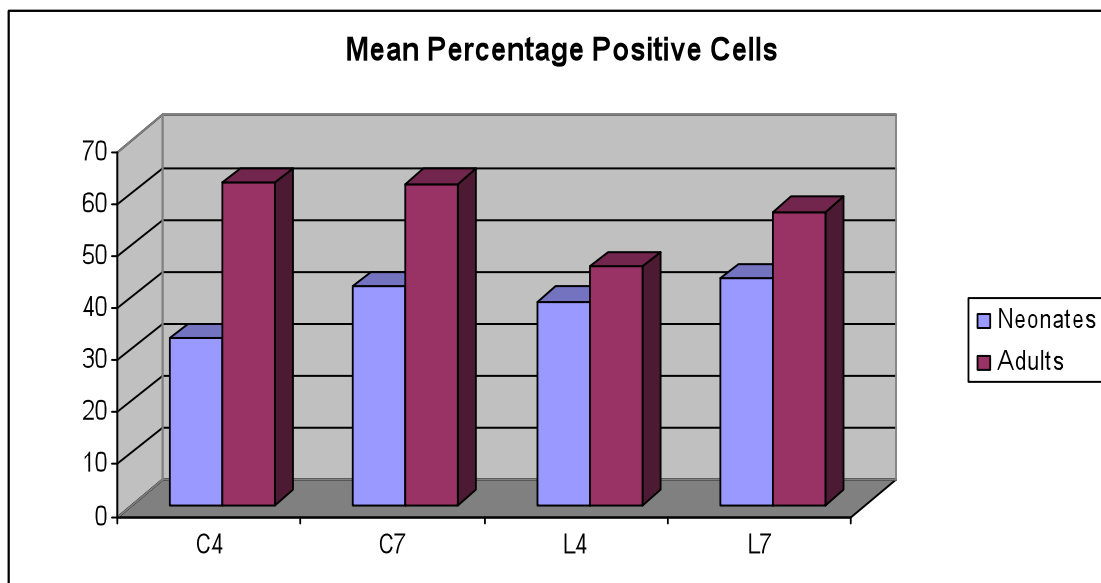


Fig 5. Comparison of the mean percentage of positive stained cells in the different segment among neonates and adults.

In the following description we are going to have a detailed look at the differences between the two groups, considering each single segment of the Dorsal Root Ganglia evaluated. In the text the values expressed in parenthesis represent the standard deviation.

Immunohistochemical analysis

The cervical segment C4 of neonates group displayed a mean of 143 stained cells (± 88.5) while in the adult group the same value accounted for 184 (± 66). The mean percentage of stained cells in this segment was 32.1% ($\pm 16,7$) in the neonates group and 62% ($\pm 15,8$) for the adults. C4 mean total value of grade 1 positive cells accounted for 95.6 (± 68.3) with a mean percentage value of 20,9% (± 11.9) in the neonates group and 86,2 (± 29.3) with a mean percentage value of 29.2% (± 7.3) in the adults group. The mean total value of grade 2 stained cells was of 33.7 (± 20.4) corresponding to a mean percentage value of 7.8% (± 4.7) in the neonates while in the adults the mean total value was 69.6 (± 29) and a mean percentage value of 23.4% (± 7.5). Grade 3 positive cells had a mean total value of 13.6 (± 10.7) with a mean percentage value of 3.4% (± 3.1) in the newborns and values of 28.1 (± 17) and of 9.4% (± 4.9) in the adults.

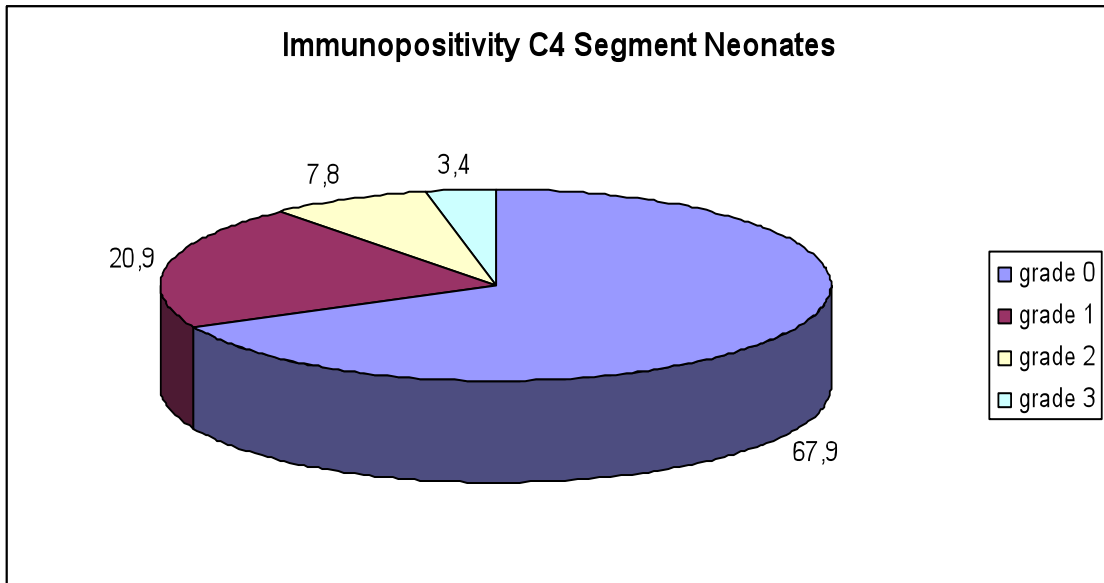


Fig. 6. Percentage distribution of the immunopositivity to VGCC alpha2 delta subunit in the cervical segment C4 in the neonates group.

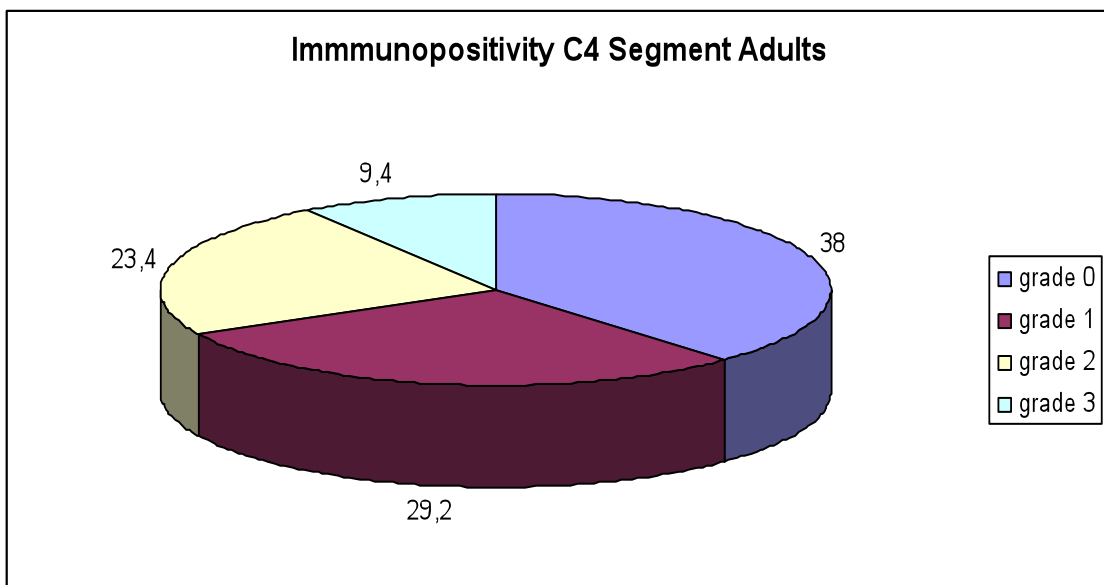


Fig. 7. Percentage distribution of the immunopositivity to VGCC alpha2 subunit in the cervical segment C4 in the adults group.

Examining the C7 segment we found a mean total number of positive cells of 262.5 (\pm 71.8) corresponding to a mean percentage value of 42.1% (\pm 6.4) in the neonates group. In the adults group these values accounted for a mean total value of 262.3 (\pm 74.6) and a mean percentage value of 61.8% (\pm 16.2). The mean total value of

grade 1 stained cells in this segment was of 175.5 (\pm 56.1) in the newborns with a mean percentage value of 28% (\pm 6) while in the adults the results were 139.3 (\pm 42.4) and 32.6% (\pm 8.8), respectively. Grade 2 positive cells in the C7 segment accounted for a mean total value of 57.5 (\pm 19) with a mean percentage value of 9.2% (\pm 2.2) in the neonates group and a mean total value of 84.4 (\pm 37.7) and a mean percentage of 20% (\pm 8.7) in the adults. The mean total value of grade 3 stained cells was 29.5 (\pm 13.4) corresponding to a mean percentage value of 4.9% (\pm 2.2) in the neonates while in the adults they were respectively 38.6 (\pm 19.7) and 9.2% (\pm 4.7).

