Capsaicin-induced activation and chemical injury of nociceptive primary sensory neurons: the role of gangliosides in the pain system

PhD Thesis

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1. INTRODUCTION

1.1. Some morphological and functional properties of primary afferent neurons

Peripheral nerve endings of primary afferent neurons of the somatosensory system are responsible for the detection of mechanical, chemical, painful and thermal stimuli of the body surface and the musculoskeletal system, whereas primary visceral nerve endings are sensitive to mechanical, chemical and painful signals arising in visceral organs. The neuronal cell bodies that give rise to primary afferents of the somatosensory system are situated in the dorsal root ganglia and in the sensory ganglia associated with the Vth, VIIth, IXth and Xth cranial nerves. Besides, visceral sensory afferents arise from cell bodies that lie in the dorsal root ganglia and in the sensory ganglia associated with the IXth and Xth cranial nerves (Grundy, 2002). These primary sensory neurons are pseudounipolar nerve cells having a peripheral and a central axon.

It is well established that there are two main morphologically defined neuronal subtypes in rat and mouse dorsal root ganglia. These two populations which have been denoted the A-type or large light neurons and the B-type or small dark neurons (Andres, 1961; Lieberman, 1976) can be distinguished on the basis of their light and electron microscopic morphology (Hossack and Wyburn, 1954). This classification has been confirmed by histochemical (Kokko, 1965) and embryological (Lawson and Biscoe, 1979; Lawson et al., 1974) properties of the neurons. Furthermore, neurofilament-rich and neurofilament-poor neurons have also been distinguished in the dorsal root ganglia based on electron microscopic examination (Friede and Samorajski, 1970) or immunohistochemical detection of 200kDa neurofilament with the RT97 antibody (Lawson et al., 1984). These cell categories coincide with the above classification of sensory neurons, since neurofilaments are abundant in A- but not B-type neurons (Lawson and Waddell, 1991; Sann et al., 1995). The peripheral and also central axons of these pseudounipolar neurons differ in their diameter, myelinisation and conduction velocity and were classified by Erlanger and Gasser (Gasser and Erlanger, 1927). Large-diameter A-type neurons have myelinated Aα-, Aß- or thin myelinated Aδ-fibres, whereas small-diameter B-type neurons have thin unmyelinated C-fibres.

Central axons of different classes of spinal primary afferent fibres terminate in a strict somatotopic and topographic manner in the dorsal horn of the spinal cord. In the early 1950's the gray matter of the spinal cord was divided into ten laminae by the Swedish neuroscientist, Bror Rexed. Rexed identified 10 distinct areas (laminae) on the basis of their cellular structure

(cytoarchitectony) in the cat spinal cord (Rexed, 1952; Rexed, 1954). Large myelinated Aβ afferents carrying mechanoreceptive information terminate within the deeper laminae (III-VI) of the spinal cord dorsal horn (Brown, 1981; Réthelyi and Szentágothai, 1973; Szentágothai, 1964; Willis and Coggeshall, 1991). Nociceptive Aδ afferents arborize mostly in laminae I and V (Light and Perl, 1979), whereas C afferents innervate the superficial laminae (I-II) of the dorsal horn (Heimer and Wall, 1968; Jancsó and Király, 1980; Szentágothai, 1964; Willis and Coggeshall, 1991).

1.2. Functional properties of C-fibre nociceptors

In 1906, Sherrington introduced the term nociceptor for primary sensory neurons involved in the processing of information on stimuli which damage or threaten to damage tissues of the body (Sherrington, 1906). According to this concept, nociceptors have characteristic thresholds or sensitivities that distinguish them from other sensory neurons. Morphologically, there are two types of nociceptors: lightly myelinated A-fibre nociceptors and unmyelinated C-fibre nociceptors. Nociceptive nerve fibres diffusely innervate virtually all organs and tissues of the body forming free nerve endings which serve as the receptive domain of the neuron. Functionally, C-fibre nociceptors can be sub-classified based on the stimulus modalities that excite them. The most widely studied types of cutaneous nociceptors are C-fibres responsive to mechanical and heat stimuli (C-mechanoheat nociceptor, CMH) (Beitel and Dubner, 1976; Bessou and Perl, 1969; LaMotte and Campbell, 1978; Meyer and Campbell, 1981; Taguchi et al., 2010). Their mechanical and heat thresholds are substantially higher than those of the low threshold mechanoreceptors and warm fibres (C-fibres sensitive to non-noxious warm stimuli). CMHs are also called polymodal C-fibres because they also respond to different types of chemical stimuli (Beck and Handwerker, 1974; Bessou and Perl, 1969). Activity in CMHs is thought to lead to the percept of burning pain. On the basis of their stimulus modalities, other subtypes of cutaneous nociceptive C afferents have been identified: C-mechanical (CM), C-mechanoheat-cold (CMHC) and C-mechanocold (CMC) nociceptors. For example, in the rat sural nerve where C-fibre nociceptors comprise about 43% of C-fibre afferents, the distribution of these receptors is the following: CMH 32%, CM 8%, CMHC 2% and CMC 1% (Leem et al., 1993; Michaelis et al., 1999).

The classes of nociceptive afferents in tissues other than skin can be highly different. Afferents that respond at high stimulus intensities are thought to encode pain sensation and the major sensation that originates from deep tissues and viscera is pain (Schaible and Grubb,

1993). Traditionally, nociceptors of deep tissues (muscles and joints) are primarily classified according to their mechanosensitivity although most of these receptors respond also to algesic chemical stimulation and some of them can be excited by high temperature (Graven-Nielsen and Mense, 2001).

1.3. Functional and neurochemical properties of capsaicin-sensitive C-fibre nociceptors

One of the most frequently used chemical agent to selectively activate CMHs is capsaicin, the principal pungent ingredient in chili peppers, which belong to the plant genus *Capsicum*. Chemically, it is a derivative of vanillyl amide, 8-methyl-N-vanillyl-6-nonenamide. When it comes in contact with the mucous membranes or the skin, capsaicin evokes a sharp sensation of burning pain and, if applied at high enough concentrations, a subsequent long-lasting unresponsiveness of the affected area to noxious stimuli. This effect has been utilized by Native Americans for controlling pain. Endre Hőgyes was the first who stated that the irritant action of *Capsicum* extracts is mediated mainly by sensory nerves (Hogyes, 1878). Later, it has been discovered by Nikolaus (Miklós) Jancsó that repeated local or systemic applications of high-dose capsaicin resulted in a unique insensitivity of the animals to painful stimuli evoked by intense heat and chemical irritants but not intense mechanical stimuli due to an impairment of chemosensitive nerve terminals. This phenomenon was termed capsaicin desensitization (Jancsó, 1960; Jancsó, 1968; Jancsó and Jancsó-Gábor, 1949; Jancsó et al., 1967; Porszasz and Jancsó, 1959). The morphology and additional functional properties of chemo/capsaicin-sensitive primary sensory neurons were revealed through the observations of G. Jancsó et al. (1977) who discovered that administration of capsaicin to newborn rats resulted in a selective degeneration of a morphologically, neurochemically and functionally well characterized population of nociceptive primary sensory neurons in spinal and cranial sensory ganglia. Acutely, neonatal capsaicin produces profound degenerative changes in the perikarya, central terminals and axons of sensory neurons, whereas chronically it produces a selective chemo- and thermal analgesia, loss of neurogenic inflammation, and a lifelong, selective loss of B-type sensory ganglion cells and, consequently, afferent C-fibres and their peripheral and central nerve endings (Jancsó and Kiraly, 1980; Jancsó et al., 1977).

It has been recognized by N. Jancsó that capsaicin-sensitive primary sensory neurons have a dual function. On the one hand, they can convey nociceptive impulses towards the central nervous system (sensory afferent function) and, on the other hand, they play a

fundamental role in the mechanisms of neurogenic plasma extravasation and antidromic vasodilatation by releasing a putative ‘neurohumour’ from their peripheral endings (Jancsó, 1960). It has been established later that these vascular reactions are mediated by vasoactive neuropeptides, such as substance P (SP) (Gamse et al., 1980; Gamse et al., 1982; Gamse et al., 1987; Germonpré et al., 1995; Lembeck, 1953; Louis et al., 1989) and calcitonin gene-related peptide (CGRP) (Brain and Grant, 2004; Brain et al., 1985; Struthers et al., 1986). The capsaicin-induced desensitization and selective chemodenervation abolished both the sensory afferent and the vascular reactions, which represent one of the major sensory efferent or local regulatory functions of the chemosensitive primary sensory neurons (Gamse et al., 1982; Holzer, 1988; Jancsó, 1992; Jancsó et al., 2009; Jancsó et al., 1980; Maggi and Meli, 1988; Such and Jancsó, 1986; Szolcsányi, 1996).

Taking advantage of the selective neurodegenerative/neurotoxic action of capsaicin, the neurochemical and morphological properties of the capsaicin-sensitive primary sensory neurons are well characterized (Gamse et al., 1980; Holzer et al., 1982; Jancsó, 1992; Jancsó and Király, 1980; Jancsó et al., 1977). Numerous neurochemical markers are selectively or at least preferably expressed by these neurons. The fast synaptic transmitter, as in most primary afferents, is glutamate (De Biasi and Rustioni, 1988) but, as stated above, vasoactive neuropeptides such as SP and CGRP are also present in a large proportion of C-fibre afferents. Among these peptides CGRP is unique because it is present in small-, medium- and large-diameter cells. In contrast, exclusively small-diameter cells display SP positivity (Hökfelt et al., 1975a; Hökfelt et al., 1975b). A small population of capsaicin-sensitive neurons contains other neuropeptides, such as neurokinin A, galanin, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK) and somatostatin (Ju et al., 1987; Lazarov, 2002). It is evident, however, that not all capsaicin-sensitive neurons synthesize neuropeptides.

A previous concept claims the existence of two populations of capsaicin-sensitive primary sensory neurons based on their peptide content (peptidergic and non-peptidergic) and other neurochemical and functional properties (Hunt and Rossi, 1985; Silverman and Kruger, 1990; Snider and McMahon, 1998; Zylka et al., 2008). The non-peptidergic population of C-fibre nociceptors can be identified by using specific chemical markers e.g. the thiamine monophosphatase (TMPase) enzyme. The previously described non-lysosomal fluoride-resistant acid phosphatase (FRAP) has the same localization pattern as TMPase. It is assumed that these are identical enzymes (Dodd et al., 1984; Inomata and Ogawa, 1979; Knyihár-Csillik et al., 1986) which present in 30-50% of spinal ganglion cells of rodents, in particular in small- to medium-sized neurons (Colmant, 1959; Silverman and Kruger, 1990). According

to recent findings, TMPase is molecularly identical with the transmembrane isoform of prostatic acid phosphatase (PAP) (Taylor-Blake and Zylka, 2010; Zylka et al., 2008) which is regarded as a marker of non-peptidergic neurons. In the central nervous system, the enzyme activity is localized mainly to the superficial laminae of the spinal cord dorsal horn and in the medulla to the subnucleus gelatinosus of the spinal trigeminal nucleus, the nucleus paratrigeminalis, the area postrema, the area subpostrema and the solitary tract nucleus similar to the distribution of degenerative argyrophilia observed after systemic capsaicin treatment of newborn or adult rats and some other species (Jancsó et al., 1985a; Jancsó and Kiraly, 1980). This population of primary sensory neurons can also be identified by binding of *Griffonia simplicifolia* isolectin B4 (IB4, (Alvarez and Fyffe, 2000; Alvarez et al., 1991), which is a plant lectin having high affinity for α-D-galactose residues in the carbohydrate chains of membrane-associated glycoconjugates (Hayes and Goldstein, 1974; Silverman and Kruger, 1990). There are also differences between the central terminations of the two types of unmyelinated afferents: the peptidergic ones terminate mainly in lamina I and outer part of lamina II, whereas those that do not contain peptides terminate mainly in the inner part of lamina II of the spinal cord dorsal horn (Hunt and Rossi, 1985; Priestley et al., 2002; Priestley et al., 1982; Snider and McMahon, 1998). Moreover, regarding the peripheral targets of these neurons it is noteworthy that the IB4-positive population innervates mainly the skin, whereas deep tissues and viscera are mainly supplied by the peptidergic population of C-fibres (Bennett et al., 1996a; Lu et al., 2001; Perry and Lawson, 1998).

The development, differentiation and mature phenotype of primary sensory neurons depends on their response/sensitivity to various growth factors the most important of which are members of the neurotrophin, glial cell line-derived neurotrophic factor (GDNF) and neuropoeitic cytokine families. In the rat and the mouse, 70-80% of dorsal root ganglion (DRG) neurons express the nerve growth factor (NGF) neurotrophic tyrosine kinase receptor type 1 (trkA) and require NGF for their survival during embryonic life (Levi-Montalcini and Angeletti, 1968; Ritter et al., 1991). However, during the early postnatal life, the sensitivity of sensory neurons for trophic factors may change, hence the individual subpopulations of DRG neurons show characteristic expression patterns of growth factor receptors. In C-fibre nociceptors the IB4- and CGRP/SP-positive populations express a different family of these receptors: those which contain CGRP and SP also co-localize with the NGF receptor trkA. In contrast, the IB4-containing population of nociceptors does not normally respond to NGF but do respond to GDNF (Averill et al., 1995; Bennett et al., 1996a; Bennett et al., 1996b; Michael and Priestley, 1999; Priestley et al., 2002; Snider and McMahon, 1998). Besides the

high-affinity NGF receptor trkA, the non-selective neurotrophin receptor p75 is also present in the CGRP-containing population of DRGs. However, p75 is not a specific marker for nociceptors because many large- and medium-sized cells with myelinated axons express this receptor (Wright and Snider, 1995). Actions of the GDNF family mediated through the receptor tyrosine kinase RET and an accessory subunit of GDNF-family receptor alpha 1-4 (GFRα1-4) (Bennett et al., 1998).

1.4. The TRPV1 receptor and its agonists

Studies on animals injected with capsaicin neonatally indicated that capsaicin evokes its effect through a specific receptor, and that "capsaicin receptor" might be involved in the development of various pathological events in mammals, such as pain, visceral hyperreflexia and neurogenic inflammation (Jancsó et al., 1977; Maggi et al., 1989; White and Helme, 1985). In 1997, an ion channel activated directly by capsaicin and other vanilloid compounds (transient receptor potential vanilloid type 1 receptor; TRPV1, previously known as vanilloid receptor subtype 1, VR1) has been identified and found to be selectively expressed in a subset of small- to medium-diameter neurons which are putative nociceptors (unmyelinated C- and thin myelinated Aδ-fibres) (Caterina et al., 1997). Previous studies have demonstrated that capsaicin application results in an accumulation of Ca^{2+} ions in the capsaicin sensitive neurons (Jancsó et al., 1984; Jancsó et al., 1978; Wood et al., 1988). This trait was utilized during the functional cloning of the TRPV1 receptor (Caterina et al., 1997).

TRPV1 is regarded as a molecular transducer of noxious heat and chemical stimuli. The functional channel apparently consists of a tetramer of subunits, each with six putative transmembrane domains. Both the amino and carboxyl termini have an intracellular location and the amino terminus has three ankyrin repeat domains. Sequence analysis revealed TRPV1 to be a part of the transient receptor potential (TRP) family of ion channels, members of which have been implicated in many sensory functions in vertebrate and invertebrate species (Montell, 2001). The TRPV1 channel is a nonselective cation channel with a preference for Ca^{2+} ions (Caterina et al., 1997). However, it is noteworthy that TRPV1 is also permeable to Co^{2+} ions which has been utilized for the *in vitro* histochemical demonstration of capsaicin sensitivity (Hogan, 1983). Opening of the TRPV1 receptor leads to inwardly rectifying Ca^{2+} and Na^{+} currents. These currents depolarize the neuronal membrane and result in action potential generation. Rising of intracellular Ca^{2+} concentration also plays a role in the exocytosis of neuropeptides. Elevated intracellular Ca^{2+} level activates several signal

transduction pathways that contribute to the desensitization of the afferent neuron (Koplas et al., 1997; Liu and Simon, 1998; Vyklicky et al., 2008) and may lead to neurotoxicity.

