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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INTRODUCTION

Pain is a common and one of the most frequent symptoms of a variety of disease states. Categories of pain involve nociceptive pain and neuropathic pain. Nociceptive pain is evoked by the activation of nociceptors and mediated by nociceptive primary sensory neurons which are specifically responsive to stimuli producing actual or potential tissue damage. Neuropathic pain develops as a consequence of lesions in the pain system, including lesions of peripheral nerves. Nociceptive primary sensory neurons comprise about half of the neurons in spinal and cranial sensory ganglia. This population of primary sensory neurons can be distinguished by their selective sensitivity and vulnerability to capsaicin, the pungent agent in red peppers (paprika). Pharmacological studies by N. Jancsó in the 1950s demonstrated that capsaicin excites and, at higher concentrations, desensitizes cutaneous and visceral pain receptors. Pórszász and Jancsó have also shown that capsaicin selectively excites C-fibre afferents. Studies by N. Jancsó and A. Jancsó-Gábor have also revealed that capsaicin-sensitive sensory nerves possess a dual function. These sensory nerve endings, on the one hand, are sensitive to and transmit noxious stimuli, and, on the other hand, if stimulated, they release vasoactive peptides which mediate neurogenic inflammation. Later studies in the 1970s by G Jancsó et al. disclosed that capsaicin is a selective sensory neurotoxin which induces a selective degeneration of small type B, in part peptide-containing, sensory ganglion neurons after systemic administration. The effects of capsaicin are mediated by the transient receptor potential vanilloid type 1 receptor (TRPV1), a non-selective cation channel cloned by Caterina et al. in 1997. The TRPV1 receptor is now regarded as the archetypal nociceptive ion channel expressed by most types of nociceptive primary sensory neurons. Hence, the study of the functional and molecular traits of the TRPV1 receptor and its involvement in the physiological and pathological processes of the pain system is in the forefront of pain research. In 1980, G Jancsó and co-workers demonstrated that a single application of capsaicin onto rat peripheral nerves produces selective and long-lasting regional thermal and chemical analgesia and abolition of the neurogenic inflammatory response without affecting the functions of non-nociceptive sensory, autonomic or motor nerves. Later studies demonstrated a dramatic reduction in the expression of the TRPV1 receptor in the relating dorsal root ganglion (DRG) neurons following perineural application of vanilloids, such as capsaicin or the highly potent TRPV1 agonist, resiniferatoxin (RTX). The defunctionalization of TRPV1 expressing primary afferent neurons through perineural capsaicin treatment has become an established experimental strategy in pain research to study nociceptive, viscerosensory and neurogenic inflammatory mechanisms. Vanilloid-induced nociceptor

analgesia may be produced in any area of the body by treating the nerve which serves it, in a variety of animal species, including primates. Perineural treatment with vanilloid compounds has therefore been considered as a possible therapeutic approach for the relief of pain of peripheral or neuropathic origin.

Perineural capsaicin treatment produces profound ganglionic and transganglionic changes in C-fibre primary afferents. By using the "capsaicin gap" technique, it has been demonstrated that capsaicin treatment of a peripheral nerve prior to a systemic administration of capsaicin prevented the argyrophylic degeneration of primary afferent terminals in the somatotopically corresponding areas of the spinal dorsal horn. Similarly, drastic reduction or complete disappearance of numerous C-fibre specific marker molecules such as fluoride-resistant acidic phosphatise/thiamine monophosphatase (FRAP/TMP), Bandeiraea simplicifolia isolectin B4 (IB4), substance P (SP) and TRPV1 can be detected in the somatotopically relating areas of the superficial laminae of the spinal dorsal horn shortly after perineural vanilloid treatment. These changes resemble alterations observed following peripheral nerve injuries, such as nerve transection. An increased transganglionic transport to the substantia gelatinosa of the spinal dorsal horn of the choleratoxin B subunit (CTB) or its conjugates injected into injured peripheral nerves is a salient feature of morphological changes which commence after peripheral nerve injuries. Since CTB and its conjugates are taken up and transported selectively only by myelinated primary afferent fibres which terminate in the deeper layers of the spinal dorsal horn in intact animals, the labelling of the substantia gelatinosa was interpreted in terms of a sprouting response of A-fibre afferents by Woolf et al. (1992). Mannion et al. (1996) demonstrated a similarly increased transganglionic labelling by CTB of the substantia gelatinosa following perineural capsaicin treatment, which was also interpreted in terms of a sprouting response of A-fibre afferents. However, studies in our laboratory have revealed that, in contrast to literature data, the nerve injury-induced increased labelling of the substantia gelatinosa by transganglionically transported CTB may be explained by a phenotypic switch of nociceptive C-fibre primary sensory neurons rather than a sprouting response of myelinated primary afferents. Sántha and Jancsó (2003) also drew attention to the fact that CTB is a highly specific and sensitive biochemical marker of the expression and cellular localisation of the GM1 ganglioside receptor. Gangliosides, including GM1, are major components of cholesterol-rich membrane microdomains which are also referred to as lipid rafts. Hence, the mechanisms underlying the increased transganglionic labelling of the substantia gelatinosa by CTB-HRP following peripheral nerve injury may be of significance not only in terms of the anatomical changes which might occur after such