The lumbar segment L4 showed a mean total value of positive cells of 173.5 (\pm 43.8) in the neonates and a mean percentage of 39.3% (\pm 6.4). In the adults these values were 120.9 (\pm 41.5) for the mean total value and 45.9% (\pm 12.2) as mean percentage. L4 mean total value of grade 1 stained cells accounted for 120.4 (\pm 34.4) with a mean percentage of 26.9% (\pm 4.2) in the neonates group and 61.6 (\pm 22.1) with a percentage value of 23% (\pm 5.3) in the remaining group. Grade2 stained cells had a mean total value of 35.8 (\pm 12.4) in the neonates, with a corresponding mean percentage of 8,2% (\pm 2.5). In the adults these values were respectively 36.9 (\pm 14.4) for the mean total value and 14.3% (standard deviation \pm 5.4) for the mean percentage. In the neonates group the mean total number of grade 3 positive cells was of 17.3 (\pm 6.9) with a mean percentage value of 4.2% (\pm 2.4), while in the other group the mean total number accounted for 22.4 (\pm 14.3) corresponding to a mean percentage of 8.5% (\pm 4.7).

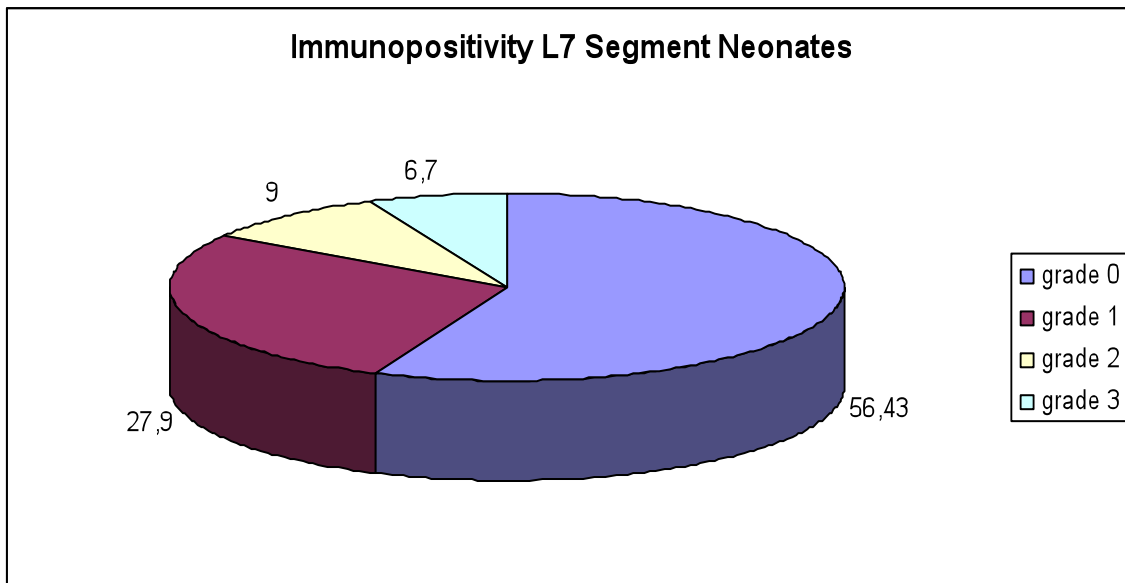


Fig. 8. Percentage distribution of the immunopositivity to VGCC alpha2 delta subunit in the lumbar segment L7 in the neonates group.

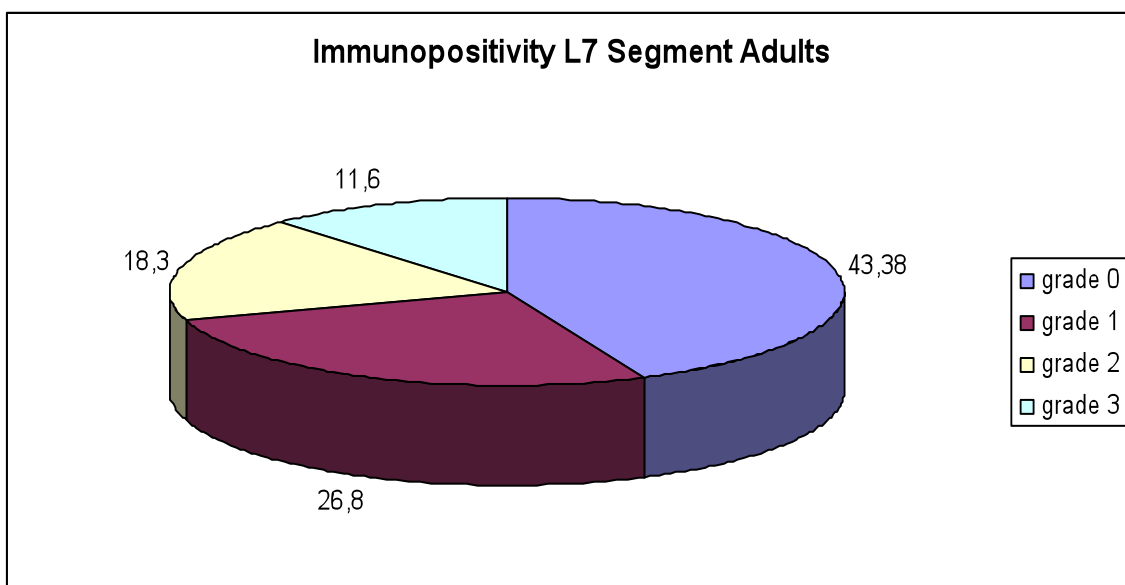


Fig. 9. Percentage distribution of the immunopositivity to VGCC alpha2 delta subunit in the lumbar segment L7 in the adults group.

Considering the latest lumbar segment L7 we found a mean total number of positive cells of 185.1 (\pm 83.5) with a mean percentage of 43.6% (\pm 7.7) in the neonates group. In the adults group the mean total number of positive cells was 161.1 (\pm 51.2) corresponding to a mean percentage value of 56.6% (\pm 13.4). Grade 1 stained cells in the lumbar segment L7 had a mean value of 124.8 (\pm 63) in the

neonates group with a mean percentage of 27.9% (± 8.4) while in the adults group they were respectively of 76.4 (± 24) and 26.8% (± 6.1). The mean total value of grade 2 stained cells was 38.4 (± 20.8) with a mean percentage of 9% (± 3.3) in the neonates group while in the remaining group it accounted for a mean total number of 50.8 (± 18.3) and a mean percentage value of 18.3% (± 7.2). Grade 3 positive cells mean total number was 21.8 (± 11.4) in the newborns with a mean percentage value of 6.7% (± 5). In the adults group the mean total number of grade 3 stained cells was 34 (± 16) corresponding to a mean percentage value of 11.6 (± 4.1).

Morphometric evaluation of the immunopositive cells

The results pertaining the evaluation of cells sizes are summarized in the table Xy and represented in the figures a and b.

Table 1: Mean total value of large and small sized cells in the four segments examined in the entire population. N= neonates, A=adults.

Cell Size		C4		C7		L4		L7	
		N	A	N	A	N	A	N	A
Small Cells	Mean	18,5	32,4	30,9	35,3	24,7	36,9	22,6	30,9
	SD	13,1	17,4	12	14,8	9	16,5	14,4	16,9
Large Cells	Mean	14	17,9	24,3	31,4	9,1	23,6	6,4	24,6
	SD	9,3	5,5	9,9	13,5	4,7	11,1	5,5	12,3

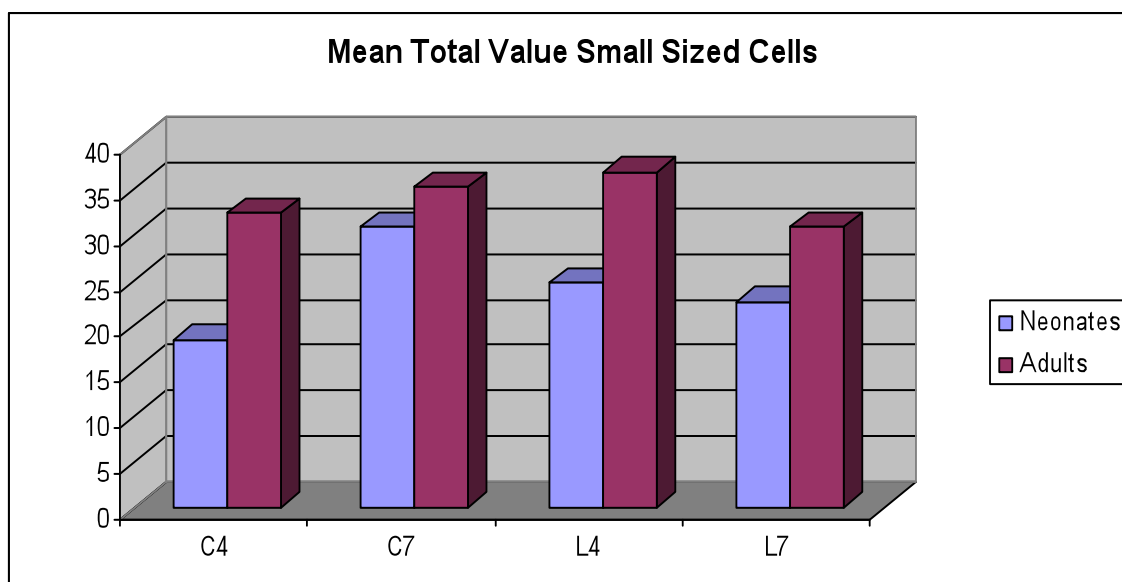


Fig. 10. Comparison of the mean total value of small sized cells in the different segment among neonates and adults.