Besides capsaicin, TRPV1 can be activated by other exogenous vanilloids such as resiniferatoxin (RTX) or endovanilloids like N-oleoyldopamine (OLDA), anandamide (arachidonylethanolamid, AEA) and N-arachidonoyldopamine (NADA). Lypoxigenase products of arachidonic acid, noxious heat (~ 43 °C) and protons also activate the capsaicin receptor (Chu et al., 2003; Hwang et al., 2000; Szallasi and Blumberg, 1989; Tominaga et al., 1998; Zygmunt et al., 1999). It has been long known that the latex of the Moroccan cactus-like plant *Euphorbia resinifera* contains an extremely irritant component (Appendino and Szallasi, 1997) which was isolated and named RTX only in 1975 (Hergenhahn et al., 1975). It is a vanilloid compound and related to phorbol substances. In several assays, RTX proved to be several thousand-fold more potent than capsaicin, thus it is regarded as an ultrapotent capsaicin analogue (Szallasi and Blumberg, 1989; Szallasi and Blumberg, 1990; Szallasi and Blumberg, 1993; Szallasi and Blumberg, 1996). However, it is only 20-fold more potent than capsaicin to activate the cloned TRPV1 (Caterina et al., 1997).

Under normal physiological conditions TRPV1 is unlikely to be activated by heat and low pH therefore, the existence of endogenous ligands (endovanilloids) was suggested (Di Marzo et al., 2001; Van Der Stelt and Di Marzo, 2004). Indeed, different classes of lipids (all derived from the metabolism of arachidonic acid) have been recently characterized as TRPV1 activators. To classify a molecule as an endovanilloid it should be formed or released in an activity-dependent manner in sufficient amounts to evoke TRPV1-mediated response by activating the receptor. The first molecule which was identified as an endovanilloid was AEA, previously discovered as an endogenous agonist of cannabinoid receptors (Devane et al., 1992). Later, other derivatives of long-chain unsaturated fatty acids were shown to act as endovanilloids of which NADA and OLDA exhibited the highest efficacy (Chu et al., 2003; Huang et al., 2002). Hwang and colleagues reported that several products of lipoxygenases were also able to activate the capsaicin-activated channel in isolated membrane patches of sensory neurons. These are 12-(*S*)- and 15-(*S*)-hydroperoxyeicosatetraenoic acid (12- and 15-HPETE) (Hwang et al., 2000). It is worthy to mention that these endovanilloids were less efficacious in activating TRPV1 than capsaicin.

Besides activation, the TRPV1 receptor can be sensitized by many chemicals liberated following tissue injury or inflammation such as bradykinin (Tang et al., 2004), prostaglandin E_2 (Moriyama et al., 2005), NGF (Bonnington and McNaughton, 2003) or histamine (Kim et al., 2004). Moreover, insulin and insulin-like growth factors (IGFs) can also increase the

sensitivity of TRPV1 and promote the translocation of the receptor from the cytosol to the plasma membrane (Nagy et al., 2004; Sathianathan et al., 2003; Van Buren et al., 2005). These substances act on guanyl nucleotide regulatory protein (G-protein)-coupled receptors. The activated G-protein activates different kinds of kinases such as protein kinase A (PKA), protein kinase C (PKC) (Bhave et al., 2003; Tang et al., 2004), phosphatidylinositol 3-kinase (PI(3)K) or Ca^{2+}/calmodulin-dependent kinase II (CaMkII) (Jung et al., 2004), which eventually led to the phosphorylation of TRPV1. Sensitization of the TRPV1 receptor renders nociceptors more sensitive toward chemical and thermal (heat) stimuli, which results in the development of chemical and heat hyperalgesia. Indeed, targeted deletion of the TRPV1 gene in mice, which completely eliminated vanilloid sensitivity, significantly attenuated inflammatory heat hyperalgesia (Caterina et al., 2000; Davis et al., 2000).

1.5. Effects of perineural capsaicin treatment on the function, morphology and neurochemistry of C-fibre nociceptors

Although systemic capsaicin treatment of adult animals or neonates provides information on the mechanisms of inflammation and pain, local treatments give the opportunity to study the functional significance of particular nociceptive afferent nerves in targeted regions of tissues or organs. There are three possible forms of local applications of capsaicin: topical, intrathecal/intracisternal and perineural treatment (Jancsó et al., 2011). Topical capsaicin application (close to the termination sites of peripheral axon terminals e.g. epicutaneous or intracutaneous application) produces regional chemo- and thermal analgesia, loss of thermal hyperalgesia and neurogenic inflammation and depletion of peripheral nerve endings together with sensory neuropeptides and other specific neuronal markers (Jancsó et al., 1985c). Injections of capsaicin into the subarachnoid space (intrathecal or intracisternal applications) affects the central terminals of the primary sensory neurons producing chemo- and thermal analgesia, transient mechanical allodynia (probably due to an acute central sensitization), central terminal degeneration and depletion of neuropeptides from the spinal cord leaving the cutaneous neurogenic inflammation response intact (Gamse et al., 1984; Jancsó, 1981).

1.5.1. Functional consequences of perineural capsaicin treatment

The selective activatory and neurodegenerative effects of perineurally applied capsaicin were first described by Gábor Jancsó and co-workers (Jancsó et al., 1980).

Electrophysiological studies disclosed that binding of vanilloids to their axonal receptors initiates a complex cascade of intracellular events which can be divided into three phases (Jancsó and Such, 1983). The first phase is excitation, which involves a selective activation of Aδ- and C-fibres (Jancsó and Such, 1983; Marsh et al., 1987; Such and Jancsó, 1986), which may evoke cardiovascular reflexes, antidromic vasodilatation and cutaneous plasma extravasation. The second phase is characterized by a blockage of impulse conduction in C- and, to a lesser extent, A-fibres (Chung et al., 1985; Jancsó and Such, 1983; Petsche et al., 1983; Pini et al., 1990; Such and Jancsó, 1986). During the third phase, direct application of high concentration of capsaicin onto the nerves produces a highly selective and apparently irreversible impairment in the function of C-fibre polymodal nociceptors (Handwerker et al., 1984; Jancsó et al., 1980; Pini et al., 1990; Pini and Lynn, 1991; Welk et al., 1983), which results in selective and permanent chemo- and thermal analgesia, loss of thermal hyperalgesia, decreased visceral reflex functions and a complete abolition of the neurogenic inflammatory response in the skin area supplied by the treated nerve (Domoki et al., 2003; Holzer, 1991; Jancsó et al., 1980; Jancsó and Such, 1983). Functional regeneration of the capsaicin sensitive nerve fibres could not be demonstrated even a year after perineural capsaicin treatment (Jancsó and Ambrus, 1994; Jancsó and Király, 1984; Jancsó et al., 1987b). In contrast to the severe functional impairment of the nociceptive afferents, the functions of the non-nociceptive cutaneous and deep afferents, skeletomotor and autonomic efferent fibres remain intact.

1.5.2. Changes in morphology and neurochemistry

Electron microscopic studies on the rat saphenous and sciatic nerves have revealed markedly swollen unmyelinated axons in the treated nerves merely 1 hour after the capsaicin treatment (Jancsó et al., 1985b; Jancsó et al., 1987a). Intra-axonal accumulation of neuropeptides and organelles indicating the blockage of axoplasmic transport of SP and somatostatin was also demonstrated (Gamse et al., 1982). This transport block eventually led to the depletion of endogenous CGRP, SP and FRAP/TMPase from the superficial dorsal horn of the spinal cord and from the periphery (Gamse et al., 1982; Gibson et al., 1982; Knotkova et al., 2008; Molander and Grant, 1986; Sántha and Jancsó, 2003; Szigeti et al., 2012). Two or three weeks later, the fine structural organization of the nerve also changed. Processes of Schwann cells, which normally enwrap the individual unmyelinated axons, seemed to be retracted. In the ganglia loss of small B-type neurons was evident. Perineural capsaicin

treatment has also an inhibitory effect on the retrograde intra-axonal transport of exogenous proteins like horseradish peroxidase (HRP). Moreover, investigations of the retrograde intra-axonal transport of HRP in nerves previously treated with capsaicin showed significant reduction in the number of retrogradely labelled DRG neurons which may occur due to a permanent loss of afferent axons from the treated nerves and/or an impairment of axon transport mechanisms (Jancsó and Lawson, 1990). Two months after perineural capsaicin treatment the numbers of unmyelinated fibres were found to be markedly reduced compared to the control nerve (Jancsó and Lawson, 1990; Pini et al., 1990). Immunohistochemical data showed that SP- and somatostatin-like immunoreactivities were also significantly decreased (Gamse et al., 1982; Gibson et al., 1982) while the overall reduction in the proportion of CGRP-immunoreactive neurons was less marked. However, there was a significant reduction in the proportion of small CGRP-immunoreactive neurons, whereas large immunopositive cells appeared to be unaffected (Jancsó, 1992). TRPV1 mRNA and protein expression of small- and medium-sized DRG neurons and TRPV1-immunoreactivity in the superficial layers of the spinal cord dorsal horn are also markedly decreased after perineural capsaicin treatment (Szigeti et al., 2012). On the periphery, an almost complete elimination of the intra-epidermal nerve fibres can be observed in the skin regions innervated by the treated nerve already 3 days after the treatment (Dux et al., 1999).

1.6. Capsaicin in pain management

Since capsaicin exerts a highly selective action on nociceptive primary afferent neurons, the possibility of a clinical use of capsaicin in pain management was proposed (Jancsó and Lynn, 1987). A number of methods of administration are possible, including local application to nerve trunks and target tissues. Topical capsaicin patches are available for treatment of neuropathic pain associated with postherpetic neuralgia. The high-concentration capsaicin (8%) Qutenza patch decreases pain sensation by reducing TRPV1 expression and decreasing the density of epidermal nerve fibres in the application area (Nolano et al., 1999). Clinical trials verify the pivotal role of these patches in pain management. The primary endpoint of the trials was the 29-32% reduction in numeric pain rating scale (NPRS) score from baseline to weeks 2 to 8. The improvement in NPRS scores persisted up to 12 weeks or more (Jones et al., 2011).

1.7. Methods for demonstration of the central projections of peripheral afferent nerves by horseradish peroxidase conjugate

Axoplasmic transport is the process responsible for the directed movement of organelles, synaptic vesicles, lipids and proteins to (retrograde transport) and from (anterograde transport) the neuronal cell body through the cytoplasm of its axon. These transport processes are mediated by the complex interactions between cytoskeletal elements (neurofilaments and neurotubuli) and anchor/motor proteins (dynein and kinesin).

To demonstrate the central representation of peripheral afferent nerves the retrograde neuronal labelling technique is used for many years. According to this method, a tracer is injected *in vivo* into the peripheral nerve or tissue innervated by a specific nerve, and it is transported retrogradely by the axons resulting in a ganglionic and, depending on the tracer and the specific pathway, transganglionic labelling of the relating afferent nerve endings. HRP, which non-selectively binds to and transported by both myelinated and unmyelinated axons of a peripheral nerve, is a widely used tracer for this purpose (Molander and Grant, 1986). Since the transport of HRP is not fibre specific, it makes impossible to distinguish the spinal projection territory of different types of sensory neurons. However, binding of lectins or other macromolecules, which can specifically recognize different membrane components of the axolemma, to HRP (HRP-conjugates) provides an opportunity to selectively label different types of sensory ganglion cells and their central terminals.

HRP conjugated to wheat germ agglutinin (WGA-HRP) is proved to be a selective marker of unmyelinated sensory neurons, hence injection of WGA-HRP into a peripheral nerve gives rise to transganglionic labelling of C-fibres, mostly nociceptors (small- and medium-sized neurons) and the somatotopically related superficial laminae (mainly lamina II) of the spinal cord dorsal horn (LaMotte et al., 1991; Molander and Grant, 1986; Shehab and Hughes, 2011). The lectin WGA specifically binds N-acetyl glucosamine residues of the carbohydrate chains of glycoconjugates of the plasma membrane (Macsween and Fox, 1975; Sharon and Lis, 1972). However, HRP conjugated to the B subunit of the holotoxin of *Vibrio cholerae* (CTB-HRP) is transported by myelinated axons of motor neurons and sensory ganglion cells and labels mainly large sensory neurons in the relating ganglia and the deeper laminae of the spinal cord dorsal horn (lamina I and The enzymatically inactive B subunit, also called choleragenoid, is responsible for the docking and internalization of the holotoxin (Stoeckel et al., 1977). This B subunit recognizes and binds to the carbohydrate chain of the

GM1 ganglioside which is present in the plasma membrane (Cuatrecasas, 1973; Robertson and Grant, 1989; Stoeckel et al., 1977).

1.8.Neuroplastic reactions of injured primary sensory neurons

1.8.1. The A-fibre sprouting hypothesis

Injuries of mechanical, toxic, metabolic and inflammatory/immune origin, affecting single or multiple peripheral nerves result in profound changes in the morphological organization and functions of the somatosensory/nocicptive system. Surgical transection of a peripheral nerve of laboratory animals is the most frequently used experimental paradigm to study these morphological and functional alterations under controlled conditions. Complete transection (axotomy) of the peripheral branches of primary sensory neurons results in degeneration of the distal segment of the severed axon known as Wallerian degeneration. Due to cessation of the retrograde transport of target-derived neurotrophins, the phenotype of the DRG cells rapidly change with the up- or down-regulation of neuropeptides (Hökfelt et al., 1994), cytoskeletal proteins (Verge et al., 1990) and growth-associated proteins (Chong et al., 1992) and a great number of regulated genes, in part with yet unidentified function and significance (Costigan et al., 2002). Two-3 days after the axon is severed the cell bodies begin to swell, the nuclei move to an eccentric position and finally the Nissl substance undergo chromatolysis in preparation for regeneration of the proximal axon segment. There are also observations that the central terminals of peripherally injured primary sensory neurons may also exhibit degenerative/atrophic changes (Arvidsson and Grant, 1979; Grant and Arvidsson, 1975; Knyihár-Csillik et al., 1987). In the spinal cord a substantial reduction in the number of synaptic contacts made by primary afferents with neurons in lamina II occurs (Castro-Lopes et al., 1990) presumably due to axonal withdrawal or degenerative changes (Knyihár-Csillik et al., 1987). To denote these morphological phenomena, the term transganglionic degeneration was introduced (Aldskogius et al., 1985; Grant et al., 1970).

In the 1990s Woolf and co-workers reported that crush or section of a peripheral nerve cause a long-lasting morphological reorganization of the central terminals of primary afferent neurons (Coggeshall et al., 1997; Lekan et al., 1996; Mannion et al., 1996; Woolf et al., 1992; Woolf et al., 1995). This observation has mainly been based on the utilization of the retrograde and transganglionic neuronal tracer CTB-HRP. As mentioned above, normally it is mainly taken up and transported retrogradely by large diameter myelinated A-fibres and labels the somatotopically related areas of the dorsal horn (laminae I and III-VI) (LaMotte et

al., 1991; Oszlács et al., 2015; Robertson and Grant, 1985; Shehab and Hughes, 2011; Trojanowski et al., 1982). However, 2 weeks after a peripheral nerve lesion CTB-HRP labelling was apparent not only in the deeper layers of the dorsal horn but also in lamina II, a region which normally receives only C-fibre input (Lekan et al., 1996; Shehab et al., 2004; Shortland et al., 1997; Woolf et al., 1992; Woolf et al., 1995). This marked (Bennett et al., 1996b) increase in CTB-labelled axon terminals of lamina II has been interpreted as a sprouting response of A fibres to peripheral nerve injury. This sprouting phenomenon was brought into connection with the degenerative changes occurring in C-fibre afferents following axotomy and with the subsequent loss of their synaptic connections with spinal interneurons (Woolf et al., 1992; Woolf et al., 1995). Woolf and his colleagues demonstrated that in the dorsal horn (lamina II) of axotomized animals 15 times more synaptic terminals were labelled by CTB-HRP 2 weeks after axotomy compared to their control side. They suggested that vacant synaptic sites on the spinal interneurons attract the Aβ terminals to sprout into lamina II and form new synapses. They also proposed that this central sprouting was initiated by an axotomy-induced conditioning of myelinated A-fibres (Woolf et al., 1992; Woolf et al., 1995).

Based on these findings Mannion and his colleagues studied whether C-fibre degeneration and A-fibre conditioning are both necessary for the sprouting of A-fibres into lamina II (Mannion et al., 1996). They took advantage of the perineural capsaicin treatment of the sciatic nerve which causes transganglionic degeneration exclusively within the C-fibre population of primary afferents (Jancsó, 1992; Jancsó and Lawson, 1990), without affecting the integrity of sciatic A-fibres. They have demonstrated that 2 weeks after capsaicin treatment of the sciatic nerve the pattern of CTB-HRP staining in the dorsal horn was identical with what was seen after axotomy. Based on these results they suggested that capsaicin-induced selective injury of C fibres alone was sufficient to evoke sprouting of A-fibre central terminals into the vacant synaptic sites in lamina II, even in the absence of the axotomy-induced conditioning affecting the same (sciatic) Aß-fibres.

