Morphometric and neurochemical characterization of primary sensory neurons expressing the insulin receptor in the rat

Ph.D. Thesis

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INTRODUCTION

Primary sensory neurons (PSNs), which are key elements in the somato- and viscerosensory functions, can be classified by their morphological, neurochemical and functional characteristics. Morphologically, spinal and cranial sensory ganglion neurons can be divided into two main categories: type A or large light, and type B or small dark neurons. Immunohistochemical and electron microscopic findings have revealed that type A PSNs are rich in neurofilaments, while type B neurons contain few neurofilaments. Neurofilament-rich, large-sized type A neurons possess myelinated fibres, whereas neurofilament-poor, small-sized type B neurons give rise to unmyelinated C-fibres. Functionally, PSNs can be divided into two main categories: first, PSNs which transmit non-noxious mechanical and thermal stimuli and largely correspond to type A PSNs with thick and thinly myelinated axons. The other category includes nociceptive PSNs, which transmit impulses evoked by non-noxious thermal and noxious mechanical, thermal and chemical stimuli and are comprised largely of type B PSNs.

Noxious stimuli inflicted onto the skin, muscles and visceral organs are detected by nociceptors. In the early 1950s, Nikolaus (Miklós) Jancsó discovered that local or systemic administrations of capsaicin (8-methyl-N-vanillyl-6-nonenamide), the main, active, highly pungent constituent of chilli peppers at high concentrations produced insensitivity to painful stimuli induced by chemical irritants, but not by mechanical stimuli. Since then, the term "capsaicin desensitization" is used to describe this phenomenon. Jancsó also observed that local application of capsaicin and mustard oil (allyl-isothiocyanate) onto the skin produces not only burning pain, but local vascular reactions such as vasodilation and plasma extravasation in rats. This observation suggested that the role of chemosensitive sensory fibres which are sensitive to capsaicin may be more complex than merely the transmission of nociceptive stimuli. It has been revealed that these nerves have a dual function: on the one hand, they convey nociceptive impulses toward the central nervous system (sensory afferent function) and, on the other hand, they play a crucial role in local tissue reactions such as vascular, contractile and inflammatory responses including, in particular, neurogenic inflammation (sensory efferent or local regulatory function).

The discovery of the selective neurotoxic action of capsaicin has permitted the identification of PSNs sensitive to capsaicin as a unique class of chemosensitive PSNs which comprise a morphologically, functionally and neurochemically distinct population of spinal and cranial C-fibre sensory ganglion cells (Jancsó G. et al., 1977). Histochemical and immunohistochemical
studies have also demonstrated that chemosensitive PSNs are made up of two distinct subpopulations: peptidergic and non-peptidergic classes. Peptidergic neurons contain sensory neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), which have been shown to mediate neurogenic plasma extravasation and neurogenic sensory vasodilatation, respectively, collectively termed the neurogenic inflammatory response. Non-peptidergic PSNs are characterized by the binding of the plant lectin, isolectin B4 (IB4) from *Griffonia simplicifolia*. Both populations of chemosensitive PSNs express the transient receptor potential vanilloid type 1 receptor (TRPV1) discovered by Michael J. Caterina and his colleagues. This non-selective cation channel, termed the capsaicin receptor or TRPV1 is specifically sensitive to capsaicin and related compounds, the vanilloids, and is also sensitive to noxious heat. It has also been demonstrated that TRPV1, the activation of which mediates the effects of capsaicin, is selectively expressed in a subset of small-medium sized PSNs.

In recent decades, TRPV1-expressing chemosensitive PSNs have gained increasing significance in the understanding of the functions of many somatic and visceral organs under physiological and pathological conditions including, for example, inflammatory changes of the skin, the dura mater, the urinary bladder and both the exocrine and endocrine pancreas. Nevertheless, it has also been shown that endogenous agents such as insulin can modulate these processes by the regulation of the TRPV1.