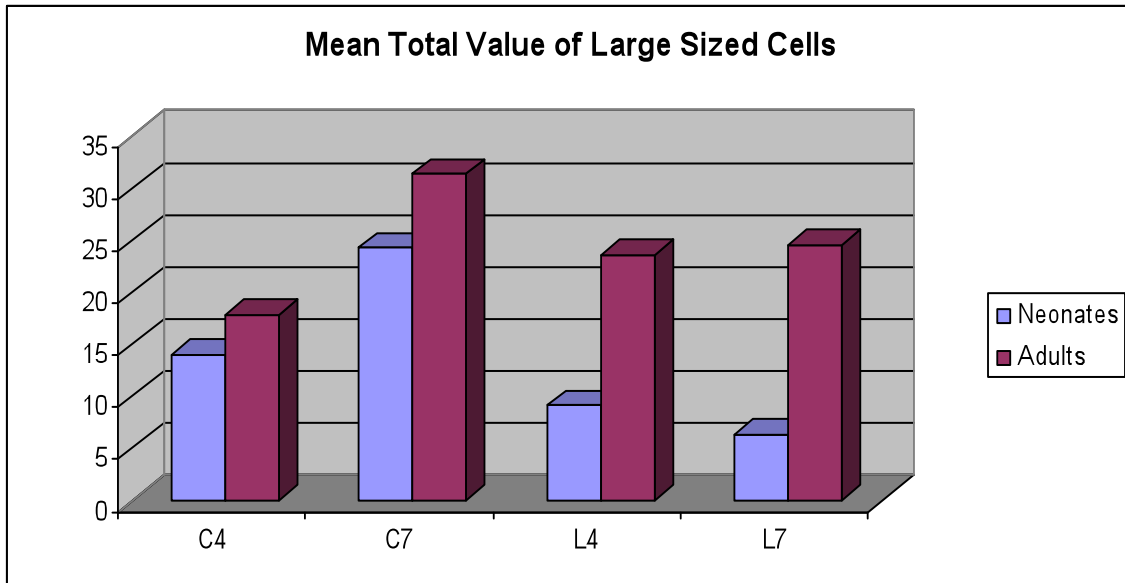


Fig. 11. Comparison of the mean total value of large sized cells in the different segment among neonates and adults.

Statistical Analysis

Cervical Segment C4

The total expression in percentage and the levels of expression of the VGCC alpha2 subunit according to the test for normality showed for both groups a normal distribution (both $p > 0.95$) in the cervical segment C4.

If compared to puppies, the adult dogs showed significantly ($p < 0.001$) increased numbers of neurons expressing the antigen. Moreover, the level of expression in the adult animals was in general higher and medium (2) and upper score (3) values were met by a significantly larger number of neurons ($p \leq 0.05$).

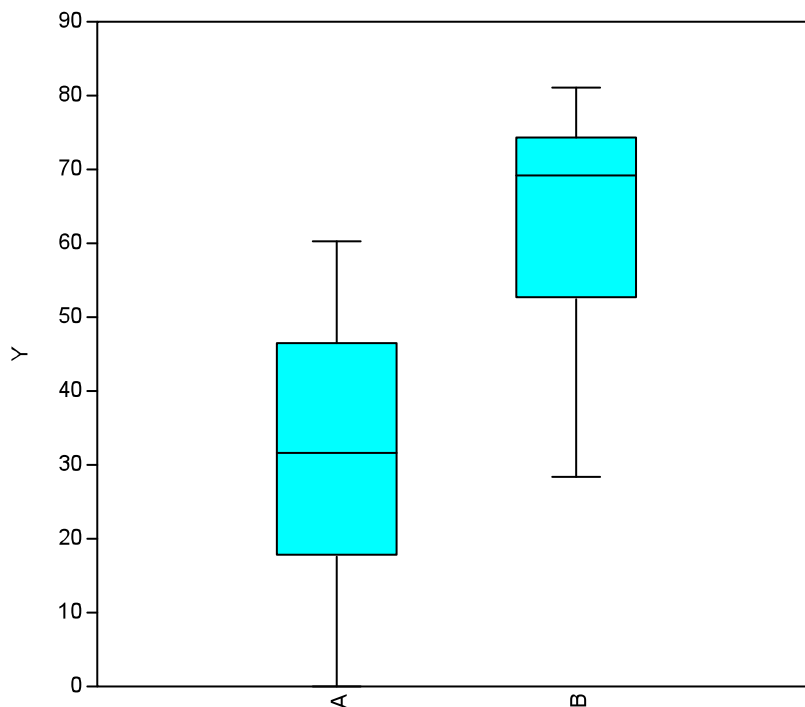


Fig. 12. Box plot showing the percentages of immunopositive cells C4 segment. A = neonates, B = adults.

Considering the ratio between Large and Small sized immunopositive neurons remarkably, the distribution of immunopositivity amongst the DRG cell populations does not vary significantly between adults and puppies ($p > 0.8$).

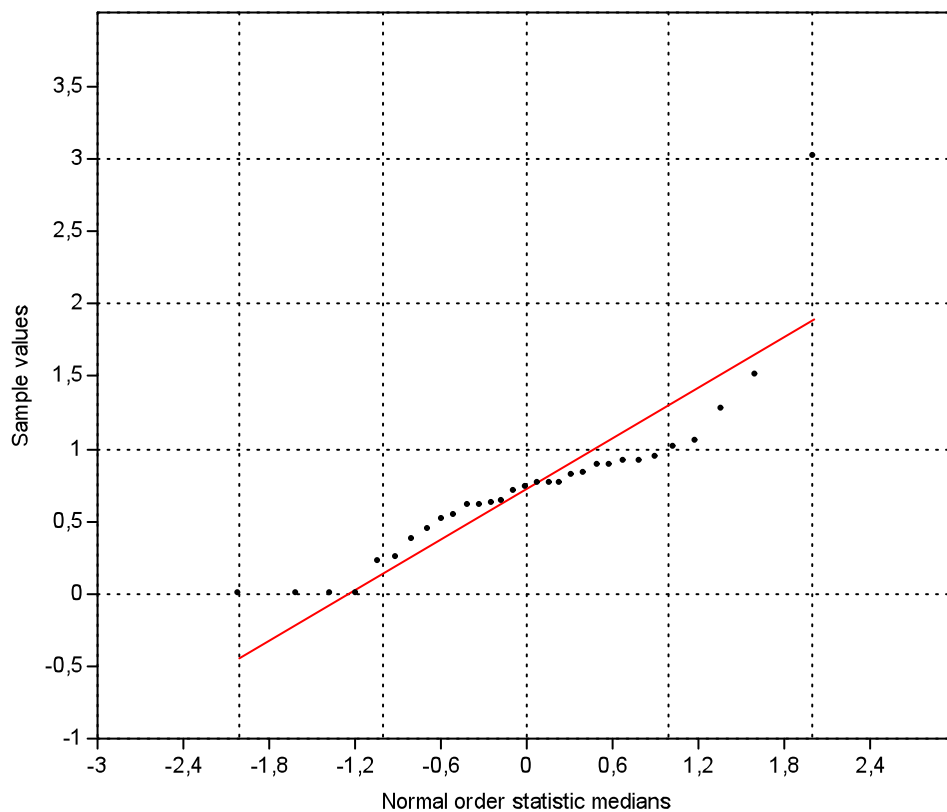


Fig. 13. Large:small sized immunopositive cell ratio in neonates at C4 level.