1.8.2. The challenge of the sprouting hypothesis

The sprouting hypothesis seemed to gain considerable attention during the past years and it has been suggested to be an anatomical basis for the development of chronic pain states (Nakamura and Myers, 1999; Nakamura and Myers, 2000; Shortland et al., 1997; White, 2000). However, some observations suggested that the lesion-induced CTB labelling of the

substantia gelatinosa may be explained by other mechanisms that do not involve the sprouting of myelinated afferents. Moreover, this hypothesis has raised some problems e.g. the supposed sprouting response of myelinated afferents is a fast process, since the first signs of it occur 1 week after the injury and reaches its maximum in 2 weeks (Mannion et al., 1996; Woolf et al., 1992; Woolf et al., 1995). Based on earlier studies, this time interval seemed to be very short for such significant anatomic rearrangements of the neuronal circuits of the spinal cord dorsal horn (Nagy and Hunt, 1983). Besides, regarding the observation of Mannion et al. (Mannion et al., 1996) it seems less probable that the extent of the sprouting response is independent of the presence or absence of the injury of the peripheral axons of Aß-fibres. Axotomy indeed, influences the regenerative capacity of these neurons therefore it was plausible to assume its importance in the proposed central sprouting, which was finally not supported by their later observations.

In 1999, Tong and his colleagues demonstrated that CTB-HRP injection into a previously axotomized peripheral nerve causes increased labelling of not only large but also many small dorsal root ganglion cells (Tong et al., 1999). This finding indicated that the binding preference of CTB to different types of DRG neurons is affected by the axotomy, which can be explained by the injury-induced neurochemical changes in these neurons. They also concluded that the observed alterations in the binding specificity of CTB might have implications in the mechanism of the injury-induced central sprouting of Aß-fibres. Besides, further studies have revealed that only limited sprouting of myelinated afferents into peripheral laminae occurred after peripheral axotomy (Bao et al., 2002; Hughes et al., 2003; Shehab et al., 2003). Moreover, specific markers of injured unmyelinated primary afferents (e.g. VIP and galanin) were found to co-localize with choleragenoid in small sensory ganglions and their central terminals (Shehab et al., 2003). Histochemical and functional morphological studies have also reported that capsaicin-sensitive C-fibre ganglion neurons respond to peripheral nerve injuries with an increased uptake and transganglionic transport of CTB-HRP (Jancsó et al., 2002; Jancsó et al., 2004; Sántha and Jancsó, 2003; Shehab et al., 2003; Tong et al., 1999). Using the so called “capsaicin block paradigm” (Gamse et al., 1982; Jancsó et al., 1980) it became evident that perineural capsaicin treatment of the previously transected sciatic nerve prevented CTB-HRP labelling of lamina II as a result of selective blockade of axonal transport in C-fibres (Sántha and Jancsó, 2003). In accord with these findings electron microscopic observations have shown a marked significant increase in the proportion of CTB-HRP-labelled unmyelinated dorsal root axons providing the first direct evidence for an increased transganglionic transport of CTB-HRP by C-fibre afferents

following peripheral nerve section (Sántha and Jancsó, 2003). In the same study these authors suggested that a profound phenotypic change occurs in C-fibres due to sciatic nerve section which involves an increase in neuronal content of GM1 ganglioside, which is the high affinity binding site of choleragenoid.

1.9. The role of GM1 ganglioside in neuronal signalling processes

Gangliosides are major components of the cell membranes in vertebrates and they are more abundant in the nervous system (Nagai and Iwamori, 1995). Membranes of neurons, especially of ganglion cells are rich in gangliosides which have a significant role in the regulation of many cellular events including neuronal differentiation, growth and regeneration. Gangliosides are glycosphingolipids containing one or more sialic acid residues in their carbohydrate chain. Being amphipathic molecules, glycosphyngolipids are composed of a hydrophilic carbohydrate chain and a hydrophobic ceramide moiety that contains a sphingosine and a fatty acid residue (Hakomori, 1990; Yu et al., 2007). In general, biosynthesis of gangliosides starts with ceramide which is synthesized in the endoplasmatic reticulum. The key step of the synthesis is the glucosylation of ceramide by glucosylceramide synthase (GCS; also called uridyl glucose-ceramide glucosyl transferase, UGCG), which takes place in the Golgi apparatus (Hanada et al., 2011). Thereafter, gangliosides are built up by the stepwise addition of carbohydrate units to glucosylceramid, catalyzed by membrane bound glycosyltransferases (Caputto et al., 1974; Keenan et al., 1974; Roseman, 1970; van Echten and Sandhoff, 1993). About 200 gangliosides are known today, differing in their carbohydrate components (Yu et al., 2007). The major gangliosides in the brain are GM1, GD1a, GD1b and GT1b (Ferrari et al., 1983).

The selective binding of CTB to the GM1 ganglioside is well established (Cuatrecasas, 1973; Holmgren et al., 1973; Robertson and Grant, 1989; Stoeckel et al., 1977). Although recent studies challenged the specificity of CTB-HRP to retrogradely label A-type primary afferent neurons under pathological conditions, CTB is still regarded as a useful tracer for demonstration of central projections of sensory neurons. Further studies have demonstrated that gangliosides, including GM1, are major components of cholesterol-rich membrane microdomains which are generally referred to as lipid rafts (Matkó and Szöllősi, 2005; Simons and Ikonen, 1997). According to a recent consensus, lipid rafts are small, heterogenous, highly dynamic, sterol- and sphingolipid-enriched domains that

compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006).

Lipid rafts and gangliosides (e.g. GM1) play a significant role in neuronal signaling processes, including mediation of the action of growth factors. It has been reported that *in vivo* exogenous gangliosides facilitate regeneration of damaged peripheral nerves (Ceccarelli et al., 1976), spinal cord (Geisler et al., 1991) and brain (Sabel et al., 1988; Schengrund, 1990; Skaper et al., 1989). Addition of gangliosides to cultured dorsal root ganglion neurons has been found to promote neurite outgrowth (Leon et al., 1984; Roisen et al., 1981). These studies suggested that neuronal gangliosides play a role as mediator molecules in trophic interactions. Indeed, GM1 ganglioside is known to be a mediator of the effects of NGF (Farooqui et al., 1997; Ferrari et al., 1983; Leon et al., 1984; Mutoh et al., 1998; Rabin and Mocchetti, 1995; Schwartz and Spirman, 1982). NGF is a critical trophic factor required during development for the growth and survival of sympathetic and sensory neurons and neurons in the central nervous system (Levi-Montalcini and Angeletti, 1968; Ritter et al., 1991). In adult animals, however, NGF is not necessary for neuronal survival (Lindsay, 1988), although it may still have a trophic role in maintaining the homeostasis of neurons or in axonal sprouting after nerve/tissue damage (Diamond et al., 1992; Verge et al., 1990). The trophic actions of NGF are largely attributed to the activation of trkA that is expressed on peripheral and central neurons. The activated trkA receptors bind to and induce the tyrosine phosphorylation and activation of several intracellular signaling proteins leading to inhibition of cell division and stimulation of neuritogenesis. Studies concerned with the connection between the trophic action of NGF and GM1 ganglioside have revealed that GM1 augments NGF-mediated dimerization of trkA monomers in trk-PC12 cells supporting the hypothesis that GM1 exerts its neurotrophic effects by facilitating the dimerization and activation of trkA (Berg et al., 1992; Farooqui et al., 1997; Ferrari et al., 1995; Ferrari et al., 1992; Mutoh et al., 1993; Mutoh et al., 1998; Rabin and Mocchetti, 1995). In accordance with these findings, Mutoh and his colleagues have demonstrated that inhibition of ganglioside synthesis by a selective inhibitor of glucosylceramide synthase (GCS) i.e. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) reduced the NGF-induced neurite outgrowth in PC12 cells (Mutoh et al., 1998). The effect of the D-PDMP-induced ganglioside depletion was reversed by supplementation of the cell cultures with GM1, further demonstrating the dependence of NGF signaling on this specific ganglioside (Mutoh et al., 1998). Biochemical analysis also revealed co-immunoprecipitation of trkA and GM1 which

strongly suggests the compartmentalization of the NGF receptor into the membrane lipid rafts.

NGF has also a significant role in the regulation of the expression of nociceptive specific ion channels and neuropeptides (Aguayo and White, 1992; Bevan and Winter, 1995; Kessler and Black, 1980; Lindsay et al., 1989; Winter et al., 1988) in primary sensory neurons. Deprivation of NGF resulted in the elimination of neuronal capsaicin sensitivity of cultured DRG neurons (Sántha et al., 2010; Winter et al., 1988). Moreover, capsaicin sensitivity (Jancsó, 1992; Jancsó and Ambrus, 1994; Jancsó et al., 1997) and TRPV1 expression (Michael and Priestley, 1999; Szigeti et al., 2012) are also reduced following nerve transection, which disturbs the supply of peripheral neurotrophic factors.

2. AIMS OF THE STUDY

The principal aim of the present thesis was the study of the capsaicin-induced structural and chemical plasticity of nociceptive primary afferent neurons with special emphasis on changes in neural ganglioside metabolism. Our observations revealing marked changes in GM1 ganglioside level of small DRG neurons following perineural treatment with capsaicin prompted us to initiate further *in vitro* experiments concerning the possible role of gangliosides in nociceptor functions. First, we aimed to confirm and re-evaluate previous findings suggesting an increased transport of CTB-HRP, in myelinated primary afferents, into the superficial layers of the spinal dorsal horn following perineural capsaicin treatment of the rat sciatic nerve. Based on the observations gained from studies in our laboratory on phenotypic changes of axotomized C-fibre primary sensory neurons regarding their CTB transport capacity, we presumed that mechanisms other than A-fibre sprouting may be implicated in the development of structural changes observed after perineural treatment with capsaicin. Since besides capsaicin, its ultrapotent analogue, RTX has been increasingly considered for human therapeutic purposes, we also planned experiments using this vanilloid compound. To further characterize and identify the types of primary sensory neurons which take up and transport CTB-HRP, the size-frequency distribution of labelled and unlabelled DRG neurons were also determined. To demonstrate the axonal transport capacity of C-fibre dorsal root axons affected by perineural capsaicin treatment, the transganglionic transport of wheat germ agglutinin-HRP conjugate (WGA-HRP), a C-fibre specific neuronal tracer was also investigated. In order to provide direct evidence for the identity of dorsal root fibres which transport CTB-HRP in intact and nerve injured animals, the localization of CTB-HRP was studied at the ultrastructural level by applying electron microscopic histochemical and quantitative morphological techniques. The recognition that the GM1 membrane ganglioside is implicated in the mediation of the cellular actions of nerve growth factor (NGF) and it is also a major component of membrane microdomains or lipid rafts, prompted us to initiate further studies to explore the possible role of gangliosides in the functions of nociceptors. Therefore, we studied the effects of the depletion of neural gangliosides on the capsaicin sensitivity, CTB-binding, NGF-mediated acute sensitization, neurite outgrowth and TRPV1 expression of DRG neurons induced by the selective inhibition of glucosyl ceramide synthase (GCS), the key enzyme of neuronal ganglioside synthesis.

3. MATERIALS AND METHODS

Adult male Wistar rats weighing 250-300 g were used for the experiments. The rats were housed with a 12 h light/dark cycle. Food and water were available ad libitum. The protocol for this study was approved by the Ethical Committee on Animal Experiments of the University of Szeged. All efforts were made to minimize the number of animals and their suffering.

3.1. Surgical procedures

3.1.1. Perineural capsaicin treatment

Animals (n=9) were anaesthetized with chloral-hydrate (400 mg/kg, i.p.) and the right sciatic nerve was exposed at mid thigh level under aseptic conditions. A plastic film was placed underneath the nerve to isolate it from the surrounding tissues and a small piece of gelfoam moistened with 0.1 mL of 1 % solution of capsaicin was put around the right nerve. On the left side the same procedure was performed except that the gelfoam was soaked in the solvent of capsaicin (6% ethanol and 8% Tween 80 in saline). After the wound had been closed and the rats had recovered from the anaesthesia, they were returned to the animal house.

3.1.2. Perineural RTX treatment

In this group of animals (n=4) the surgery was performed as described above except that a gelfoam previously soaked in 0.1 mL of 0.005% solution of RTX was wrapped around the right sciatic nerve. On the left side, the nerve was treated with the solvent of RTX (1 mg/mL absolute ethanol in 0.9% NaCl and 0.3% Tween 80).

3.1.3. Intraneural injection of CTB-HRP or WGA-HRP

In order to label the sensory fibres located in the sciatic nerves 2 weeks after the perineural capsaicin or RTX treatment rats were anaesthetized with chloral-hydrate and 1 μL 1.5% solution of CTB-HRP (n=7) or WGA-HRP (n=2) was injected into both the left and the right sciatic nerves by using a Hamilton microsyringe. The tracer was injected slowly into the nerve distal to the site of the previous capsaicin treatment. The wound was closed and the rats

were returned to the animal house. For rats treated with RTX the same procedure was applied 2 weeks after the surgery.

3.2. Histological procedures

3.2.1. Tissue preparations and sectioning

In order to collect tissue samples for further histological analysis the animals were terminally anaesthetised 3 days after the injection of the tracers (CTB-HRP or WGA-HRP) and were perfused transcardially with a fixative containing 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). This was immediately followed by 400 mL of 4°C phosphate buffer containing 30% sucrose. The L4 and L5 dorsal root ganglia and the lumbar spinal cords were removed and stored in the sucrose-buffer solution at 4°C until sectioning. Serial frozen sections of dorsal root ganglia (15 μm in thickness) and spinal cords (20 and 60 μm in thickness) were cut on a cryostat and a freezing microtome, respectively.

3.2.2. Histochemical demonstration of peroxidase enzyme activity

To demonstrate the distribution of transported tracer molecules in different tissues, peroxidase activity was detected according to Mesulam (1978). The ganglia and spinal cord sections were pre-incubated for 20 minute in a solution containing sodium nitroprussid (100 mg/100 mL) and 3,3',5,5'-tetramethylbenzidine (TMB; 5 mg/100 mL) dissolved in absolute ethanol (final concentration was 0.2%) and diluted with acetate buffer, (0.1 N; pH 3.3). Then the incubation solution was replaced with fresh solution supplemented with hydrogen peroxide (H_2O_2; final concentration was 0.3%). The development of the reaction product was observed with a low magnification microscope and the reaction was stopped by transfer of the sections into diluted acetic acid solution (5%). Spinal cord sections were drawn up from alcoholic solution of gelatin to gelatin-covered slides, let dry for 24 hours, dehydrated in upstream dilutions of ethanol, cleared through xylene and mounted by di-n-butyle phthalate in xylene (DPX) mounting medium. Before mounting, sections of dorsal root ganglia were counterstained with neutral red.

3.2.3. Demonstration of thiamine monophosphatase (TMP) activity

TMP activity was detected by using a modified method originally described by Colmant (1959) on both ganglia and spinal cord sections. This staining method is based on the

Gömöri acid phosphatase technique. The incubation solution contains 0.3% thiamine monophosphate, 20 mL 0.2% lead nitrate and 5.5% sucrose in Tris maleate buffer (pH 5.2-5.4). During incubation, sections were kept at 37°C for 90 minutes. The TMP activity produced insoluble lead phosphate ions. After a brief wash, the colourless reaction product was detected by staining of the sections with 5% potassium-bichromate for 3-5 minutes. The sections were then washed again, dehydrated in increasing concentrations of ethanol, cleared with xylene and mounted by DPX. Before mounting, sections of dorsal root ganglia were counterstained with cresyl violet.

3.2.4. Electron microscopic histochemistry

Electron microscopic histochemistry was performed to identify the dorsal root afferents which transport CTB-HRP 2 weeks after perineural treatment of the sciatic nerve with capsaicin. In 3 animals, the L5 dorsal roots were ligated 12 hours after the injection of CTB-HRP into the intact and capsaicin-treated nerves. Thirty-six hours later, the animals were perfused as described above and the L5 dorsal roots were removed. To prepare the specimen for histochemistry 80 μm thick vibratome sections were cut from the L5 dorsal root segments located just distal to the site of ligation. The peroxidase activity was demonstrated by using a sensitive detection method utilizing TMB as a chromogene and sodium tungstate as a stabilizer (Gu et al., 1992). The samples were then reacted with a diaminobenzidine and cobalt chloride containing solution, developed with H_2O_2, post-fixed with osmium tetroxide, dehydrated and embedded in Araldite. Ultrathin sections were cut on an Ultracut ultramicrotome, contrasted with uranyl acetate and lead citrate and viewed under a Jeol Jem 1010 electron microscope. Dorsal root axons were counted on-screen in randomly selected sections.