Over the past few decades, several studies have shown that insulin, apart from being a pivotal regulator of body metabolism, is significantly involved in various neuronal processes such as neural survival, initiation of neurite outgrowth and regulation of neuronal activity. It has also been revealed that neural effects of insulin are mediated by the insulin receptor (InsR), which has been demonstrated in the nerve tissue, too. Experiments on cultured rat PSNs demonstrated that insulin enhances the capsaicin-induced cobalt uptake resulting from the activation of the TRPV1. Further, it has been suggested that interaction among insulin, the InsR and the TRPV1 expressed in PSNs may contribute to physiological and pathophysiological processes. Immunohistochemical studies provided further support to this notion by showing a substantial co-localization of the TRPV1 and the InsR in rat and mouse PSNs of unidentified target innervation territories.

**AIMS OF THE STUDY**
Previous observations demonstrated a functional interaction between the InsR and the TRPV1 in vitro, and a substantial co-localization of the InsR with the TRPV1 in rat and mouse native dorsal root ganglion (DRG) neurons. Although these studies have provided clear evidence for the localization and functionality of the InsR in PSNs, information is not available as to the target specificity of InsR-positive PSNs. The exploration of the target organs innervated by PSNs which express the InsR is of critical importance for the understanding of the possible role of the InsR in the mechanism of physiological and pathological processes.

Therefore, the principal aim of the present Thesis was the morphometric and neurochemical characterization of InsR-expressing nociceptive PSNs innervating somatic (skin and skeletal muscle) and visceral (pancreas and urinary bladder) organs with particular emphasis on the co-localization of the InsR with the TRPV1 by using retrograde neuronal tracing techniques combined with quantitative morphometry and immunohistochemistry.

Recent observations indicated that an interplay among insulin, the sensory neuropeptides SP and CGRP and pancreatic PSNs which express the nociceptive ion channel TRPV1 may significantly contribute to pathological processes affecting both the exocrine and the endocrine pancreas. Therefore, an additional aim of the present Thesis was the further phenotypic characterization of pancreatic nociceptive DRG and nodose ganglion (NG) neurons by using quantitative morphometric and immunohistochemical techniques to demonstrate the expression and co-expression patterns of the InsR, TRPV1, SP and CGRP.

**MATERIALS AND METHODS**

**Surgical procedures**

Adult male Wistar rats (n=17) weighing 300-350 g were used in this study. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) or with isoflurane. For the identification of spinal cutaneous, muscle, urinary bladder, and spinal and vagal pancreatic PSNs, biotin-conjugated wheat germ agglutinin (bWGA; 1% in distilled water) was injected into the target organs using a Hamilton microsyringe. For retrograde labelling of cutaneous, muscle, urinary bladder and pancreatic PSNs, bWGA was injected into to the dorsal hind paw skin, the gastrocnemius muscle, the wall of the urinary bladder and the head, the body and the tail of the pancreas. After recovery from anaesthesia, the rats were returned to the animal house.

**Histological methods**
Three days after the injection of bWGA, the rats were terminally anesthetized with an overdose of thiopental sodium (150 mg/kg i.p.) and were perfused transcardially first with physiological saline (100 ml), immediately followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). To examine neurons retrogradely labelled from the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder and the pancreas, the L₃-L₅, L₄-5, L₅-S₁ and Th₁₀-₁₃ DRGs, respectively, were removed on both sides. In the case of the pancreas, the right and left NGs were also removed. The samples were post-fixed for 2 h and stored in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose at 4°C until sectioning. Serial frozen sections of the DRGs and the NGs, 15 µm in thickness, were cut on a cryostat.