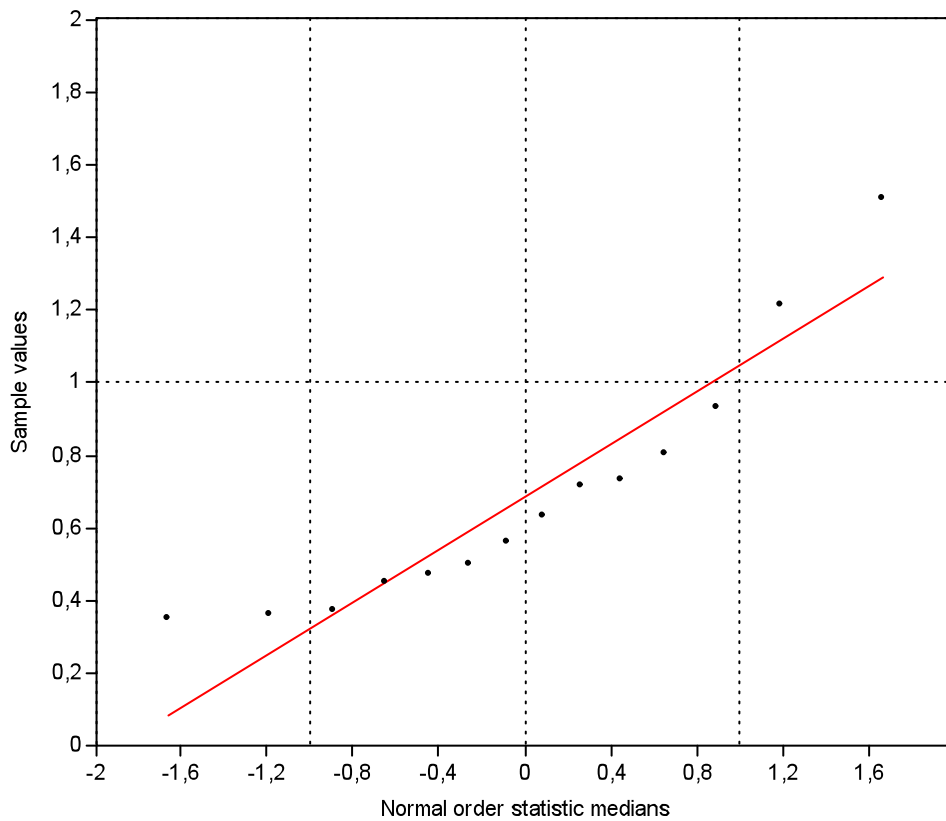


Fig. 14. Large:small sized immunopositive cell ratio in Adults at C4 level.

Cervical segment C7

For both groups the total expression percentage and the expression levels values show approximately a normal distribution (puppies $p > 0.98$ / adults $p > 0.93$).

If compared to puppies, the adult dogs showed significantly ($p < 0.001$) increased numbers of neurons expressing the antigen. Compared to C4 a smaller percentage of neurons in adult dogs reached expression score 3. Still the overall pattern differed significantly between puppies and adults ($p = 0.014$).

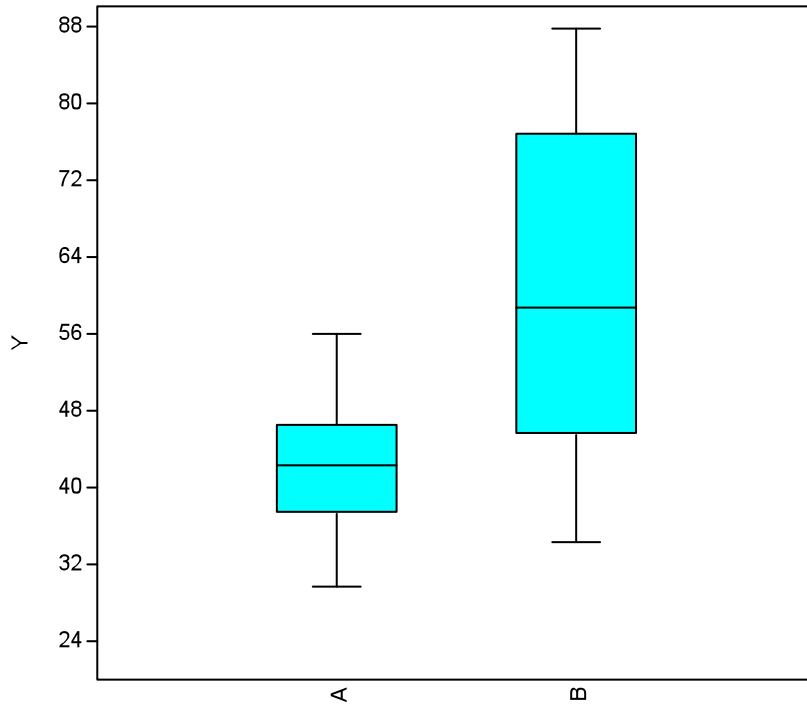


Fig. 15. Box plot showing the percentages of immunopositive cells at the level of C7. A = neonates, B = adults.

Considering cells sizes the distribution of immunopositivity amongst the DRG cell populations does not vary significantly between adults and puppies (both $p > 0.8$).

Lumbar segment L4

The total expression percentage and the expression levels of immunopositive neurons show for both groups values approximately of a normal distribution (both $p > 0.96$).

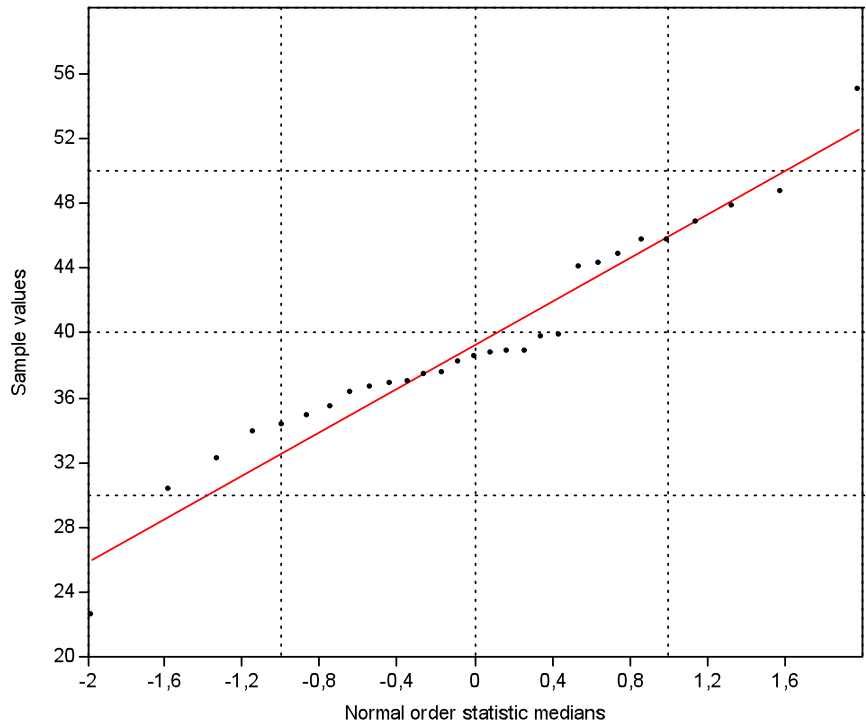


Fig. 16. Normal probability plot – Neonates L4 level.

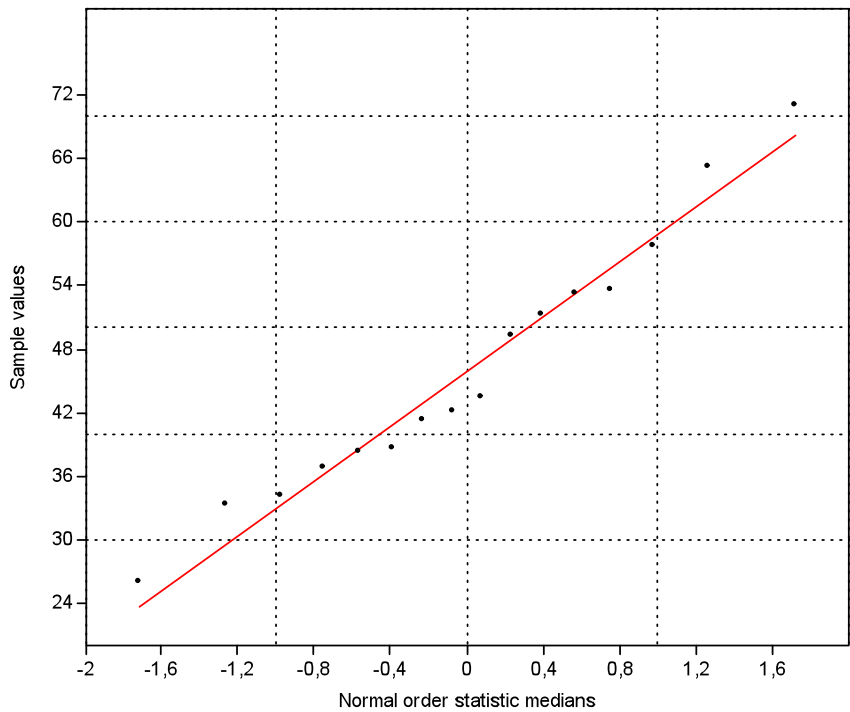


Fig. 17. Normal probability plot – Adults L4 level.

Compared to the cervical segments, the difference between puppies and adults regarding their percentage of immunopositive cells is less, albeit still significant ($p = 0.04$). Even though the general tendency of adult dogs to exhibit strong expression levels is preserved, nearly equal percentages of immunonegative and grade 1 cells render the p-value subsignificant ($p = 0.1$ for all cells / $p = 0.06$ for immunopositive population). However, there still remains a larger proportion of score 2 and score 3 expressing cells within these lumbar DRGs.