3.2.5. Data analysis

Cross-sectional areas of CTB-HRP-labelled and unlabelled DRG neurons with clear-cut nuclei (minimum 200/group) were measured in representative serial sections of L4 and L5 dorsal root ganglia by means of a light microscope (under 40x magnification) equipped with a camera lucida and a digitizing tablet connected to a computerized system. The averages of the cross-sectional areas of total and labelled populations were determined. Size-frequency distribution histograms were generated by the SigmaPlot 8.0 program. Based on previous

analysis (Soares et al., 2002) neurons having a cross sectional area < 600 μm^2 were classified as small-type cells.

To compare the size distributions of different DRG neuron populations the size-frequency distribution histograms were generated as described above. The mean values of neuronal cross-sectional areas and the relative proportions of the different sub-populations were expressed as means ± S.E.M. Parametric statistical analyses were performed by using the Student's *t*-test and one-way ANOVA of the Statistica program. For *post hoc* comparisons Dunnett's test was applied and $p < 0.05$ was regarded as a statistically significant difference between groups.

3.3. Preparation of cultures of DRG neurons

Adult male Wistar rats (n=24) weighing 250-300 g were deeply anaesthetized with ether, decapitated and the spinal columns were removed under aseptic conditions. Primary neuron cultures from DRGs were prepared as described by Winter et al. with slight modifications (Winter et al., 1988). The spinal cords were exposed and C1-L6 ganglia were then removed and collected in a culture medium (Dulbecco's Modified Eagle's Medium/Nutrient mixture F-12 HAM) supplemented with 1 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and 4% Ultroser G. The connective tissue was digested with collagenase type IV (2000 U/mL) for 3 hours at 37°C in a humidified atmosphere containing 5% CO_2. The ganglia were then washed 3 times with F-12 HAM medium and dissociated by trituration through a fire-polished Pasteur pipette, spun through 15% bovine serum albumin, and the pellet was resuspended in the culture medium. The concentration of the neurons was measured by using Bürker's counting chamber. DRG cells (1600 per well) were plated on laminin and poly-DL-ornithine-coated glass coverslips and kept at 37°C in a 5% CO_2 atmosphere for 4 hours. Thereafter, the medium was replaced with fresh medium, containing 50 ng/mL NGF.

3.3.1. D-PDMP treatment of primary cell cultures

Cultures were incubated with different concentrations (10, 20 or 50 μM) of D-PDMP a selective inhibitor of glucosylceramide synthase, or its vehicle for 4 days at 37°C in a 5% CO_2 atmosphere. In other experiments the cultures were exposed to D-PDMP (20 or 50 μM) for 4 days and then further maintained in fresh medium without D-PDMP for another 4 days. A stock solution of D-PDMP was prepared with the aid of ethanol in saline and further diluted

with the culture medium to obtain final concentrations of 20 or 50 μM D-PDMP and 0.05% ethanol.

3.3.2. Demonstration of CTB binding of cultured DRG neurons

Control and D-PDMP treated DRG cultures were incubated with fluorescein isothiocyanate-conjugated CTB (FITC-CTB; 10μg/mL) for 10 minutes at 37°C, whereafter they were washed with fresh medium and fixed with 10% buffered formalin for 10 minutes (Mutoh et al., 1998). After washing, the cultures were mounted with ProlongGold on glass slides and examined with a CARV II spinning disc confocal fluorescence imaging system. Z-stack image series were collected from 50 neurons of each treatment group by using a 40x magnification oil immersion objective. The relative fluorescence intensity over the cell membrane was determined on confocal images by using the line profile measurement tool of the ImagePro Plus 6 image processing and analysis software. The relative intensity values were compared and percentage changes were calculated. Data are expressed as means ± S.E.M.

3.3.3. Demonstration of capsaicin-sensitive DRG neurons with the cobalt uptake assay

Cultured DRG cells attached to cover slips were washed in buffer solution (57.5 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mg HEPES, 12 mM glucose and 139 mM sucrose; pH 7.4) twice for 2 minutes at room temperature and then incubated for 5 minutes at 37°C in the same buffer to which 5 mM $CoCl_2$ and 1 μM capsaicin were added. In control experiments, the cells were incubated without capsaicin for 5 minutes. After the incubation of the cobalt or cobalt and capsaicin containing buffer, the cover slips were quickly washed twice in cobalt-free buffer and transferred into the buffer containing 2% mercaptoethanol, which produced a brownish precipitation in the neurons loaded with cobalt. After 2 minutes staining, the cells were fixed in 70% ethanol, dehydrated in absolute ethanol, cleared in xylene and mounted on glass slides by using DPX mounting medium.

3.3.4. Demonstration of the acute sensitizing effect of NGF on cultured DRG neurons

Control and D-PDMP-treated (20 μM for 4 days) cultured DRG cells were exposed to NGF (200 ng/mL) for 10 min prior to the challenge with capsaicin (1 μM). Capsaicin-induced activation of nociceptive DRG neurons was detected with the cobalt uptake technique as

described above. The number of neurons was counted in ten randomly selected microscopic fields by using a 20x objective and the proportions of cobalt-labelled neurons were calculated. Data are given as means ± S.E.M.

3.3.5. TRPV1 immunohistochemistry

Cells attached to cover slips were fixed with 4% formaldehyde in 0.1M phosphate buffer (pH 7.4) for 10 minutes and then washed twice in phosphate-buffered (0.01M) isotonic NaCl solution (phosphate-buffered saline, PBS). Rabbit polyclonal anti-TRPV1 primary antibody (1:1500) were dissolved in a PBS buffer containing 0.3% Triton X-100 and applied to the fixed cultures overnight at 4°C. After rinsing the cover slips three times in PBS, the Cy3-conjugated donkey anti-rabbit IgG secondary antibody (1:1500) was dissolved in PBS-Triton buffer supplemented with 3% normal donkey serum. After incubations for 2h, the cover slips were rinsed for 3 x 5 minutes in PBS. For fluorescence microscopy, the specimens were mounted with Vectashield mounting medium.

3.3.6. Measurement of the total neurite length of individual DRG neurons

The neurite lengths of DRG neurons were measured in cultures stained with a monoclonal mouse anti-neurofilament 200 kDa antibody. Microphotographs were taken of individual DRG neurons (of 2- or 4-day cultures) with clearly identifiable axonal arborization from vehicle or D-PDMP-treated (20 or 50 μM) cultures and were processed and evaluated by using the ImagePro Plus image analysis software. Briefly, after adjustment of the brightness and contrast of the grayscale images, the total neurite arborization was demarcated by using the segmentation tool and manual thresholding and cut out from the original image. The resulting binary image was further processed by using the thinning morphological filter menu until the neural processes were skeletonized to obtain single pixel lines representing the original neurites. The total length of the arborization was determined automatically by using the length measurement tool (perimeter length) of the program. Fifty neurons in each treatment group were analyzed. Data are expressed in μm and given as means ± S.E.M.

3.3.7. Data analysis

DRG cultures subjected to the cobalt uptake assay and to TRPV1 immunohistochemistry were visualized by using a 20x objective of a Leica DMLB

microscope equipped with a Retiga 2000R digital camera connected to a computer running the ImagePro Plus 6 analysis software. Random serial images were captured from each culture and at least 150-200 neurons/culture were measured. Cobalt-labelled, TRPV1-labelled and unlabelled neurons were identified on screen and their sizes and optical densities were measured with the image analysis software. The relative proportions of the cobalt-labelled, and immunopositive neurons were calculated, and cell-size distribution histograms were generated. Data were expressed as means ± S.E.M.

Effects of capsaicin and D-PDMP treatment on the proportions and areas of the different populations of cultured DRG neurons were studied by one-way ANOVA. For *post hoc* comparisons between the different groups, Dunnett's test was applied. Differences were regarded as significant at $p < 0.05$.

4. RESULTS

4.1. Effects of perineural capsaicin treatment on the distribution of primary afferent terminals in the spinal dorsal horn

4.1.1. Changes in the CTB-HRP labelling

CTB-HRP is a neuronal tract tracer, which is preferentially taken up and transported by myelinated primary afferents in intact rats (Robertson and Grant, 1985). Similarly to previous observations, injection of CTB-HRP into the left, vehicle-treated sciatic nerve resulted in a transganglionic labelling of the deeper layers of the somatotopically related regions of the dorsal horn of the lumbar spinal cord (Mannion et al., 1996; Sántha and Jancsó, 2003; Woolf et al., 1992; Woolf et al., 1995). Injection of CTB-HRP into the (right) sciatic nerve 2 weeks after perineural treatment with capsaicin resulted in a marked labelling not only of the deeper layers of the spinal dorsal horn, but also of the marginal zone and the substantia gelatinosa (**Fig. 1**).

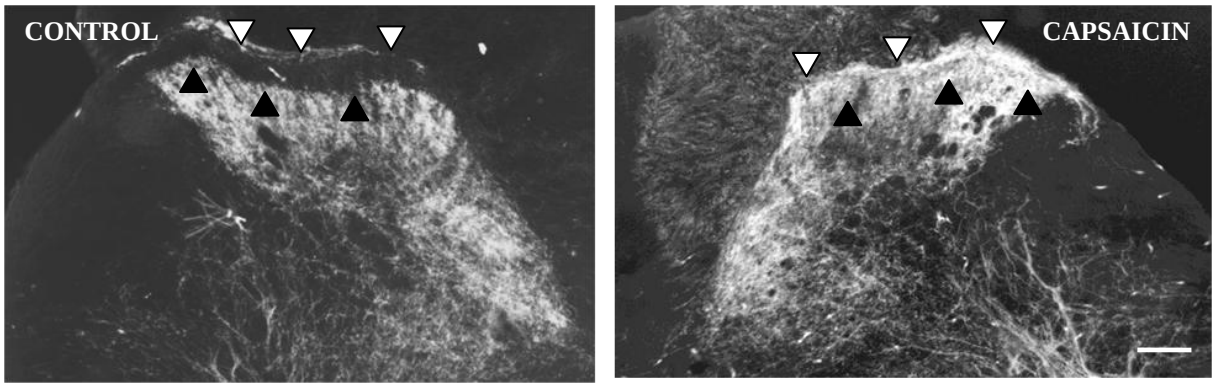

Figure 1. Photomicrographs illustrating the spinal distribution of CTB-HRP-labelled primary afferent fibres following perineural capsaicin treatment of the right sciatic nerve. Capsaicin was applied 2 weeks before the CTB-HRP injection. Scale bar indicates 50 μm and applies for both photomicrographs.

4.1.2. Changes in WGA-HRP labelling

In agreement with previous reports (LaMotte et al., 1991), intraneural injection of WGA-HRP, a selective marker of unmyelinated, C-fibre afferents, into an intact peripheral nerve, resulted in a selective labelling of the most superficial layers of the spinal dorsal horn. The reaction product formed a continuous band in the marginal zone and the substantia gelatinosa in spinal cord segments relating to the injected nerve. Injection of WGA-HRP into a nerve which was treated perineurally with capsaicin 2 weeks earlier resulted in an

essentially similar labelling of the somatotopically relating areas of the marginal zone and the substantia gelatinosa (**Fig. 2**).

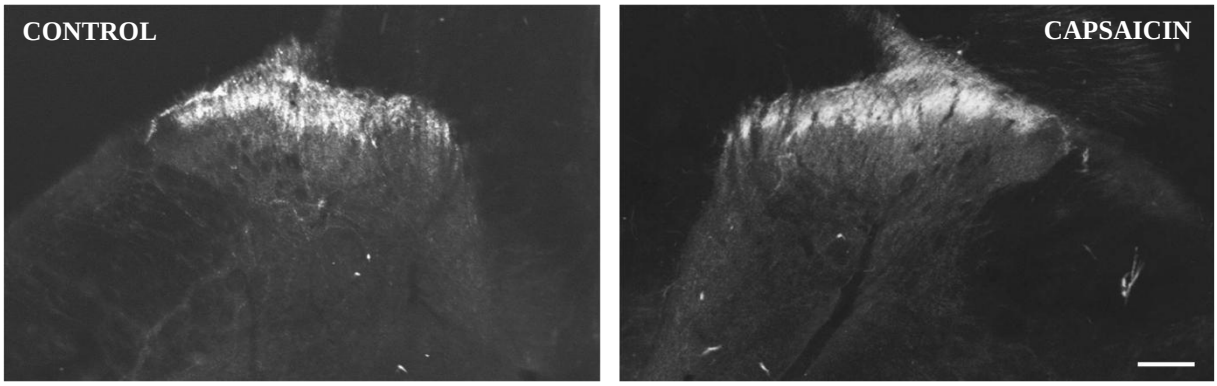

Figure 2. Spinal distribution of primary afferent fibres which transport WGA-HRP 2 weeks after perineural treatment of the sciatic nerve with capsaicin. Scale bar indicates 50 μm and applies for both photomicrographs.

4.1.3. Changes in TMP activity

TMP which is present in about 30-50% of the DRG neurons of rodents (in particular in small-diameter cells with unmyelinated axons), is known as a marker of small primary afferent neurons (Colmant, 1959; Inomata and Ogawa, 1979; Knyihár-Csillik et al., 1986). On the control side the enzyme activity was present in the substantia gelatinosa of the spinal dorsal horn. Two weeks after perineural capsaicin treatment of the right sciatic nerve, a marked reduction of TMP activity in the medial 2/3 of the substantia gelatinosa was observed in the L4/5 spinal cord segments. This region corresponds to the termination site of sciatic C-fibre afferents somatotopically relating to the capsaicin treated sciatic nerve (**Fig. 3**).

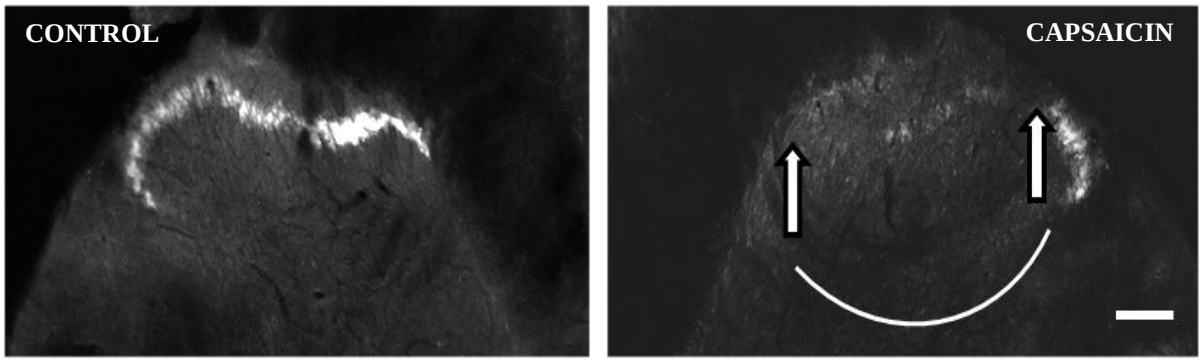

Figure 3. Spinal distribution of primary afferent terminals showing TMP activity 2 weeks after perineural capsaicin treatment of the sciatic nerve. Arrows indicate the decreased TMP activity in the substantia gelatinosa. Scale bar denotes 50 μm and applies for both microphotographs.

4.2. Effects of perineural capsaicin treatment on the CTB-HRP labelling of L5 DRG neurons

The retrograde labelling of the parent cell bodies by CTB-HRP in the L5 DRGs was demonstrated with the histochemical detection of peroxidase activity (**Fig. 4**). In the L5 DRGs many neurons showed fine granular perikaryal peroxidase staining. In the ganglia relating to the vehicle-treated nerves, mainly the large and medium-sized neurons were labelled. In contrast, in ganglia relating to the capsaicin-treated nerve, many small neurons were labelled which amounted 66.41 ± 0.64 per cent of the total neuronal population (**Fig. 4**). Size-frequency distribution histograms revealed an obvious increase in the proportion of labelled small neurons in DRGs relating to the treated nerve as compared with DRGs relating to the control nerve (**Fig. 5**). This was also evident from comparisons of the cross-sectional areas of labelled neurons: in ganglia relating to the control, vehicle-treated nerves the mean area of labelled cells was 916.16 ± 40.07 μm^2, whereas ipsilateral to the capsaicin-treated nerves the mean area of labelled cells was significantly smaller (698.16 ± 27.59 μm^2; n=4, $p<0.05$, **Fig. 8**). There was no difference in the mean cross-sectional area of the total neuronal populations (control: 657.6 ± 26 μm^2 vs. capsaicin treated: 652.02 ± 48 μm^2). The proportion of labelled nerve cells was 40.71 ± 2.21% in the control ganglia, while it significantly increased in ganglia relating to the capsaicin treated nerve (63.44 ± 1.17%; n=4, $p<0.05$). It has been demonstrated that the proportion of small, CTB-HRP-positive neurons with a cross-sectional area <600 μm^2 was also significantly increased: in the control ganglia it was amounted to 45.35 ± 1.58%, while in the DRGs of the treated side it increased to 66.41 ± 0.64% (n=4, $p < 0.05$, **Fig. 9**).