**Immunohistochemistry**

Sections were rinsed twice for 10 minutes in phosphate-buffered (0.01 M) isotonic NaCl solution (phosphate-buffered saline, PBS), and incubated in PBS containing 1% donkey serum and 0.1% Triton X-100 for 12 hours and processed for staining with the indirect immunofluorescence technique using the following antibodies: rabbit anti-InsRα subunit antibody (1:500), guinea pig anti-TRPV1 antibody (1:1500), mouse anti-CGRP antibody (1:1500), and guinea pig anti-SP antibody (1:1500). Donkey anti-rabbit IgG labelled with DL488 (1:500), donkey anti-guinea pig IgG labelled with Cy3 (1:500) and donkey anti-mouse IgG labelled with Cy3 (1:500) were used as secondary antibodies. bWGA lectin was detected by using an extravidin-AMCA conjugate. Sections were incubated in the presence of the primary antibodies overnight at 4°C and, after washing with PBS for 3x10 min, incubated for 2 hours with the secondary antibodies. Control procedures for immunolabelling were performed by replacing the primary antisera with normal donkey serum. No immunostaining was observed in control experiments. For fluorescence microscopy, the specimens were covered with Prolong Gold antifade mounting medium.

**Data analysis**

DRG and NG neurons with clear-cut nuclei were selected and analysed in systemic random serial photomicrographs taken with a Leica DMLB fluorescence microscope under x40 magnification, 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 selected sections of the ganglia. bWGA-positive neurons with visible nuclei were selected and analysis of retrogradely labelled neurons was performed by using the ImageJ image analysis software.
The mean cross-sectional areas of the total and the labelled populations were determined. Size-frequency distribution histograms were created by using the ImageJ software. The relative proportions of retrogradely labelled InsR-, TRPV1-, CGRP- or SP-immunoreactive (IR) DRG and NG neurons were calculated in each ganglion, and pie charts and tables were generated.

All data are presented as mean ± standard deviation (SD). Statistical comparisons of the data were performed using the Fisher’s exact probability test utilizing the Dell Statistica software. A p value of ≤0.05 was considered as a statistically significant difference between groups.

RESULTS

Retrograde labelling of DRG and NG neurons innervating somatic (skin, muscle) and visceral (urinary bladder, pancreas) organs

After the injection of retrograde tracer into the target organs, the tracer is rapidly taken up by sensory nerves and transported centripetally to the parent cell bodies. In this study, by using bWGA as a retrograde tracer, we identified numerous labelled neurons in the DRGs and NGs relating to the injected organ. Our findings showed that neurons retrogradely labelled with bWGA belonged to the small to medium sized populations of DRG and NG neurons.

Injection of bWGA into the dorsal hind paw skin resulted in the labelling of numerous neurons in the L3-L5 DRGs with a peak at the L4 DRGs. 147 retrogradely labelled DRG neurons innervating the dorsal hind paw skin were identified and analysed from three animals. Analysis of size-frequency distribution histograms revealed that the mean cross-sectional area of the retrogradely labelled neurons amounted to 311.1±43.4 µm².

Quantitative analysis revealed that after the injection of bWGA into the gastrocnemius muscle, 138 neurons were retrogradely labelled in the L4-5 DRGs with a peak at L5 DRGs. The mean cross-sectional area of the retrogradely labelled DRG neurons amounted to 345.8±55.9 µm².

Injection of the retrograde tracer, bWGA into the wall of the urinary bladder resulted in the labelling of a great number of neurons in the L3-S1 DRGs with a peak at L6 DRGs on both sides. We identified and analysed 225 retrogradely labelled DRG neurons from four animals. Quantitative morphometry revealed that the mean cross-sectional area of the retrogradely labelled DRG neurons amounted to 339.2±54.8 µm².
The DRG and NG neurons innervating the pancreas were identified by multiple injections of bWGA into the head, body and tail of the pancreas. Numerous retrogradely labelled neurons were found in the Th-L DRGs on both sides with a peak in Th11, and in the left and right NGs. Quantitative analysis revealed that 263 and 167 neurons were retrogradely labelled with bWGA in the DRGs and NGs from four animals. The analysis of the size frequency distribution of the retrogradely labelled DRG and NG neurons revealed that these neurons are small to medium sized with mean cross-sectional areas of 447.2±39.4 µm² and 585.4±44.9 µm², respectively.