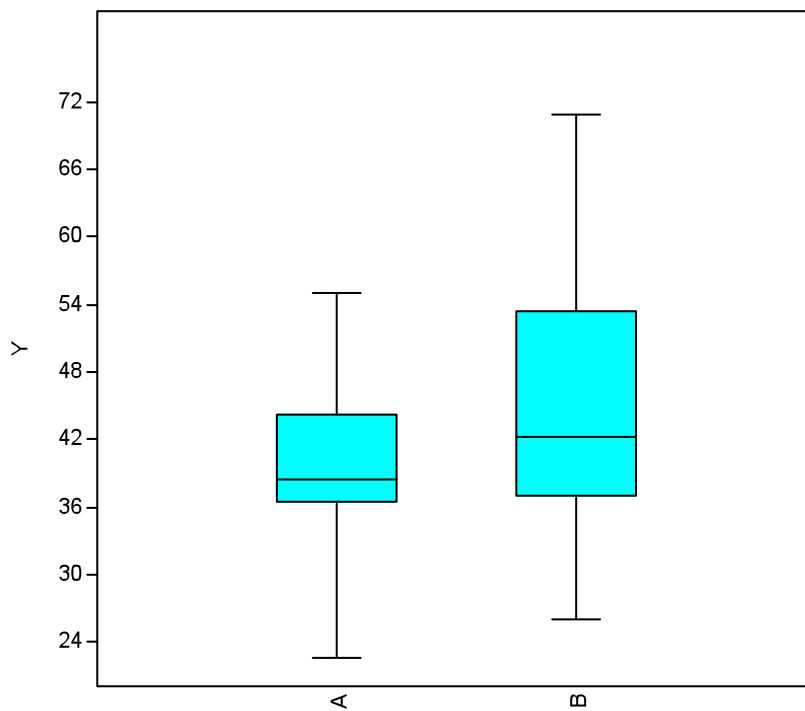


Fig. 18. Box plot showing the percentages of immunopositive cells.

The distribution of immunopositivity amongst the DRG cell populations is normal throughout (puppies $p = 0.89$ / adults $p = 0.92$). On the other hand, the ratios differ significantly ($P = 0.007$) since the proportion of immunopositive large neurons is remarkably higher in adults.

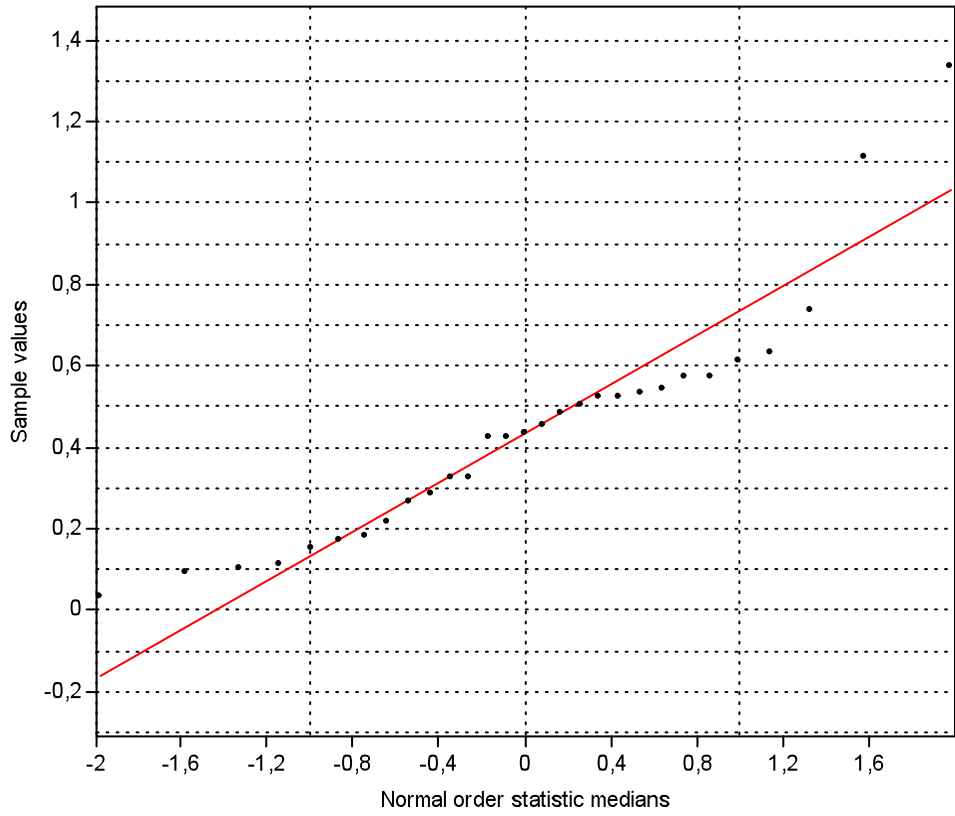


Fig. 19. Large:small sized cells ratio in Neonates at L4 level.

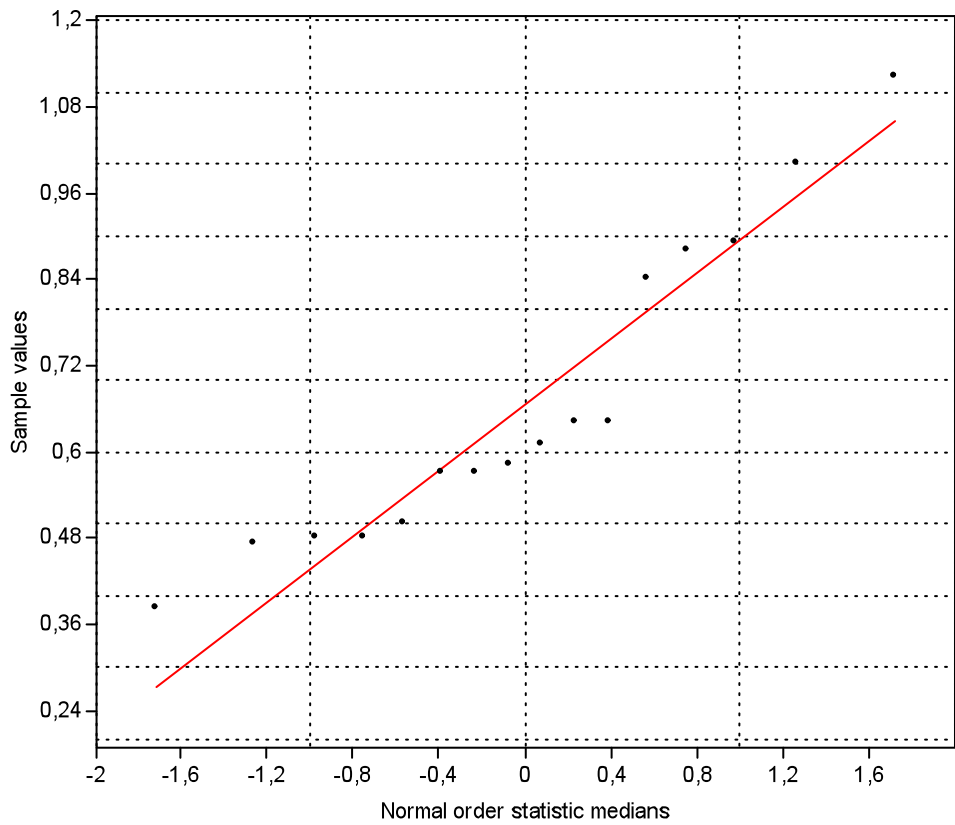


Fig. 20. Large:small sized cells ratio in Adults at L4 level.

Lumbar Segment L7

The test for normality of the total expression percentage and the expression levels show for both groups values of approximately a normal distribution (puppies $p = 0.97$ / adults $p = 0.94$).

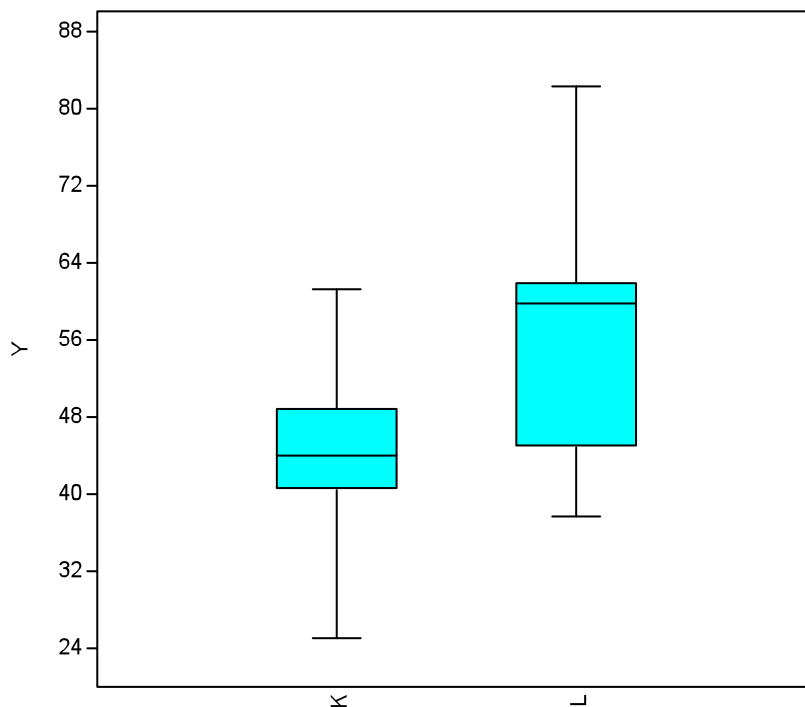


Fig. 21. Box plot showing the percentages of immunopositive cells at L7 level. K= neonates L= adults.