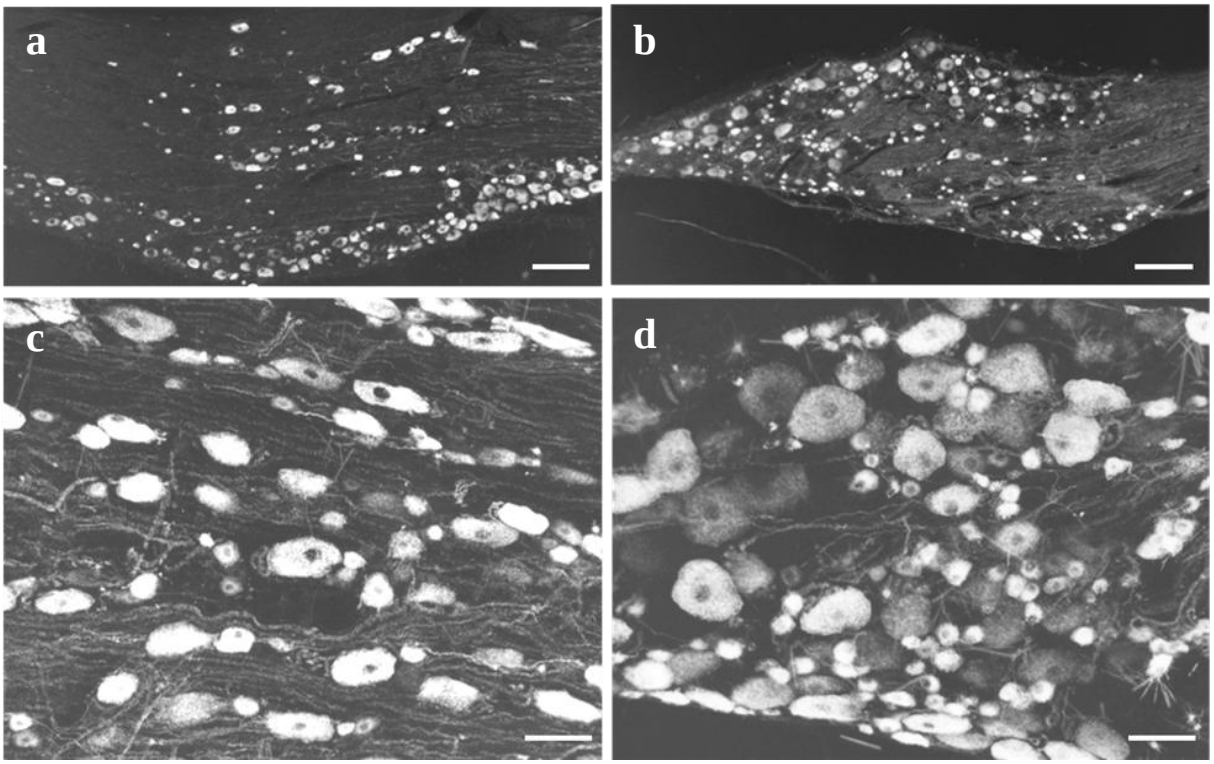

Figure 4. Inverse photomicrographs of labelled neurons in L5 dorsal root ganglia following the injection of CTB-HRP into intact (**a, c**) and capsaicin- treated (**b, d**) sciatic nerves of a rat. Note the marked increase in the number of labelled small neurons after capsaicin treatment. The scale bar indicates 200 μm in **a, b** and 50 μm in **c, d**.

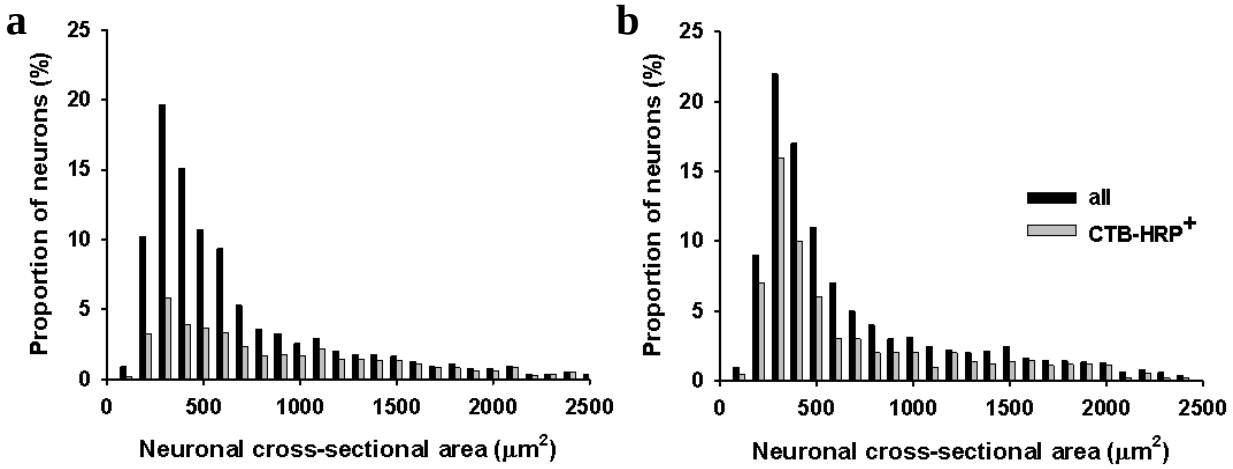

Figure 5. Effects of vehicle (**a**) and perineural capsaicin (**b**) treatment on the size-frequency distribution of CTB-HRP-labelled neuronal populations in L5 DRGs 2 weeks after capsaicin treatment.

4.3. Effects of perineural RTX treatment on the labelling of spinal ganglion cells and spinal primary afferents with CTB-HRP

4.3.1. Changes in the CTB-HRP labelling of the lumbar spinal dorsal horn after RTX treatment

Perineural application of RTX, which is an ultrapotent analogue of capsaicin, produced a spinal CTB-HRP labelling pattern similar to that seen after perineural capsaicin. Intraneural injection of this tracer 2 weeks after perineural treatment of the sciatic nerve with RTX resulted in a strong somatotopically appropriate (transganglionic) labelling of primary afferent fibres terminating in the most superficial layers of the spinal dorsal horn, in particular the substantia gelatinosa. In contrast, the superficial layers on the vehicle-treated side remained free of labelling (**Fig. 6**).

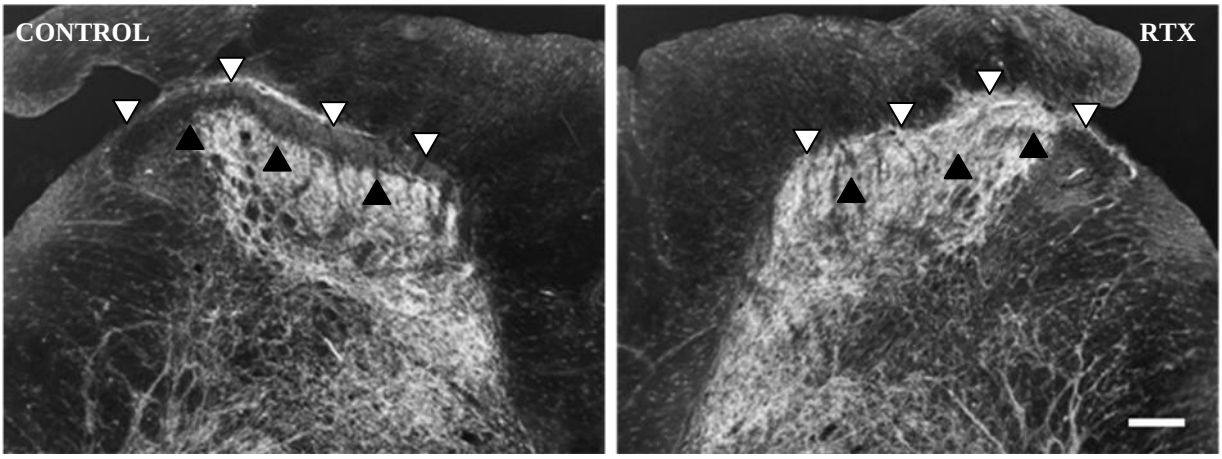

Figure 6. Inverse photomicrographs illustrating the effect of perineural treatment with resiniferatoxin on the distribution of labelled primary afferents in the 4th lumbar spinal cord segment of the rat. CTB-HRP was injected into the intact sciatic nerve (control) and into the contralateral sciatic nerve treated perineurally with resiniferatoxin 2 weeks previously (RTX). Note the massive labelling of the substantia gelatinosa (marked by arrowheads) following treatment with resiniferatoxin. The scale bar indicates 50 μm and holds for both microphotographs.

4.3.2. CTB-HRP labelling of L5 DRG neurons after RTX treatment

The size-frequency distribution histogram of L5 DRG neurons revealed that perineurally applied RTX markedly and significantly increased the proportion of CTB-HRP-labelled small cells (**Fig. 7**). The proportion of labelled neurons increased from 40.71 ± 2.21% to 54.29 ± 3.21% (n=4; $p < 0.05$) in the ganglia related to the treated sciatic nerve 2 weeks after the treatment. Moreover, RTX treatment resulted in an increase in the proportion of labelled small neurons with cross-sectional area <600 μm^2 (control: 45.35 ± 1.58%, RTX:

65.62 ± 1.03%; p <0.05, n=4) (**Fig. 9**). Similarly to the effects of the capsaicin treatment, the mean cross-sectional area of CTB-HRP-labelled L5 DRG neurons decreased from 916.16 ± 40.07 μm^2 (vehicle-treated) to 673.57 ± 43.44 μm^2 (RTX-treated; p <0.05, n=4, **Fig. 8**). There was no difference in the mean cross sectional area of the total neuronal population (control: 657.6 ± 26 μm^2, RTX: 657.78 ± 57 μm^2).

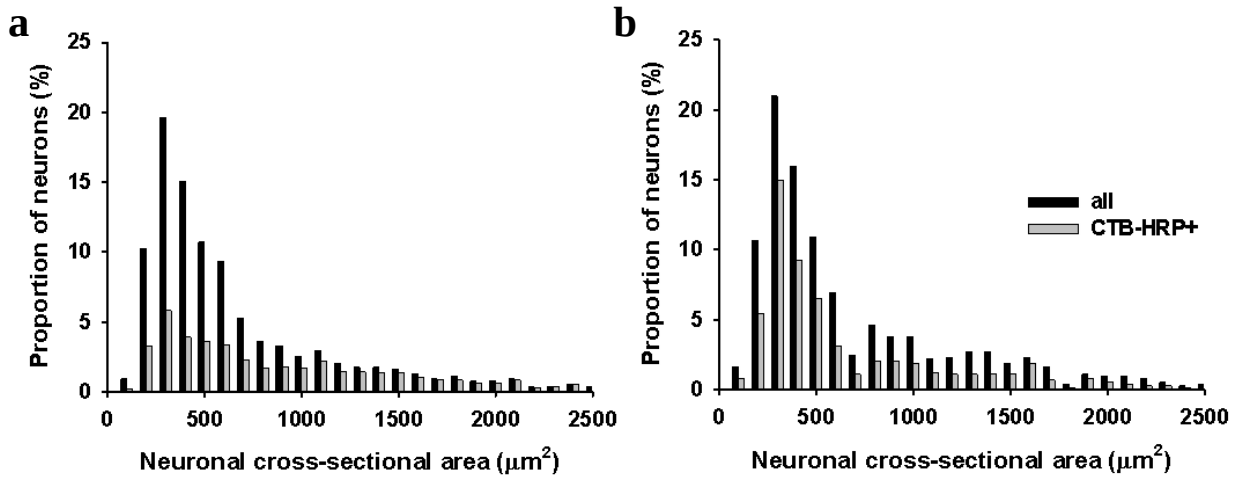

Figure 7. Effects of vehicle (**a**) and perineural RTX (**b**) treatment on the size-frequency distribution of CTB-HRP-labelled neuronal populations in L5 DRGs 2 weeks after RTX treatment.

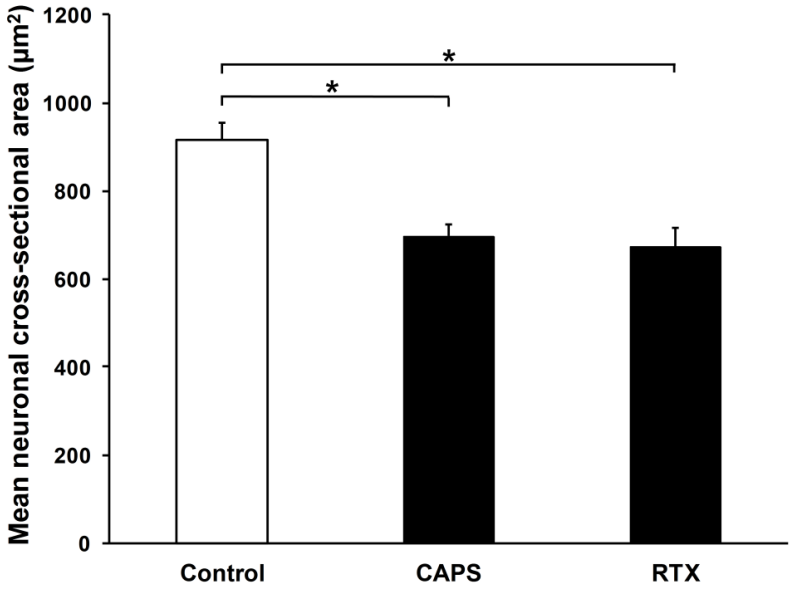

Figure 8. The effects of perineural treatment with capsaicin and RTX on the mean cross-sectional area of CTB-HRP-labelled L5 DRG neurons 2 weeks after perineural application of capsaicin or RTX (mean ± S.E.M., n=4, * p < 0.05).

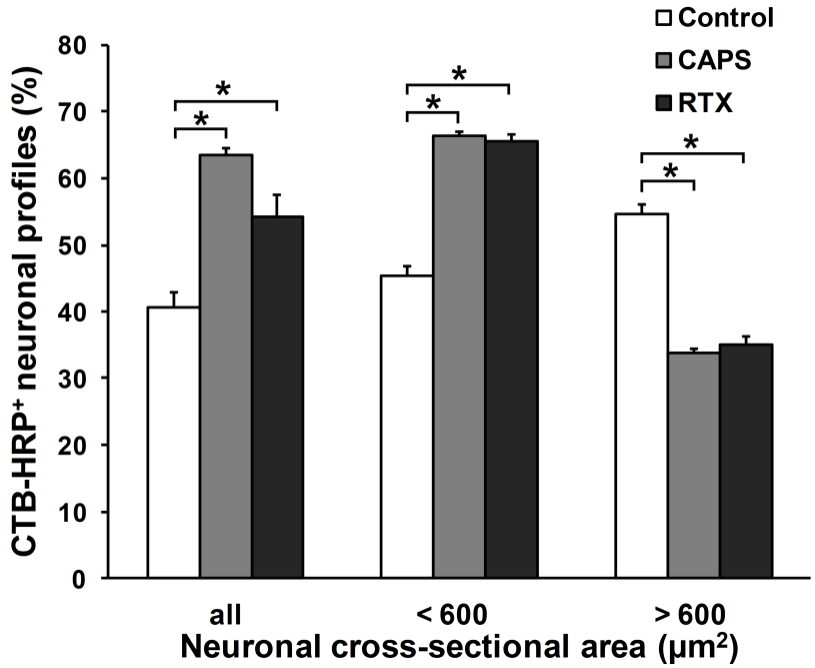

Figure 9. The effects of perineural treatment with capsaicin and RTX on the mean neuronal cross sectional area of different populations of L5 DRG neurons 2 weeks after treatment (mean ± S.E.M., * $p < 0.05$).

4.4. Ultrastructural localization of CTB-HRP in spinal dorsal roots relating to intact and capsaicin-treated peripheral nerves

Electron microscopic examination of the intact L5 spinal dorsal roots revealed that many myelinated fibres (50.60 ± 6%), but very few unmyelinated fibres (3.00 ± 1%) showed CTB-HRP positivity (**Fig. 10**). The electron dense peroxidase reaction product indicating the presence of CTB-HRP was localised either diffusely in the axoplasm and/or associated with the axolemma (**Fig. 10**). In contrast, 2 weeks after perineural capsaicin treatment, the proportion of CTB-HRP-positive unmyelinated axons increased significantly to 47.80 ± 6%, in the L5 spinal dorsal roots relating to the capsaicin-treated nerve, whereas the proportion of labelled myelinated fibres (57.20 ± 6.5%) was similar to the control.