TRPV1 expression in identified somatic and visceral DRG and NG neurons

The large majority of bWGA-labelled cutaneous, muscle, urinary bladder and pancreatic DRG and pancreatic NG neurons showed TRPV1 immunoreactivity. In the DRGs, 63.1±3.4% 62.5±2.7%, 65.0±1.8% and 68.2±4.8% of the retrogradely labelled neurons innervating the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder and the pancreas displayed TRPV1 immunoreactivity. In the NGs, 64.0±3.9% of the identified pancreatic neurons showed TRPV1 immunopositivity.

InsR expression in retrogradely labelled DRG and NG neurons

A relatively high proportion of DRG neurons labelled retrogradely with bWGA innervating the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder and the pancreas displayed InsR immunostaining. Similarly, many retrogradely labelled pancreatic NG neurons showed InsR immunoreactivity. Our quantitative data indicate that 22.4±2.8% of cutaneous, 21.8±1.9% of muscle, 53.4±3.1% of urinary bladder, 48.3±2.6% of pancreatic DRG and 49.1±2.5% of pancreatic NG neurons displayed InsR immunoreactivity. The statistical analysis revealed that there were no significant differences in the proportions of the InsR immunoreactive neurons either between cutaneous and muscle DRG neurons or between pancreatic and urinary bladder spinal afferents. However, a highly significant difference between the proportions of the InsR-immunoreactive somatic and visceral DRG neurons was revealed (p < 0.05).

Co-localization of the InsR with the TRPV1 in retrogradely labelled somatic and visceral DRG and NG neurons

To reveal the co-localization of the InsR with the TRPV1, the co-expression of the InsR and the TRPV1 in the bWGA-labelled DRG and NG neuron populations, the expression of the TRPV1 in the InsR-immunopositive bWGA-labelled DRG and NG neuron populations and the
expression of the InsR in the TRPV1-immunopositive bWGA-labelled DRG and NG neuron populations were examined.

First, the co-localization of the InsR with the TRPV1 was analysed in retrogradely labelled DRG and NG neurons. Our data indicated that 16.56±0.6% of cutaneous, 15.33±1.1% of muscle, 30.34±2.1±% of urinary bladder, 23.2±2.2% of pancreatic DRG and 35.3±1.7% of pancreatic NG neurons displayed both InsR and TRPV1 immunoreactivity, respectively. Hence, these quantitative data suggested a higher co-localization rate of the InsR and the TRPV1 in visceral than somatic PSNs. Statistical analysis revealed significant differences (p < 0.05) in the proportions of somatic and visceral DRG neurons which co-expressed the InsR and the TRPV1.

The proportions of the TRPV1-immunopositive neurons in the bWGA-labelled InsR-immunoreactive neuron population were also assessed. 72.7±3.4%, 73.3±2.6%, 57.1±3.6%, and 50.1±3.0% of the bWGA-labelled InsR-immunoreactive DRG neurons innervating the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder and the pancreas displayed TRPV1 immunoreactivity, respectively. Furthermore, our data indicate that 71.0±5.0% of the bWGA-labelled InsR-immunoreactive pancreatic NG neurons showed TRPV1-immunoreactivity. The statistical analysis revealed that there were no significant differences in the expression of TRPV1 among the five different populations of neurons.