Despite a considerable overlap of values, the adult animals still show a significantly higher number of immunopositive neurons ($p < 0.001$). The overlap again mainly results from a similar percentage of immunonegative and score 1 neurons while a significantly higher number of neurons in adult DRGs express scores 2 and 3.

Considering the sizes of the immunopositive cell population statistic evaluation of L7 DRG neurons in neonates shows a high degree homogeneity even though normality testing still simulates a normal distribution ($p = 0.29$).

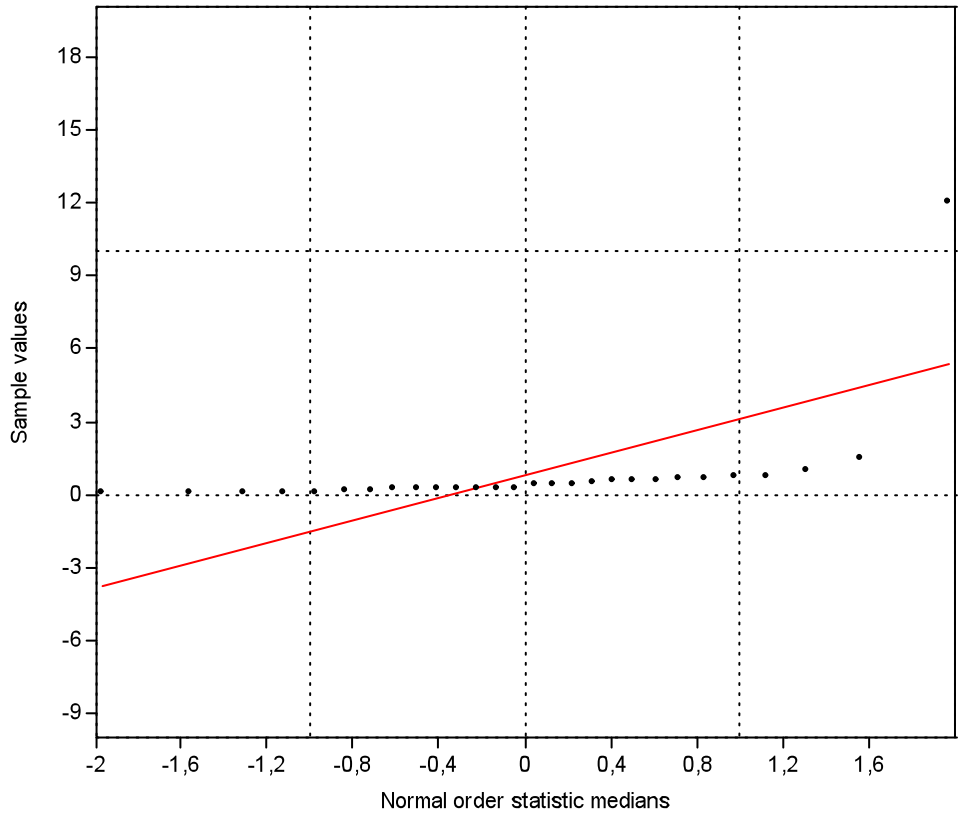


Fig. 22. Large:small sized cells ratio in neonates at L7.

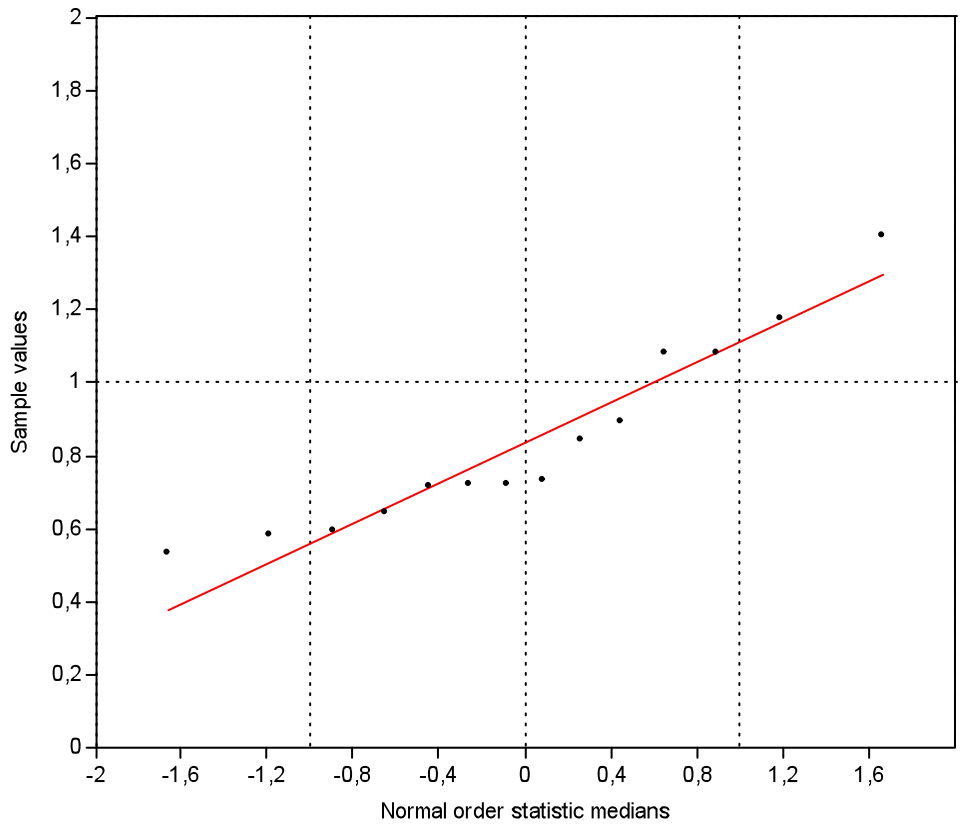


Fig. 23. Large:small sized cells ratio in neonates at L7.

The normal distribution is much more convincingly displayed in the adult animals ($p = 0.9$). Notably, discrimination analysis did not reveal significant changes in between the different age groups regarding the relative involvement of large versus small DRG neurons.

Comparison of expression parameters in between the different spinal segments

Within the dogs there is a tendency of lumbar DRGs to exhibit a lower percentage of immunopositive neurons (see Box plot). If lumbar and cervical data are pooled, this tendency becomes statistically significant ($p = 0.005$).

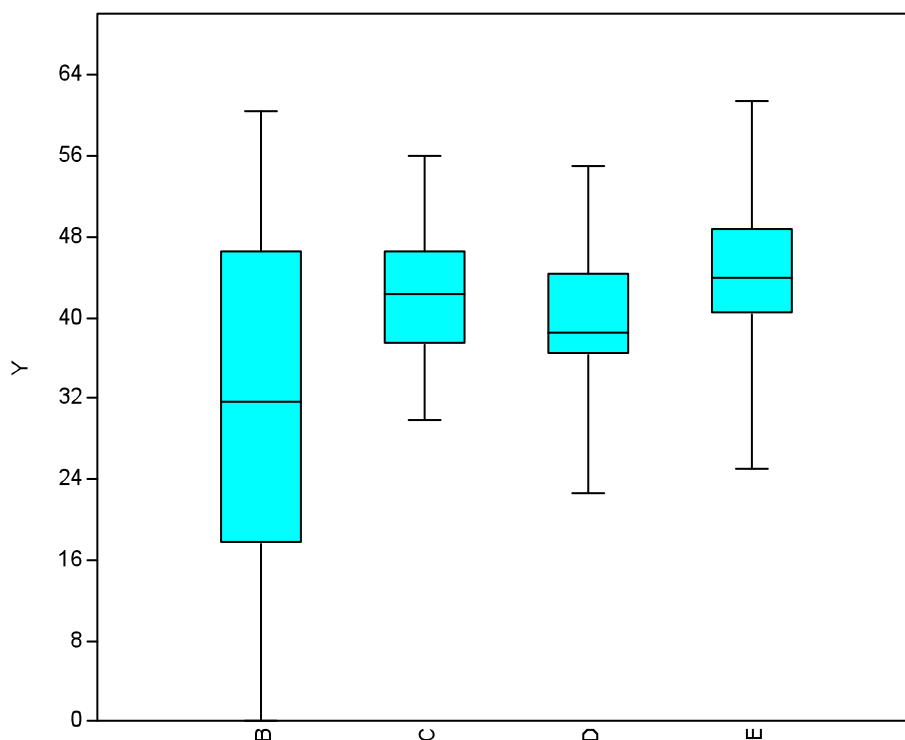


Fig. 24. Comparison of the immunopositive neurons amongst neonates in the different segment. B = C4, C = C7, D = L4, E = L7.