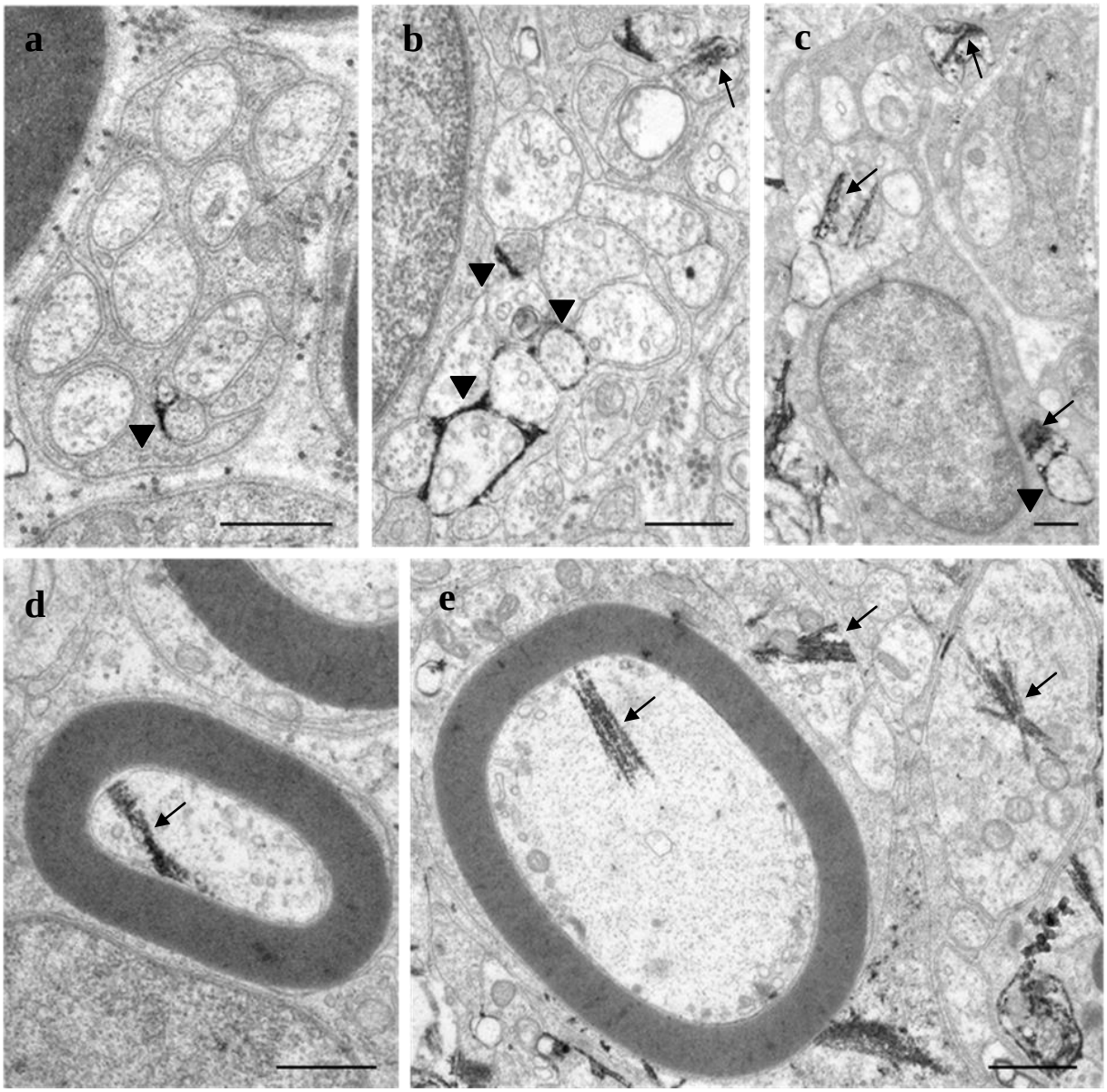

Figure 10. Electron micrographs illustrating the localization of CTB-HRP in myelinated and unmyelinated dorsal root axons relating to the intact (**a**, **d**) and capsaicin treated (**b**, **c**, **e**) sciatic nerves. The electron dense product of the peroxidase reaction is either finely distributed and associated with the axolemma (arrowheads) and/or localized as amorphous material within the axoplasm (arrows). The scale bars indicate 0.5 μm in all figures.

4.5. Effects of D-PDMP treatment on the capsaicin sensitivity of cultured DRG neurons

4.5.1. Effect of D-PDMP on the CTB binding of DRG neurons

To demonstrate the effects of D-PDMP treatment on the neuronal level of GM1 ganglioside, the binding of CTB-FITC was examined. In DRG neurons cultured for 2-4 days, the neuronal plasma membrane showed intense CTB-FITC labelling whereas the perikarya exhibited various staining intensities. However, pretreatment with D-PDMP resulted in a

dose- and time-dependent decrease in CTB-FITC binding (**Fig. 11**). The quantitative data demonstrate that, as compared with the controls, the relative fluorescence intensity of the cell membrane in labelled neurons displayed significant reductions already after a 2-day exposure of the cultures to D-PDMP, reaching maximum reductions of about 60% after 4 days of treatment (**Fig. 11 d**).

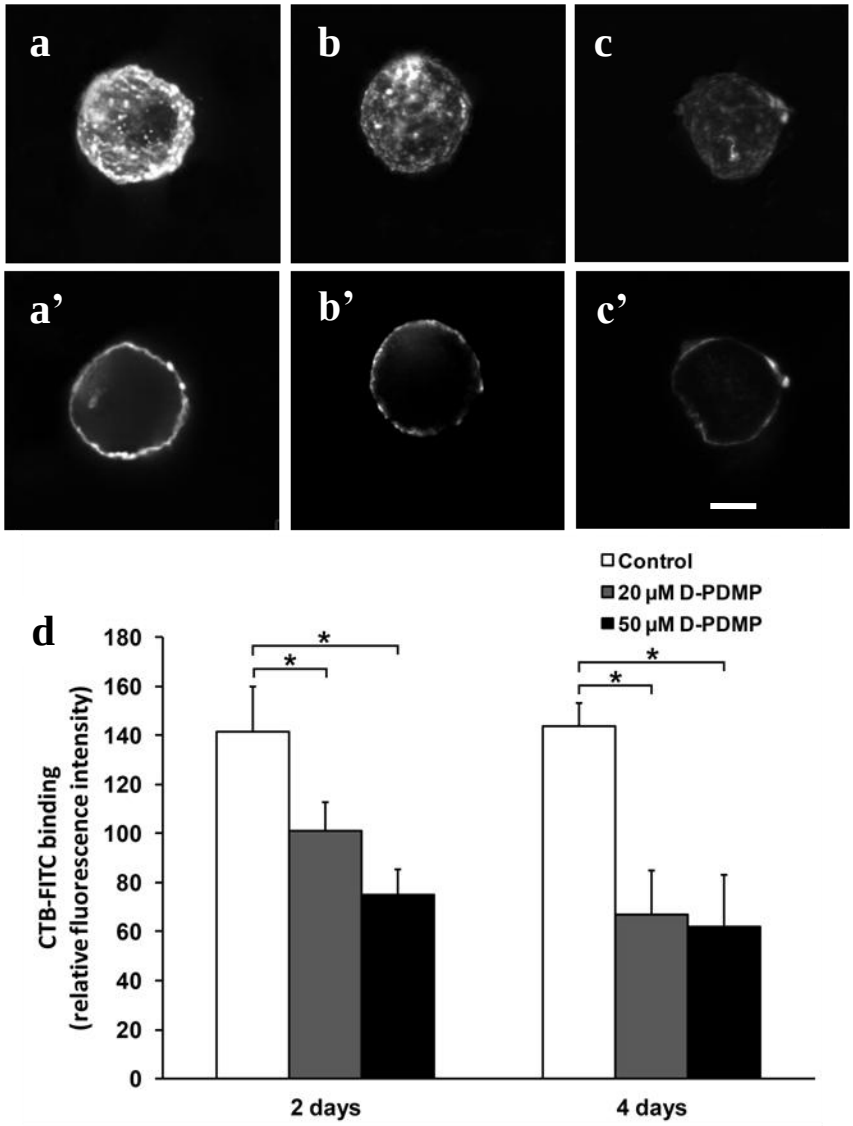

Figure 11. Confocal microscopic images of CTB-FITC binding of control (**a** and **a'**) and D-PDMP-treated (**b** and **b'**: 20 µM; **c** and **c'**: 50 µM, for 4 days) cultured DRG neurons. Images (**a**-**c**) are projections of 25 optical sections taken at 1µm intervals. Images (**a'**-**c'**) are single confocal images taken from neurons shown in (**a**–**c**). The histogram (**d**) represents the quantitative data of relative fluorescent intensity values of the membrane labelling of CTB-FITC-stained neurons in control cultures and in cultures exposed to D-PDMP at concentrations of 20 and 50 µM for 2 and 4 days. Scale bar denotes 20 µm and applies for all photomicrographs (* $p < 0.05$).

4.5.2. Effects of D-PDMP on the capsaicin-induced cobalt uptake of DRG neurons

Selective activation of TRPV1 with capsaicin results in a strong influx of cobalt ions, which can be detected by histochemical methods. Neurons responsive to capsaicin display a brownish cobalt precipitate of varying intensity. Cobalt-positive neurons can be clearly identified and also characterized by their staining intensity through measurement of their optical density. In the absence of capsaicin, very few cells (< 5%) displayed the brownish reaction product in either the untreated cultures or in cultures previously treated with D-PDMP for 4 days (**Fig. 12 a, b**). Similar to previous studies, in control experiments 29.74 ± 2.53% of the cultured DRG cells exhibited moderate to intense staining after incubation with the cobalt uptake assay buffer in the presence of capsaicin (1 μM). The effect of D-PDMP was concentration-dependent (**Fig. 12 c)**. In DRG cultures treated with 10 μM D-PDMP for 4 days the proportion of cobalt-labelled ganglion cells slightly decreased as compared to control cultures (26.8 ± 1.9% vs. 29.74 ± 2.53%). However, administration of 20 or 50 μM D-PDMP produced significant changes in the proportion of cobalt-labelled neurons (20.57 ± 0.93% and 11.63 ± 1.22%; n=24, $p<0.05$ for both treatment groups). Analysis of the size-frequency distribution histograms revealed similar distribution patterns of neurons in the control and D-PDMP (50 μM) treated cultures (**Fig. 13)**. Further, measurement of the relative optical densities of the labelled neurons indicated a significant overall reduction in staining intensity. In the D-PDMP-treated cultures, the mean optical density of capsaicin-induced neuronal cobalt staining was reduced by 47.8 ± 6.4% as compared to the control.

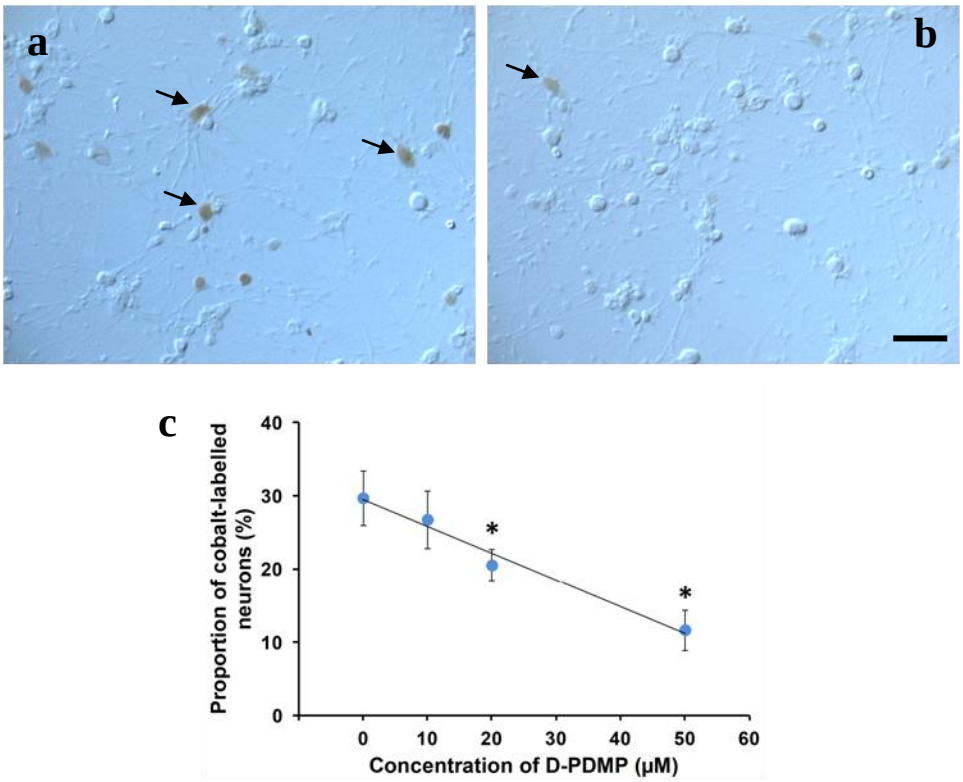

Figure 12. Light-microscopic photomicrographs represent DRG cultures grown in the absence (**a**) and presence of 50 μM D-PDMP (**b**) for 4 days followed by capsaicin-induced cobalt uptake assay. Arrows indicate cobalt-labelled neurons. Diagram (**c**) shows percent ratios of capsaicin activated neurons in control and DRG cultures pre-treated with different concentration of D-PDMP (mean ± S.E.M.; n=3-6; * $p < 0.05$). The scale bar, which denotes 100 μm applies to both pictures.

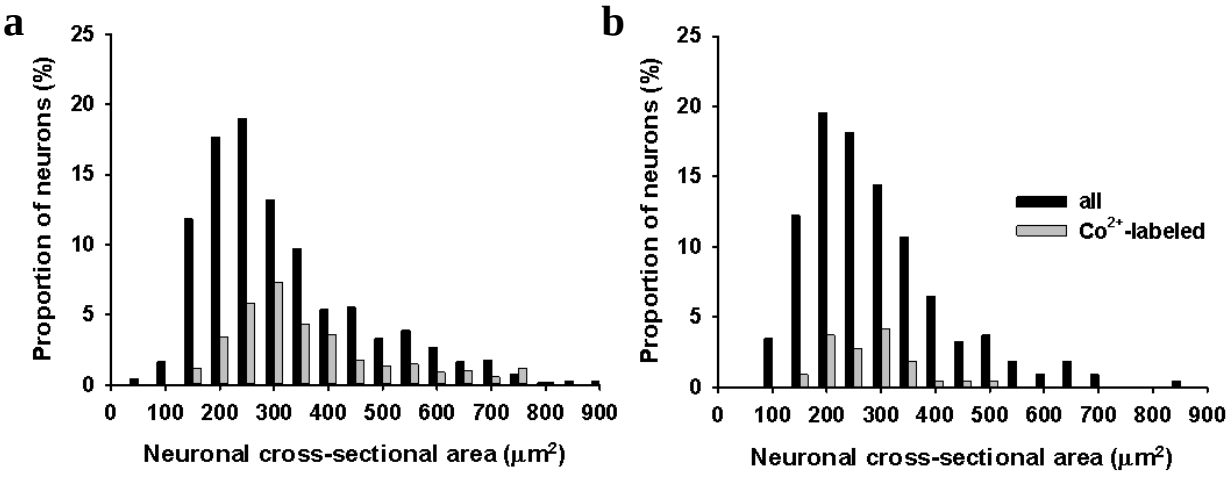

Figure 13. Size-frequency distributions of the total (open bars) and the cobalt-labelled (filled bars) populations of cultured adult rat DRG neurons following the exposure of control (**a**) and D-PDMP-treated (**b**, 50 μM) cultures to 1 μM capsaicin.

4.5.3. Recovery of the capsaicin sensitivity of cultured DRG neurons after withdrawal of D-PDMP

Possible reversibility of the effect of D-PDMP was assessed after the treatment of DRG cultures with 20 and 50 μM D-PDMP for 4 days, after which the cultures were maintained in fresh culture medium for another 4 days. As a result of administration of D-PDMP at concentrations of 20 or 50 μM for 4 days the proportion of cobalt-labelled neurons was significantly reduced to 65.4 ± 3.3% and 44.6 ± 1.4% of the control in response to the capsaicin application (for each group n=5; $p<0.05$). However after the withdrawal of D-PDMP and incubation in fresh medium for another 4 days, the sensory ganglion neurons had regained their sensitivity to capsaicin: similarly as for the control cultures (29.74 ± 2.53%), the proportion of cobalt-labelled neurons in response to the capsaicin challenge amounted to 30.8 ± 4% and 26.6 ± 3.4% (for each group n=5; $p < 0.05$). Cessation of D-PDMP treatment had a similar effect on the proportion of TRPV1-immunoreactive neurons. Treating the cultures with 20 or 50 μM D-PDMP for 4 days resulted in a significant decrease in the proportion of TRPV1-immunopositive neurons from 35.2 ± 2.5% to 18.2 ± 1.9% and 14.3 ± 1.7% (n=5 for both groups; $p<0.05$), respectively. After incubation of the D-PDMP-treated cultures in fresh medium for another 4 days, the proportion of TRPV1-immunoreactive neurons was comparable to that observed in the cultures maintained without D-PDMP for 8 days (30.3 ± 1.9% and 27.6 ± 2.3%).