The expression of the InsR in the retrogradely labelled TRPV1-immunopositive DRG and NG neuron populations was also revealed. In the DRGs, 25.8±2.2%, 25.5±2.4%, 43.9±2.3% and 34.0±1.96% of the bWGA-labelled TRPV1-immunoreactive neurons innervating the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder and the pancreas showed InsR-immunoreactivity, respectively. Of the bWGA retrogradely labelled TRPV1-immunoreactive pancreatic NG neurons, 55.4±2.0% showed InsR immunoreactivity. The statistical analysis revealed that there were no significant differences in the proportions of the InsR-immunoreactive neurons between either the cutaneous and the muscle DRG neurons or between the pancreatic DRG and NG and the urinary bladder DRG neurons. However, the differences between the cutaneous and pancreatic DRG neurons, the cutaneous and bladder DRG neurons, the muscle and pancreatic DRG neurons and the muscle and urinary bladder DRG neurons were significant (p < 0.05).
Co-localization of the InsR with sensory neuropeptides in pancreatic DRG and NG neurons

Considering the importance of sensory neuropeptides and the possible role of insulin and the InsR in pathologies of the pancreas, the co-localization patterns of the InsR, CGRP and SP, were analysed in pancreatic DRG and NG neurons.

Our data revealed that 33.2±3.7% and 54.3±4.4% of retrogradely labelled DRG neurons innervating the pancreas displayed SP and CGRP immunoreactivity. In the NGs, 40.0±2.1% and 25.1±2.9% of retrogradely labelled pancreatic neurons showed SP and CGRP immunoreactivity.

In the DRGs and the NG 14.4±1.2% and 24.2±1.0% of retrogradely labelled neurons showed InsR and SP co-localization. Further, 28.4±1.3% and 46.2±1.9% of the retrogradely labelled pancreatic InsR-immunopositive DRG and NG neurons displayed SP immunoreactivity. Conversely, 42.0±4.8% and 60.2±4.2% of the labelled SP-IR DRG and NG neurons were IR for the InsR.

The co-localization of the InsR with CGRP was also examined. Our data revealed that 28.4±2.7% and 8.0±0.9% of the retrogradely labelled pancreatic DRG and NG neurons exhibited both InsR and CGRP immunoreactivity. Further, of the retrogradely labelled InsR-immunopositive DRG and NG neurons, 58.3±5.3% and 17.4±3.6% displayed CGRP immunoreactivity. Conversely, 52.1±4.4% and 32.0±2.5% of the retrogradely labelled pancreatic CGRP-IR DRG and NG neurons showed InsR immunopositivity.

CONCLUSIONS

The studies presented in this Thesis have revealed the morphometric and neurochemical characteristics of InsR-expressing PSNs innervating somatic and visceral organs. The phenotypic features of pancreatic nociceptive DRG and NG neurons were assessed in detail.

The experiments summarized in this Thesis provided quantitative data, for the first time, on the expression of InsRs in PSNs innervating specific organs, including the skin, skeletal muscle, urinary bladder and the pancreas. We demonstrated that a high proportion of small to medium sized InsR-expressing PSNs display TRPV1-IR indicating that these DRG and NG neurons are nociceptive in character. Moreover, our quantitative immunohistochemical data revealed a preponderance of InsR-immunoreactivity among PSNs which innervate visceral organs. These
findings suggest that visceral PSNs are more likely to be exposed to the modulatory effects of insulin on sensory functions, including neurotrophic, nociceptive and inflammatory processes. Considering the newly found co-localization of the InsR with the TRPV1 in identified visceral DRG and NG neurons and the functional interplay between these receptors, a significant role of InsR-expressing visceral PSNs in the development of visceral inflammatory processes could be hypothesized.

In recent years, several studies have suggested that peptidergic TRPV1-expressing sensory nerves are critically involved in the development of pancreatic pathologies. Further, it has also been demonstrated that interactions of insulin and the pancreatic nociceptive afferent nerves contribute to pathological processes affecting both the exocrine and the endocrine pancreas. Our quantitative data provide morphological basis for possible functional interactions among the nociceptive ion channel TRPV1, the InsR, and the proinflammatory neuropeptides SP and CGRP expressed by pancreatic DRG and NG neurons. Based on these observations, a new, proinflammatory role of insulin in the pathomechanism of pancreatic pathologies may emerge.
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