lesions, but also as regards the possible functional significance of altered levels of neural gangliosides, and in particular GM1. However, despite the apparent close association of GM1 ganglioside with pathobiological phenomena affecting nociceptive primary afferent neurons, gangliosides have not been implicated in pain mechanisms, yet.

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.

MATERIALS AND METHODS

Surgical Procedures

Adult male Wistar rats weighing 250-300 g were used for the experiments. Animals were anaesthetized with chloral-hydrate (400 mg /kg, i.p.) and the sciatic nerves were exposed at mid thigh level on both sides under aseptic conditions. A plastic film was placed underneath the nerve to isolate it from surrounding tissues and a small piece of gelfoam moistened with 0.1 mL of either a 1% solution of capsaicin (n=9) or a 0.005% solution of RTX (n=4) was wrapped 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.

In order to label the sensory fibres located in the sciatic nerves 2 weeks after the perineural capsaicin (n=9) or RTX (n=4) 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.

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.

Histological procedures

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 or a freezing microtome, respectively.

Histochemical demonstration of peroxidase enzyme activity

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.

Demonstration of 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 cresylechtviolet.

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. 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. The ultrathin sections were contrasted with uranyl acetate and lead citrate and viewed under an electron microscope. Dorsal root axons were counted on-screen in randomly selected sections.

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. Based on previous analysis neurons having a cross sectional area $< 600\ \mu m^2$ were classified as small-type cells. 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.

Preparation of cultures of DRG neurons and D-PDMP treatment

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. (1988) with slight modifications. 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 serum supplement. 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 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.

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. 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.

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. 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 ImageProPlus 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.

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.

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 by using a 20x objective and the proportion of cobalt-labelled neurons was calculated. Data are given as means ± S.E.M.

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 2 hours, the cover slips were rinsed for 3 x 5 minutes in PBS. For fluorescence microscopy, the specimens were mounted with Vectashield mounting medium.

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. Fifty neurons in each treatment group were analyzed. Data are expressed in μm and given as means ± S.E.M.

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 ImageProPlus 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 $p < 0.05$.

RESULTS

The effects of perineural treatment with capsaicin or RTX on the distribution of primary afferent terminals in the spinal dorsal horn

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. Similarly to previous observations, injection of CTB-HRP into the left, vehicle-treated sciatic nerve resulted in the transganglionic labelling of the deeper layers of the somatotopically related regions of the dorsal horn of the lumbar

spinal cord. Injection of CTB-HRP into the (right) sciatic nerve 2 weeks after perineural treatment with either capsaicin or RTX, 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.

Changes in WGA-HRP labelling

In agreement with previous reports, intraneural injection of WGA-HRP, a selective marker of unmyelinated, C-fibre afferents, into an intact peripheral nerve, resulted in aselective 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.

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. On the control side the enzyme activity was present in the substantia gelatinosa of the spinal dorsal horn. Two weeks after perineural capsaicin or RTX treatment of the right sciatic nerve, a marked reduction of TMP activity in the medial 2/3 of the substantia gelatinosa was observed. This region corresponds to the termination site of sciatic C-fibre afferents somatotopically relating to the capsaicin treated sciatic nerve.

Effects of perineural capsaicin or RTX 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. 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- or RTX-treated nerves, many small neurons were also labelled. 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. 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 cross-sectional area of the labelled cells was 916.16 ± 40.07 μm^2, whereas ipsilateral to the capsaicin- or RTX-treated nerves the mean cross-sectional area of labelled cells was significantly smaller (capsaicin-treated: 698.16 ± 27.59 μm^2 ; RTX-treated: 673.57 ± 43.44 μm^2; for both groups n=4, $p < 0.05$). There was no difference in the mean cross-sectional area of the total neuronal populations (control: 657.6 ± 26 μm^2, capsaicin treated: 652.02 ± 48 μm^2; RTX-treated: 657.78 ± 57 μm^2). The proportion of labelled nerve cells was 40.71 ± 2.21% in the control ganglia, while it significantly increased in the ganglia relating to the capsaicin- or RTX-treated nerve (capsaicin: 63.44 ± 1.17%; RTX:54.29 ± 3.21%; for both groups 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% and 65.62 ± 1.03% following capsaicin and RTX treatment, respectively (for both groups n=4, $p < 0.05$).