4.5.4. The acute sensitizing effect of NGF on cultured DRG neurons

Acute sensitization, i.e. enhancement of the capsaicin-induced activation of the TRPV1 channel by acute, brief administrations of NGF is a well-established and important trait of nociceptive DRG neurons (Bonnington and McNaughton, 2003; Shu and Mendell, 1999). In the present experiments, the acute exposure of DRG neurons to high NGF significantly increased the number of cobalt-labelled neurons following the capsaicin challenge. In control cultures, 27.7 ± 1.3% of the neurons displayed cobalt labelling, which was increased to 36.5 ± 2.4% following a brief exposure to NGF (200 ng/mL; $n = 7$, $p < 0.05$). In contrast, in the cultures pretreated with D-PDMP only 18.1 ± 2.9% of the neurons responded to capsaicin, and the proportion of cobalt-labelled neurons after the administration of NGF was 17.3 ± 2.4% ($n = 7$).

4.5.5. Effect of pretreatment with D-PDMP on the TRPV1-immunoreactive DRG neurons

In the control DRG cultures neurons of various sizes displayed TRPV1-immunoreactivity (**Fig. 14 a**). Neurons showing TRPV1 staining comprised 37.5 ± 1.4% of the total neuronal population (**Fig. 14 c**). The effect of D-PDMP was concentration dependent. In DRG cultures treated with 10 µM D-PDMP for 4 days the proportion of TRPV1-immunoreactive ganglion cells were only slightly decreased as compared to the control (37.5 ± 1.4% vs. 32.34 ± 2.45%). However, administration of 20 or 50 µM D-PDMP to DRG cultures for 4 days resulted in a significant reduction in the proportion of TRPV1-immunoreactive neurons, which then amounted to 23.07 ± 1.93% (20 µM) and 18.23 ± 2.1% (50 µM) of the total neuronal population (**Fig. 14 c**). Analysis of the size-frequency distribution histograms revealed similar distribution patterns of the total and TRPV1-immunoreactive populations of neurons in the control and the D-PDMP-treated cultures (**Fig. 15**).

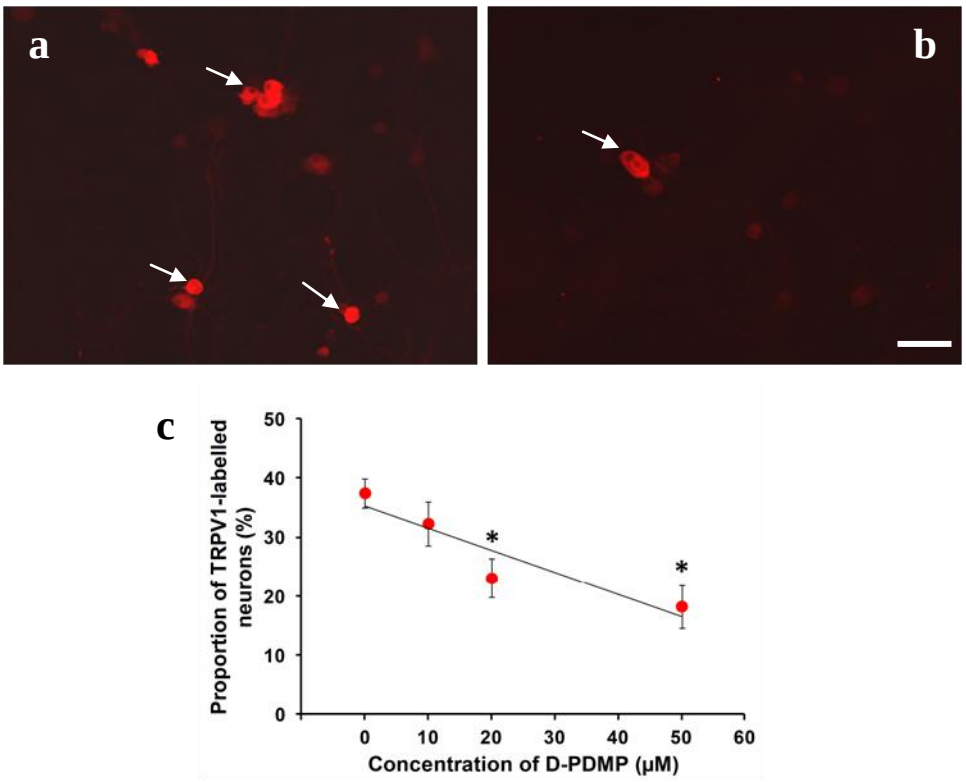

Figure 14. Fluorescence photomicrographs illustrating the decrease in the proportion of TRPV1-immunoreactive neurons following pretreatment with D-PDMP (50 µM; **a**: control, **b**: D-PDMP-treated). The scale bar, which denotes 50 µm, applies to both micrographs. Diagram (**c**) demonstrates the dose-dependent effect of D-PDMP on TRPV1-immunoreactive cultured DRG neurons (mean ± S.E.M.; n=3-6; * $p < 0.05$).

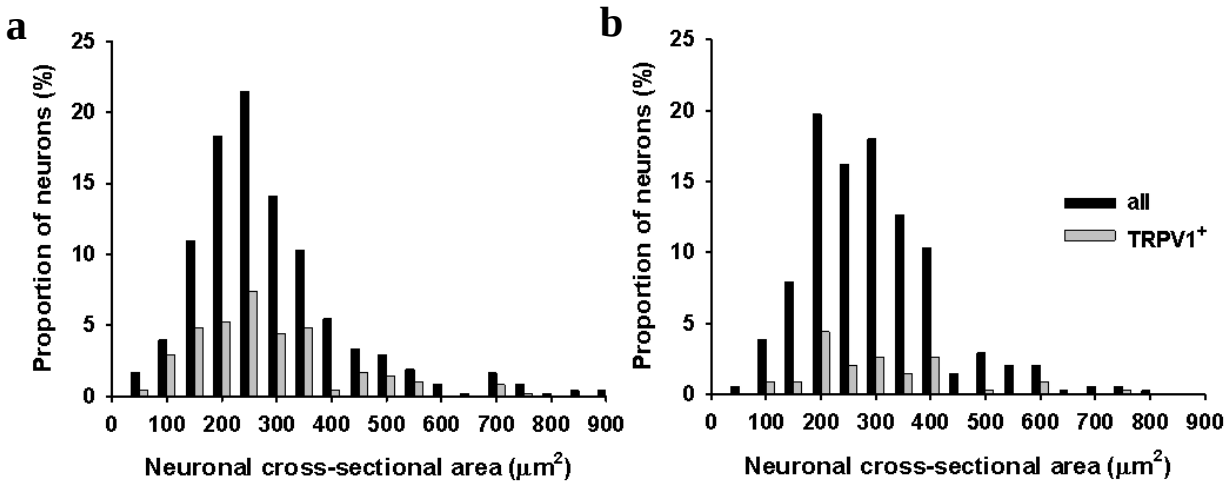

Figure 15. Size-frequency distribution histograms of the total (black bars) and the TRPV1-immunoreactive (gray bars) populations of DRG neurons in control (**a**) and D-PDMP-treated (**b**, 50 µM) cultures.

4.5.6. Effects of D-PDMP treatment on the neurite outgrowth of cultured DRG neurons

In the control cultures, the total neurite length of the individual DRG neurons after 2 or 4 days of culturing were 956.3 ± 54.3 µm and 1557.4 ±75.8 µm. As illustrated on the original microphotographs of neurons stained with the monoclonal mouse anti-neurofilament 200 kD antibody and their skeletonized counterparts in **Fig. 16**, treatment with D-PDMP resulted in a significant dose-dependent decrease in neurite length. The quantitative data shown in **Fig. 16 d** support these findings and are suggestive of a marked reduction in neurite outgrowth after D-PDMP treatment (2-day-culture: 20 µM D-PDMP 737.82 ± 35.62; 50 µM D-PDMP 578.39 ± 53.42 and 4-day-culture: 20 µM D-PDMP 616.46 ± 31.62; 50 µM D-PDMP 312.22 ± 21.81; n=50, $p < 0.05$).

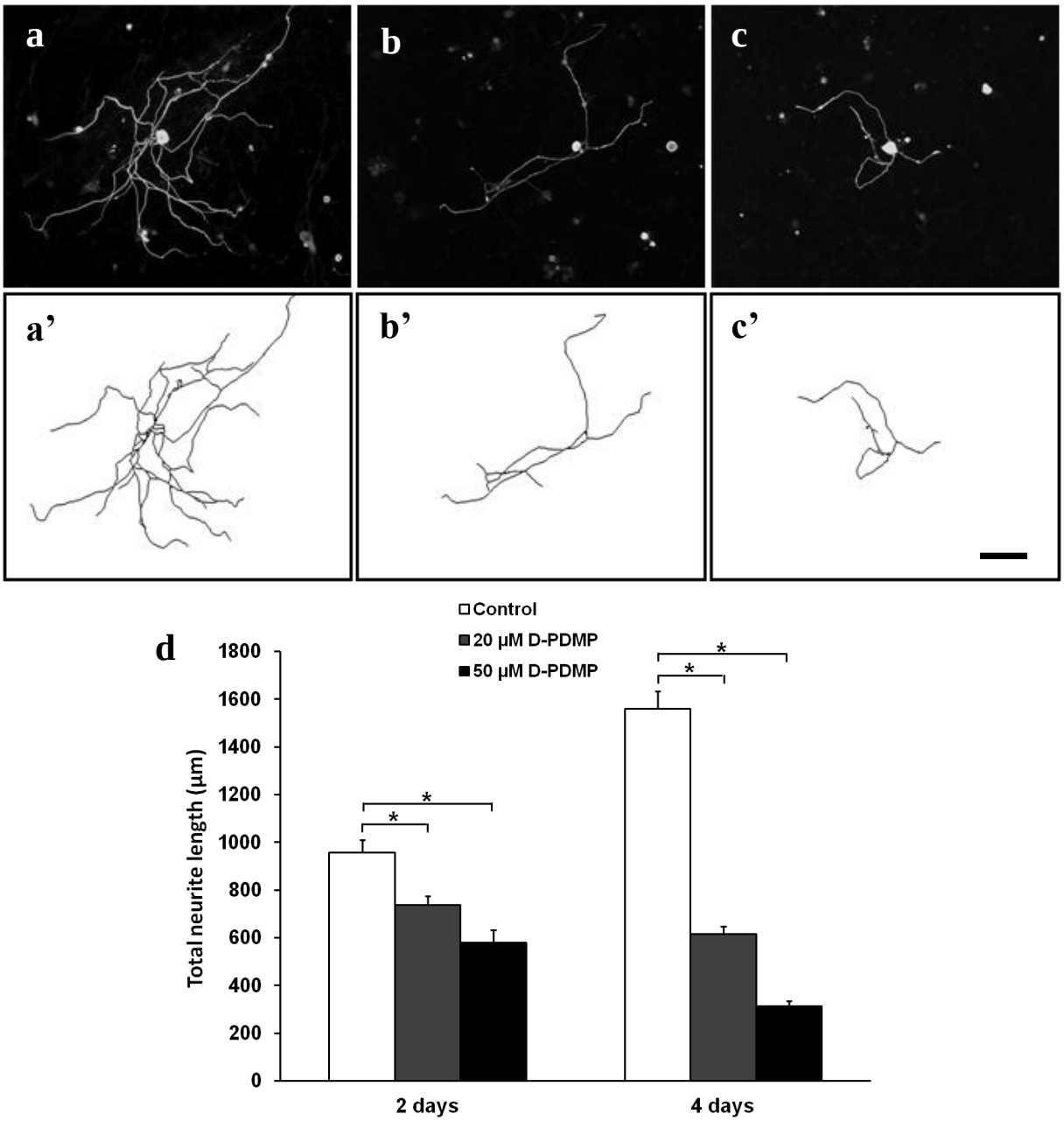

Figure 16. a-c and **a'-c'**: Fluorescence photomicrographs of DRG neurons taken from cultures exposed to D-PDMP and stained with the monoclonal mouse anti-neurofilament 200 kD antibody. Neurons and their skeletonized counterparts are shown from control (**a, a'**) and D-PDMP-treated (**b, b'**: 20 µM; **c, c'**: 50 µM) cultures. Note the marked reduction in neurite outgrowth after D-PDMP treatment. The scale bar in **c'**, denoting 100 µm, applies to all micrographs. **d**: Quantitative data demonstrating the effects of D-PDMP treatments on the neurite length of cultured dorsal root ganglion neurons.

5. DISCUSSION

In the presented experiments we studied the morphological and neurochemical alterations exerted by perineural vanilloid treatment of peripheral nerves regarding the transganglionic transport of neuronal tracer molecules CTB-HRP and WGA-HRP. Our results indicate, that shortly after the perineural capsaicin (or RTX) treatment the affinity of C-fibre afferents toward CTB-HRP binding and transport increases significantly. At the same time, these afferents retain the capability to transport WGA-HRP. These findings indicate a dynamic regulation of the CTB binding of capsaicin-sensitive primary sensory neurons following selective chemical injury, which might affect the GM1 ganglioside metabolism in the affected neurons. In order to clarify the functional implications of these changes, the possible role of the GM1 gangliosides in the regulation of the chemosensitivity and TRPV1 expression of primary sensory neurons was also tested in vitro by using a selective inhibitor of the ganglioside synthesis. Our results indicate a significant role of membrane GM1 in the maintenance of the activity and expression of the capsaicin receptor TRPV1 channel.

5.1. Mechanisms of vanilloid-induced changes in the distribution of primary afferent terminals in the rat spinal dorsal horn

Previous reports have claimed a substantial reorganization of myelinated primary afferent terminals in the spinal dorsal horn after both peripheral nerve transection (Bennett et al., 1998; Coggeshall et al., 1997; Lekan et al., 1996; Mannion et al., 1996; Shehab et al., 2004; Woolf et al., 1992; Woolf et al., 1995) and perineural treatment with capsaicin (Mannion et al., 1996). CTB and its conjugates, e.g. CTB-HRP were used in these experiments to trace the terminations of primary afferent fibres in the spinal dorsal horn. Based on previous observations by Robertson and Grant (1985), CTB was regarded as a neuronal tracer selective for myelinated afferent nerves. Injection of CTB or CTB-HRP into an intact peripheral nerve resulted in a selective labelling of spinal afferent fibres terminating in the deeper layers of the dorsal horn of the rat spinal cord. In contrast, injection of these tracers into the central trunk of a previously transected nerve resulted in a massive labelling of the most superficial layers of the spinal dorsal horn, in particular the substantia gelatinosa (Woolf et al., 1992; Woolf et al., 1995). This sprouting response was explained by a substantial injury-induced reduction of C-fibre synaptic contacts with lamina II neurons possibly due to transganglionic or transsynaptic degenerative changes. They also proposed, that the vacant synaptic sites in lamina II may be occupied by invading sprouts arising from

axotomized (“conditioned”) myelinated afferent fibres. Hence, injury-induced increased labelling of the substantia gelatinosa was explained by the central sprouting of myelinated primary afferents, which normally terminate only in the deeper layers of the dorsal horn, in the substantia gelatinosa. This sprouting hypothesis was based on the assumption that CTB specifically and exclusively labels myelinated primary afferent fibres (Robertson and Grant, 1985). The authors suggested that A-fibre sprouting may have considerable clinical significance in terms of the pathogenesis of neuropathic pain, in particular touch-evoked pain and allodynia (Doubell et al., 1997; Lekan et al., 1996; Mannion et al., 1996; Nakamura and Myers, 1999; Nakamura and Myers, 2000; Shortland et al., 1997; White, 2000; Woolf and Doubell, 1994; Woolf et al., 1992; Woolf et al., 1995). Mannion and co-workers performed further studies to support the sprouting hypothesis utilizing perineural application of the C-fibre specific sensory neurotoxin, capsaicin (Mannion et al., 1996). After finding a similar reorganization of CTB-HRP labelling in the spinal dorsal horn following capsaicin treatment, they suggested that central sprouting can occur even after C-fibre injury alone, without a conditioning lesion of the A-fibres by axotomy. However, they did not examine the CTB-HRP labelling of the primary afferent neurons in the related dorsal root ganglia.