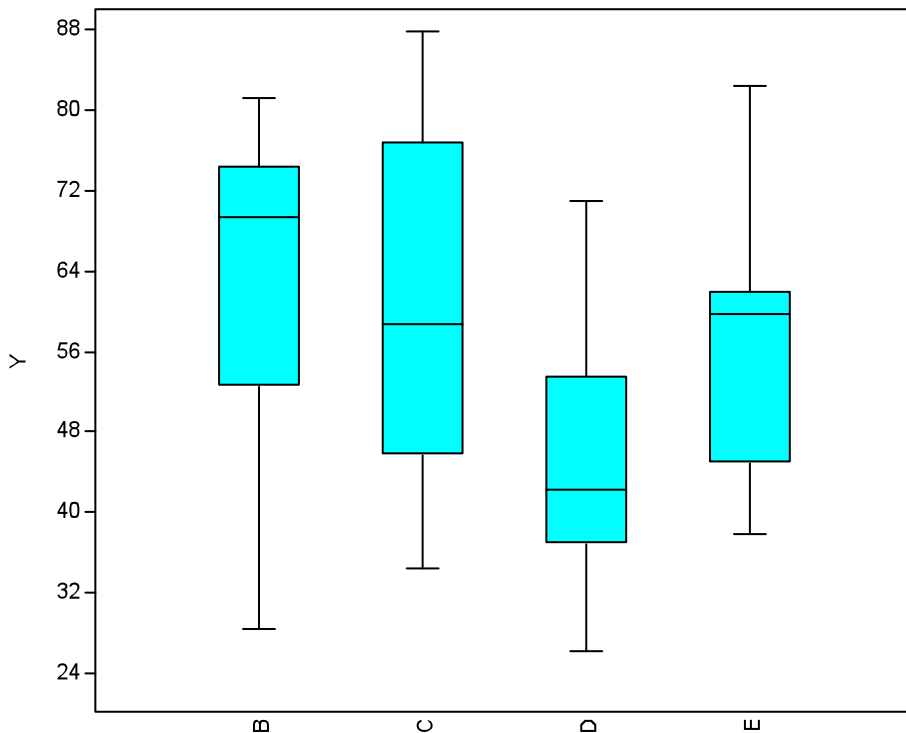


Fig. 25. Comparison of the immunopositive neurons amongst Adults in the different segment. B= C4, C= C7, D= L4, E= L7.

When comparing the expression levels, there is no significant change in between the DRGs of the puppies or adults ($p = 1.0$).

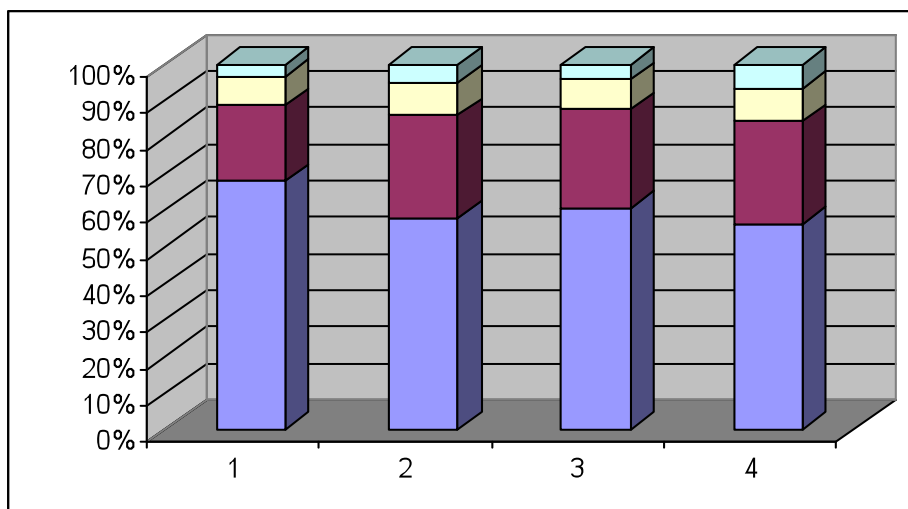


Fig. 26. Bar diagrams showing the scored cell populations in the different

segments amongst puppies.

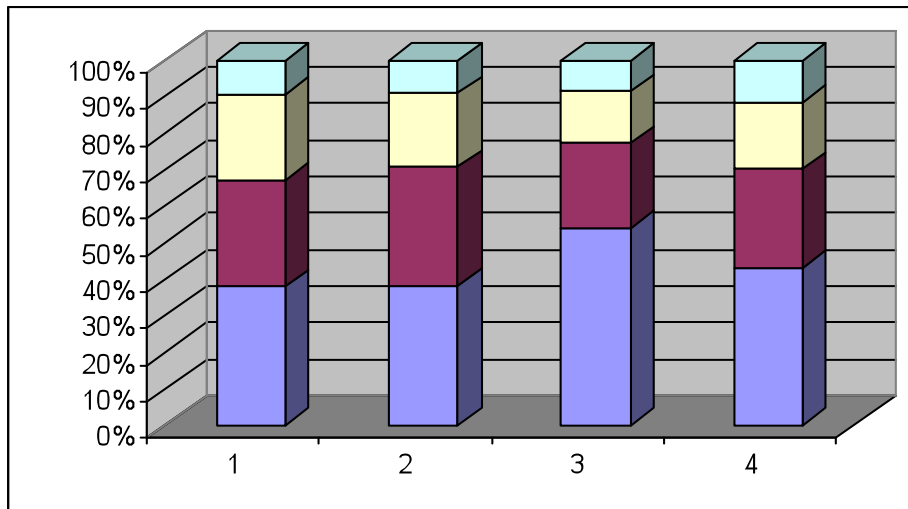


Fig. 27. Bar diagrams showing the scored cell populations in the different segments amongst adults.

Discussion

The present study revealed that all tested dorsal root ganglia of non-neurologic dogs are immunopositive for the Voltage Gated Calcium Channel (VGCC) alpha 2 delta subunit, which appears to be relevant for sensory signal transmission similar to other reported species including humans (Zeilhofer, 2005). With expression rates of about 40% in the neonates and 56% in the adults subjects, the dog ranges close to DRGs of rats, in which gene transcription had been demonstrated by in situ hybridization study for mRNA of VGCC alpha 2 delta 1 and VGCC alpha 2 delta 2 . Canine values, on the other hand are slightly different from the situation reported in the chicken, where at least 65% of all DRG neurons stained immunopositive for VGCC alpha 2 subunit . The discrepancy between mammals and volatile species maybe due to the different modalities of sensory transmission related to locomotion, with two footed and flying animals in one side versus four footed animals on the other.

This is the first systematic investigation of this particular subunit of the VGCC in the dog species using immunohistochemistry. In fact, looking at the literature the only published studies pertaining VGCC in dogs focus their attention on other subunits of this molecule and/or in other tissues than the nervous system, and employed other techniques than immunohistochemistry such as Western blotting and in situ hybridization . From a technical point of view, demonstration of VGCC alpha2 delta subunit by immunohistochemistry using monoclonal mouse antibodies is fast, reproducible and sensible enough to clearly distinguish three different degrees of immunopositivity among large and small DRG neurons. These facts allows for simple application of semiquantitative scoring with intra- and interrater variability of less than 5% for repeated measurements.

Hence, the presented approach had been considered feasible for

establishment of base data on the expression of this presynaptic channel subunit and implies possible insights in the molecular mechanisms of neuropathic pain in the canine species.

The study consequently draws close to previous research in other mammalian species, like rat, and non-mammalian species, like chicken, where researches have been carried on since long on the DRG and Calcium channel in relation with neuropathic pain .

Establishment of normal values, however also has to take the neuronal subtypes into consideration, that are mirrored by the size/volume of neuronal perikarya. Neuronal cell body size increases in the DRG cells during physiological growth and ageing, as it does in the brain and spinal motoneurons of mammals and amphibians . Throughout the different ages, the functional diversification is closely related to either large or small neurons. DRG small neurons develop peripheral C fibres and A δ fibres which are responsible for the transmission of thermoception and nociceptive information, while large neurons develop peripheral A α and A β fibres responsible for the so-called large fibre senses such as proprioception, pressure/vibration and tactile processing . Even though the absolute cell size in puppies is much lower than in adolescent and adult individuals, a certain cut-off between the two main populations can be easily identified within single samples. Therefore it has been decided to relay the immunohistochemical data to these two categories rather than to establish morphometric profiles of the individual cells. Thereby, general rules for simple stereologic assessments of the DRG have been followed such as choosing nonoverlapping serial sections and establishing the cell size category only in full perikaryon sections displaying nucleus plus nucleolus .