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. 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. 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.

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

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. 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.

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 either in the untreated cultures or in cultures previously treated with D-PDMP for 4 days. 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. 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 the D-PDMP (50 μM) treated cultures. 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.

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. 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 neurones responded to capsaicin, and the proportion of cobalt-labelled neurons after the administration of NGF was 17.3 ± 2.4% ($n = 7$).

Effects of pretreatment with D-PDMP on the TRPV1 immunoreactivity of DRG neurons

In the control DRG cultures neurons of various sizes displayed TRPV1-immunoreactivity. Neurons showing TRPV1 staining comprised 37.5 ± 1.4% of the total neuronal population. 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. 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.

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

In the control cultures, the average of 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. Treatment with D-PDMP resulted in a significant dose-dependent decrease in neurite length (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; $n=50$, $p < 0.05$).

CONCLUSIONS

Previous studies have shown an increased transport of CTB-HRP into the substantia gelatinosa of the spinal dorsal horn following perineural capsaicin treatment. Since in intact animals CTB-HRP is taken up and transported by A-fibre, myelinated primary afferents which terminate in the deeper layers of the dorsal horn but not the superficial substantia

gelatinosa, this phenomenon was interpreted in terms of a sprouting response of myelinated afferents. The observations presented in this thesis have revealed that, in contrast to previous reports, the transganglionic labelling of the substantia gelatinosa by CTB-HRP following perineural vanilloid (capsaicin or resiniferatoxin) treatment is brought about by an increased uptake and transport of CTB-HRP by chemically injured chemosensitive unmyelinated, C-fibre afferents, rather than a structural re-arrangement of Aβ primary afferents involving axonal sprouting. This may be accounted for by a capsaicin-induced phenotypic switch of C-fibre primary sensory neurons resulting in an increase in neuronal GM1 ganglioside level and, consequently, CTB binding. Examination of dorsal roots relating to vanilloid-treated peripheral nerves using electron microscopic histochemistry provided direct evidence for the transport of CTB-HRP by unmyelinated dorsal root axons. These findings furnished direct evidence for an increased uptake and transport of CTB-HRP by C-fibre primary afferents following perineural vanilloid treatment and cast serious doubts on the sprouting hypothesis. Importantly, these observations pointed to a hitherto unrecognized role of neural ganglioside metabolism in nociceptor function. To further evaluate the possible role of gangliosides in the function of nociceptive primary afferent neurons, we examined the effects of inhibition by D-PDMP of glucosylceramide synthase, the key enzyme in neuronal ganglioside synthesis, on the function and neurochemical traits of cultured DRG neurons. Inhibition of glucosylceramide synthase resulted in a significant and reversible reduction in neuronal capsaicin sensitivity and TRPV1 immunoreactivity. Treatment with D-PDMP also impaired neurite growth and NGF-induced acute sensitization. These findings may be explained by a decreased NGF-dependent expression of the TRPV1 receptor resulting from an impairment of the NGF signalisation pathway and/or an altered function of membrane lipid rafts involved in TRPV1 receptor activation. It is well established that the GM1 ganglioside is involved in NGF signalisation and it is an integral component of membrane lipid rafts.

Finally, these observations demonstrate that pharmacological perturbation of neuronal ganglioside metabolism may affect the nociceptor function significantly and therefore point to a novel therapeutic approach to interfere with the mechanisms of pain sensation.

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Publications related to the thesis

I. Oszlács O, Jancsó G, Kis G, Dux M, Sántha P. (2015) Perineural capsaicin induces the uptake and transganglionic transport of choleratoxin b subunit by nociceptive C-fiber primary afferent neurons. Neuroscience 311:243-252.

II. Sántha P, Oszlács O, Dux M, Dobos I, Jancsó G. (2010) Inhibition of glucosylceramide synthase reversibly decreases the capsaicin-induced activation and TRPV1 expression of cultured dorsal root ganglion neurons. Pain 150:103-112.

III. Jancsó G, Dux M, Oszlács O, Sántha P. (2008) Activation of the transient receptor potential vanilloid-1 (TRPV1) channel opens the gate for pain relief. Br J Pharmacol 155:1139-1141.

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