Further studies, however, have failed to support the sprouting hypothesis by showing a marked labelling of peripherally injured small dorsal root ganglion neurons with CTB and/or CTB-HRP (Bao et al., 2002; Hughes et al., 2003; Sántha and Jancsó, 2003; Shehab et al., 2003; Tong et al., 1999). Since small DRG neurons give rise to mostly unmyelinated axons (Lawson and Waddell, 1991), which terminate in the superficial layers of the spinal dorsal horn, the sprouting hypothesis was called into question. Studies by Sántha and Jancsó (2003), by using selective chemical lesion techniques and a quantitative electron microscopic histochemical approach, provided direct evidence for the transport of CTB-HRP by unmyelinated dorsal root axons and labelling of the substantia gelatinosa by C-fibre primary afferent terminals following peripheral nerve transection (Jancsó et al., 2002; Jancsó et al., 2004; Sántha and Jancsó, 2003; Szigeti et al., 2012). Based on these observations, we re-investigated the hypothesized spinal sprouting phenomenon after perineural vanilloid treatment by studying the size-frequency distribution of CTB-HRP-labelled dorsal root ganglion cells and the localization of CTB-HRP in spinal dorsal roots relating to intact and vanilloid-treated sciatic nerves using quantitative enzyme histochemistry and electron microscopy.

Our findings support the notion that perineural capsaicin treatment resulted in significant morphological and neurochemical alterations in the spinal cord dorsal horn. In

agreement with previous observations (Ainsworth et al., 1981; Gamse et al., 1982; Jancsó, 1992; Jancsó and Lawson, 1990; Wall, 1987) we found that, in the substantia gelatinosa, TMP activity was virtually completely depleted from the somatotopically related areas of the treated sciatic nerve 2 weeks after the treatment. Other nociceptor specific markers i.e., somatostatin, IB4, SP and TRPV1 also showed marked decreases due to perineural capsaicin treatment (Gamse et al., 1982; Gibson et al., 1982; Knotkova et al., 2008; Molander and Grant, 1986; Sántha and Jancsó, 2003; Szigeti et al., 2012) and similar changes were observed in the spinal dorsal horn after axotomy (Atkinson and Shehab, 1986; Bennett et al., 1998; Jancsó, 1992; Jessel et al., 1979; Michael and Priestley, 1999). Observations demonstrating the lack of argyrophil degeneration in the somatotopically relating regions of the substantia gelatinosa of a capsaicin-treated peripheral nerve, elicited by a systemic administration of capsaicin, suggested that central terminals of chemosensitive C-fibres are also profoundly affected by perineural capsaicin treatment (Jancsó and Ambrus, 1994; Jancsó and Lawson, 1990). It was suggested that the development of the "capsaicin gap" might be a consequence of either the elimination of the central terminals due to a transganglionic degenerative process, or a decrease in their sensitivity to capsaicin, possibly due to a phenotypic change. There are observations supporting the latter mechanism indicating a strong decrease in the expression of the TRPV1 channels in the affected DRG neurons both after axotomy (Michael and Priestley, 1999) and after perineural capsaicin treatment (Szigeti et al., 2012). We have also demonstrated that the chemosensitive unmyelinated nerve fibres of the affected sciatic nerve are still capable of the uptake and transganglionic transport of WGA-HRP, a marker of unmyelinated sensory neurons, 2 weeks after the treatment. The preserved transport of WGA-HRP into the superficial dorsal horn 2 weeks after the local application of capsaicin indicates that neither a complete blockade of the cellulifugal axonal transport, nor a substantial degeneration or loss of unmyelinated sensory nerve fibres and their central terminals occur at this time. These findings indicate that the transport blockade induced by perineural capsaicin treatment is transient in nature, and at a later time point (2 weeks in our experiment) a large population of C-fibre afferents projecting to the superficial dorsal horn is able to transport retrograde tracer molecules. However, the severe structural and functional impairments of the peripheral (cutaneous) endings of the chemosensitive nerves are well documented following perineural capsaicin treatment (Gamse et al., 1982; Jancsó, 1992; Jancsó et al., 1980; Such and Jancsó, 1986). The significance of the existence of non-functional but still preserved nociceptive neurons/nerves and the possible functional implications of this condition remain to be determined.

In accord with previous findings (Mannion et al., 1996) we demonstrated a marked transganglionic CTB-HRP labelling of the somatotopically relating areas of the spinal dorsal horn 2 weeks after perineural capsaicin treatment of the sciatic nerve. Perineural treatment with resiniferatoxin, an ultrapotent analogue of capsaicin, produced a similar labelling pattern, too. The quantitative morphometric analysis of L5 dorsal root ganglion neurons revealed many, mainly large CTB-HRP-labelled neurons in the ganglia relating to the vehicle treated sciatic nerve 2 weeks after the treatment. In contrast, in ganglia relating to the capsaicin-treated nerve, the proportion of labelled small cells (cross sectional area <600 μm^2) was significantly increased. These findings suggested that C-fibre primary sensory neurons may be implicated in the mechanism of "A-fibre sprouting". This assumption was strongly supported by the demonstration of the very pronounced and highly significant increase in the proportion of CTB-HRP labelled unmyelinated axons in the dorsal roots relating to a capsaicin-treated nerve. Additionally, we have demonstrated that WGA-HRP labelling of the central terminals of C-fibre primary afferents remained apparently unaffected 2 weeks after perineural capsaicin treatment indicating the viability and preserved transport capacity of the affected C-fibres. These observations lead us to conclude that the perineural capsaicin induced CTB-HRP labelling of the substantia gelatinosa may be accounted for by a phenotypic switch of C-fibre primary afferent neurons rather than a sprouting response of spinal Aβ-afferent fibres. Transganglionic transport of WGA-HRP indicated that even if some degeneration of the central terminals of C-fibres might occur, the majority of these terminals are still preserved at that time point of the experiment. These findings strongly suggested that CTB-HRP, injected into a nerve treated perineurally with capsaicin (or resiniferatoxin) 2 weeks earlier, is taken up and transported by injured C-fibre afferents to the substantia gelatinosa. This is at variance with the earlier interpretation of this finding by Mannion et al (Mannion et al., 1996) who suggested that the increased labelling of the substantia gelatinosa following perineural capsaicin may be attributed to a sprouting response of A-fibre afferents. This conclusion was based on the assumption that CTB specifically binds to myelinated fibres. Our findings, however, indicate that besides intact myelinated afferent fibres, chemically injured unmyelinated sensory axons may also bind CTB. We suggest therefore that perineural application of vanilloid compounds, such as capsaicin and resiniferatoxin induce a phenotypic switch in C-fibre primary sensory neurons resulting from an increased expression of GM1 ganglioside, the receptor of CTB (Cuatrecasas, 1973; Robertson and Grant, 1989; Stoeckel et al., 1977). Hence, increased labelling of the substantia gelatinosa following perineural application of vanilloids may be attributed to a phenotypic switch of C-fibre afferent neurons

rather than a sprouting response of A-fibre primary afferents. This may bear important functional implications concerning pain mechanisms, since GM1 ganglioside plays a pivotal role in the regulation of the expression of nociceptive ion channels (Mutoh et al., 1998). Further, GM1 ganglioside is an important constituent of the plasma membrane, in particular of lipid rafts, and may affect neuronal signaling processes. Taken together, these findings suggest that changes in neuronal ganglioside, in particular GM1 ganglioside level may interfere with the nociceptor function of C-fibre primary afferents.

5.2. Inhibition of glucosylceramide synthase reduces neuronal capsaicin sensitivity and the expression of the TRPV1 receptor in DRG neurons

The TRPV1 receptor is considered as a molecular integrator for a broad range of noxious stimuli. The mechanisms regarding the modulation of the expression and function of the receptor have been in the centre of interest for many years. Several molecular pathways have revealed which regulate the sensitivity of the TRPV1 receptor to capsaicin and other modulators (Bonnington and McNaughton, 2003; Chuang et al., 2001; Julius and Basbaum, 2001; Nagy et al., 2004; Tominaga et al., 1998) but the possible role of gangliosides in the regulation of capsaicin sensitivity has not been considered, yet. The morphological and neurochemical changes occurring in primary afferent neurons after peripheral nerve injuries led us to suggest that changes in the ganglioside metabolism of these neurons may modulate their nociceptive function (Jancsó et al., 2008; Jancsó et al., 2002; Sántha and Jancsó, 2003).

To reveal the possible role of gangliosides, in particular GM1 ganglioside, in nociceptor function, we studied the effects of inhibition of glucosylceramide synthase, the key enzyme of the neuronal ganglioside metabolism, on the capsaicin sensitivity of cultured sensory ganglion cells. We have demonstrated a reversible reduction of the capsaicin sensitivity of DRG neurons following the inhibition of glucosylceramide synthase by D-PDMP. Using a quantitative cobalt uptake assay we found that pre-treatment with D-PDMP significantly reduced both the proportion and the intensity of the neurons stained with cobalt, i.e. activated by capsaicin. We also observed a significant reduction in the proportion of TRPV1-immunoreactive neurons following the inhibition of ganglioside synthesis. Although different mechanisms may be involved, most probably an altered regulation of TRPV1 expression due to an impairment of an NGF signaling pathway may underlie these effects. Hence, GM1 ganglioside has been shown to be critically involved in the mediation of the cellular effects of NGF (Mutoh et al., 1998). Importantly, NGF plays a crucial role in the

regulation of the expression of the capsaicin/TRPV1 receptor in sensory ganglion neurons (Aguayo and White, 1992; Bevan and Winter, 1995; Winter et al., 1988). Deprivation of cultured DRG neurons of NGF or a reduction of the availability of peripherally derived NGF by the inhibition of retrograde axonal transport in sensory axons *in vivo* (Bevan and Winter, 1995; Jancsó et al., 1997; Winter et al., 1988) results in a marked loss of neuronal capsaicin sensitivity (Jancsó and Ambrus, 1994). Gangliosides and in particular GM1, play a crucial role in the mediation of the trophic effects of NGF in PC12 cells by promoting neurite outgrowth (Ferrari et al., 1983; Mutoh et al., 1998). Moreover, the inhibition of glucosylceramide synthase inhibits the action of NGF on neurite outgrowth of PC12 cells and DRG neurons (Lainetti et al., 1998; Mutoh et al., 1998; Schwartz and Spirman, 1982).

The acute sensitizing effect of NGF on the capsaicin sensitivity of cultured DRG neurons is well established (Shu and Mendell, 1999). Investigating the effect of D-PDMP on the acute sensitising effect of NGF in DRG cell cultures we observed that NGF failed to produce this response after D-PDMP treatment. We also observed that similar to the PC12 results (Mutoh et al., 1998) the axonal growth decreased in cultured DRG neurons after D-PDMP treatment (Sántha et al., 2010). Collectively, these findings and previous observations strongly suggest that gangliosides, in particular GM1, play an important role in the regulation of neuronal capsaicin-sensitivity through an action on TRPV1 expression mediated by the GM1-NGF signaling pathway.

Although an interaction of GM1 with the NGF signaling pathway seems to be the most likely explanation of our observations on the effect of inhibition, by D-PDMP, of glucosylceramide synthase on neuronal sensitivity to capsaicin, the role of possible changes in the functions of membrane lipid microdomains (rafts) cannot be excluded. Indeed, quantitative analysis of CTB-binding in cultured DRG neurons revealed a marked and significant, dose-dependent decrease in membrane GM1 level after D-PDMP treatment which may affect the membrane localization of and signaling through the TRPV1 receptor (Holzer, 1991; Jancsó et al., 2008). Similar studies focusing on the depletion of membrane cholesterol and sphingomyelin, other important components of lipid microdomains, have also been shown to reduce the sensitivity of cultured DRG neurons to capsaicin (Liu et al., 2006; Szoke et al., 2010).

6. CONCLUSIONS

The observations presented in this thesis have revealed that in contrast to previous reports (Mannion et al., 1996; Woolf et al., 1992; Woolf et al., 1995), the transganglionic labelling of the substantia gelatinosa by CTB-HRP after perineural vanilloid (capsaicin or RTX) treatment may be brought about by an increased uptake and transport of CTB-HRP by injured chemosensitive C-fibre afferents, rather than a sprouting response of Aβ afferents. This may be accounted for by a phenotypic switch of injured C-fibre primary sensory neurons resulting in an increased neuronal level of GM1 ganglioside which specifically binds CTB. Electron microscopic examination of dorsal roots relating to a vanilloid-treated peripheral nerve provided direct evidence for the transport of CTB-HRP by unmyelinated dorsal root axons. To reveal the significance of the phenotypic change of C-fibre primary sensory neurons and the possible role of membrane gangliosides, in particular GM1 ganglioside in this phenomenon, we examined the functional and morphological consequences of the inhibition of glucosylceramide synthase, the key enzyme in neuronal ganglioside synthesis, in DRG cultures. We observed a significant and reversible reduction in neuronal capsaicin sensitivity induced by the glucosylceramide synthase inhibitor D-PDMP. Immunohistochemical findings showing a significant reduction in the proportion of TRPV1-immunoreactive neurons after the administration of D-PDMP indicated that the decreased responsiveness of nociceptive DRG neurons to the TRPV1 receptor agonist capsaicin may be largely accounted for by a reduction in the expression of TRPV1 receptors. These observations have demonstrated that pharmacological perturbation of neuronal ganglioside metabolism may affect the nociceptor function significantly and point to a novel therapeutic approach to interfere with the mechanisms of pain sensation.

7. SUMMARY

Chemosensitive sensory ganglion neurons comprise a well defined population of nociceptive primary sensory neurons which express the capsaicin/transient receptor potential vanilloid type 1 (TRPV1) receptor, a molecular integrator of nociceptive stimuli. A single perineural application of capsaicin produces defunctionalization of chemosensitive primary sensory neurons resulting in a selective and long lasting regional thermal and chemical analgesia and a complete abolition of the neurogenic inflammatory response. The analgesic and anti-inflammatory actions of perineural capsaicin treatment may be attributed, at least in part, to a selective chemodenervation of the nerve producing pathological alterations in both the relating sensory ganglia and the spinal dorsal horn. Previous studies demonstrated marked transganglionic labelling, by choleratoxin B – horseradish peroxidase conjugate (CTB-HRP), of the substantia gelatinosa of the spinal dorsal horn following perineural capsaicin. Since CTB-HRP is a specific marker of myelinated nerve fibres in intact peripheral nerves, this phenomenon was interpreted in terms of a sprouting response of Aß myelinated primary afferents which normally terminate only in the deeper layers of the dorsal horn, but not in the substantia gelatinosa, the main projection field of nociceptive primary afferents.

The main objective of this Thesis was to re-examine the mechanisms of the apparent structural re-arrangement of the spinal dorsal horn, in particular the substantia gelatinosa, observed after perineural capsaicin treatment. In accord with previous findings, perineural application of capsaicin, or the ultrapotent vanilloid analogue, resiniferatoxin, resulted in a massive transganglionic labelling of the substantia gelatinosa by CTB-HRP. Electron microscopic histochemistry revealed many CTB-HRP-labelled myelinated but not unmyelinated axons in spinal dorsal roots relating to vehicle-treated or intact sciatic nerves. In contrast, besides labelled myelinated axons, many unmyelinated axons were also labelled in the dorsal roots relating to the capsaicin- or resiniferatoxin-treated sciatic nerves. Therefore, we concluded that, in contrast to previous reports, the capsaicin-induced marked transganglionic labelling of the substantia gelatinosa by CTB-HRP may be accounted for by a phenotypic switch of C-fibre primary afferent neurons, rather than an A-fibre sprouting response. This interpretation necessitated not only a substantial re-evaluation of previous views on the mechanisms of capsaicin-induced plastic changes in the spinal cord, but it also pointed to the possible significance of neural gangliosides, in particular GM1 which specifically binds CTB, in pain mechanisms. Therefore, we initiated experiments to examine the putative role of gangliosides in the function of cultured nociceptive primary sensory

neurons. Inhhibition of glucosyl ceramide synthase, the key enzyme in neuronal ganglioside synthesis, resulted in a significant decrease in neuronal ganglioside level and in the capsaicin-induced activation of the TRPV1 receptor, as assessed with the quantitative cobalt uptake assay. Inhibition of glucosyl ceramide synthase reduced the proportion of TRPV1-positive ganglion cells, too. Nerve growth factor-induced acute sensitization and neurite outgrowth were also markedly reduced following the inhibition of ganglioside synthesis. Collectively, these findings suggest that neuronal gangliosides, in particular GM1 may be intimately involved not only in the mechanisms of neurochemical changes induced by perineural capsaicin, but they may also play a significant role in the function of pain-sensing primary sensory neurons.

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