The purpose of this investigation was to establish base values for the expression of VGCC alpha 2 delta subunit under physiologic conditions in the DRG of dogs. Consequently neurologically affected animals or those with a history consistent with lameness or spinal disease had been excluded. Furthermore all the animals included in

the study underwent a full post mortem examination consisting of gross inspection and histologic evaluation of all organs and tissues including brain, spinal cord, peripheral nerves and muscles. During the selection of suitable DRGs on HE stained slides as well as after immuno-labelling and counterstaining, the investigator was looking out for all histomorphological changes indicating a non-physiologic situation.

Regarding the histological evaluation of the different sections within one group of animals, interestingly there were no significant differences. From the functional point of view C7, L4 and L7 convey sensory information from the limbs to the cervical (C7) or lumbosacral intumescence (L4 and L7), while C4 collects somatosensory and visceral afferents from the neck rather than the limbs. Thus one would rather expect differences in the expression pattern between C4 and the intumescences, even that of the lower cervical cord.

Comparing the two groups from the histological point of view it is easy to recognize the difference in cell size, for the adults have in general larger perikarya throughout all neuronal subgroups than neonates. This finding is similar to that reported by Martinelli et al. on rabbit Dorsal Root Ganglia where the mean volume of the nerve cell bodies increased progressively with the advancing of the age. Investigations have revealed that the body cell volume of primary sensory neurons correlates with the size of the peripheral field of innervation, thus it correlates with the body size and surface of the animal which change considerably from birth to adulthood (Martinelli et al., 2006). Hence, the neuronal density per high power field differed also between the two groups.

In spite of close expression rates, the immunoreactivity of VGCC alpha 2 delta subunit was significantly stronger in the adults. It may be that the given pathways were not yet all steadily employed in the neonates, in which synapsing in the dorsal horn remains plastic for a considerable postnatal period (Fitzgerald, 2005). Moreover, ion channel regulation is strongly influenced by trophic factors such as

nerve growth factor (NGF), glial derived neurotrophic factor (GDNF) and epidermal growth factor (EGF), which underly developmental changes until the animals are fully developed (Woodal et al., 2008). This difference in expression mirrors the extensive induction, structural and functional integration and plastic reorganisation of sensory processing during the neonatal period, which are characterised by longitudinal variations regarding expression of key molecules, receptors and channels, including those associated with nociception. For these reasons both nociceptive processing and experience of pain in newborns and therefore also the detection, monitoring and management has to be seen in a completely separate setting .

During the embryologic development nociceptive neurons are specified early, even before neuronal crest cells become destined to become neuron or glial cells. In the DRG, sensory neurons are generated in two waves: large diameter neurons appear first, followed by the smaller population . In the rat, the innervation of peripheral and central target occurs before birth and reflects this two distinct waves of neurogenesis. The A fibres penetrate the dorsal horn grey matter of the spinal cord creating synapses at first, during embryonic day 15-17 which are then followed by C fibres at day 18-20 . Furthermore C fibres complete their connections with the laminae I and II of the spinal cord around post natal day 5, thus this relatively late formation of central C fibre synapses means that, despite the ability to detect noxious stimuli at the periphery, the central processing is still immature in the early post natal period in the rat . The A fibres in the early stages reach predominantly laminae I and II, with a subsequent withdrawal toward laminae III and IV over the first 3 postnatal weeks . Apart from the physiologic modification that occurs during growth, the nervous tissue is capable of further adaptation thanks to neuroplasticity. In particular diseases affecting the sensory pathways, molecules, receptors, channels and even synapses can undergoes significant changes . For example, if a neuropathic pain has been induced, these changes impact

primarily on C and A δ fibres, small fibres which are responsible of pain transmission. In addition, large fibres such as A β fibres, which normally transmit proprioceptive information, may experience a variation in their phenotype with increased production of neurotransmitters implied in pain transmission, such as substance P (Jones et al., 2007). These changes come into action by reorientation of their synapses in the dorsal horn from laminae III and IV to laminae I and II those responsible for the transmission of nociceptive informations. . According to our results there was not a statistically significant difference between small and large cells, both in neonates and adults. Hence we can assume that calcium currents are used in the entire sensory transmission and are not restricted to a specific function segregating with one single cell type. This finding is different from those previously reported in rats and chickens where the distribution of the VGCC alpha 2 delta subunit was predominantly related to small and medium size neurons (Newton et al., 2001, Li et al., 2007). Further interpretation of this difference requires comparison of the cut-off criteria. Our tissues did not exhibit a trimodal neuron size histogram. The large cell population therefore may contain cells that in the other named species present as a third ganglion cell subgroup. Morphometry is unlikely to shed further light onto this subject. Multilabelling for neurotransmitters and functional phenotyping, on the other hand, could help subcategorising the groups.

Within the dogs there is a tendency of lumbar DRGs to exhibit a lower percentage of immunopositive neurons, and this is particularly evident in the adults where C4 segment has 62% of positive stained cells while L4 accounts only for a 44%. A possible explanation for this craniocaudal decrement of expression along spinal ganglia may be related to functional differences between front limbs and hind limbs. Even in the quadrupede dog the front limbs are not used merely for walking, but they can do further specialized movements which in turn require the transmission of a major sensory amount of information. Whether this really is the cause, remains to be tested

further.

Another possible explanation could rely on the fact that at the different segment there could be a preference in signalling transmission, and maybe in the lumbar segment other molecules such as glutamate or Substance P can be prevalent. In order to increase the accuracy of the detection and to better understand the functional relationships with calcium channel and other molecules on the dynamics of sensory transmission, multilabelling studies such as those made by Li et al. in .2007 can be of great interest .

The synaptic transmission of noxious and non-noxious signals in neuropathic condition partially depends on presynaptic calcium channels that contain VGCC alpha 2 delta subunit. Therefore it has been proven that rats show a marked increase in the expression of this molecule when a state of neuropathic pain has been established in experimental conditions (Newton et al., 2001, Luo et al., 2002).

Dogs can experience neuropathic pain because several conditions such as trauma, surgical procedures, pelvic fractures, limb nerve entrapment, amputation, lumbosacral lesions, spinal cord injury, intervertebral disc herniation, fibrocartilaginous embolic myelopathy, syringomyelia, discospondylitis, polyradiculoneuritis, tumors of the central nervous system, inflammatory bowel disease and pancreatitis can determine this pathologic condition of the sensory pathway (Mathews, 2008, Grubb, 2010).

To confirm whether a neuropathic pain condition goes along with increased expression of the VGCC alpha 2 delta subunit in dogs as it has been observed in rats requires further investigation on animals with well characterised nerve lesions. Furthermore from the clinical point of view, the presence of a different pattern of expression in the front limb and hind limb for the VGCC alpha 2 delta subunit that we found, can make us wonder whether a dog can have a different response to the administration of certain class of drugs like gabapentinoids in relation to the site of nerve damage. Investigations and clinical trials on this direction in the future will help us to better understand and control neuropathic pain.

Giving the high number of pathological states that can produce neuropathic pain, the clinician must be able to recognize his symptoms in a patient that can not express himself verbally and treat this debilitating condition appropriately. As previously mentioned in this work, in human medicine neuropathic pain treatment is a challenge from the clinical point of view because despite the efforts made in developing rationale therapeutic approach, the response to most treatment is unpredictable with moderate or minimal relief using standard analgesic drugs . In the recent literature VGCC alpha 2 delta subunit has revealed to be a binding site for Gabapentin (GBP) and Pregabalin (PGB) . The mechanism of action is still not completely understood but it has been reported that these drugs are able to reduce Calcium current in the DRG and in the dorsal horn of the spinal cord, thus inhibiting the release of excitatory neurotransmitters from presynaptic terminals of the DRG neurons . In the studies conducted by Luo et al. 2001 and 2002 has been observed an increase in the expression of VGCC alpha 2 delta subunit in the DRG and spinal dorsal horn of animals presenting neuropathic pain that were sensitive to gabapentin thus providing evidence for the antineuropathic pain effect of this drug.

The European Federation of Neurological Societies published in 2009 the guidelines for neuropathic pain management in humans and Gabapentin (GBP) and Pregabalin (PGB) have been elected as first drugs of choice for the treatment of this condition . In veterinary medicine the treatment of neuropathic pain is as challenging as it is for people, with few published data in the literature on the topic. As it happens for many drugs, veterinary surgeons has the tendency to utilize on the patients drugs that are designed for human treatments and are thus used off label for companion animals. This means a complete disregard of the principles of Evidence Based Medicine, which aims to apply the best available evidence gained from the scientific method to clinical decision making. Gabapentinoids are currently used in veterinary practice with success for the treatment of neuropathic pain, but there are no clinical trial published, and the

data to their efficacy in the dog account for few reports in a small dog population . Considering the clinical importance and the economical consequences implied by the introduction of a new drug for a disease treatment, a more rationale approach should be recommended. Therefore our investigation, demonstrating the presence of the binding site of gabapentinoids, represents a fundamental step toward an Evidence Based Medicine approach in the treatment of neuropathic pain in the dog.

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