PACAP and orphanin FQ/nociceptin -Distribution, importance and regulation in sensory neurons and spinal cord

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PACAP and orphanin FQ/nociceptin - Distribution, importance and regulation in sensory neurons and spinal cord

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Contents

Original Papers

The thesis is based on the work in the following papers, which will be referred to by their roman numerals.

I. **Pettersson LME**, Sundler F, Danielsen N. Expression of orphanin FQ/nociceptin and its receptor in rat peripheral ganglia and spinal cord. *Brain Res* 945:266-275, 2002.

II. Jongsma***** H, **Pettersson* LME**, Zhang YZ, Kvist Reimer M, Kanje M, Waldenström A, Sundler F, Danielsen N. ***Contributed equally to the manuscript: shared first authorship**. Markedly reduced chronic nociceptive response in mice lacking the PAC_1 receptor. *Neuroreport* 12:2215-2219, 2001.

III. **Pettersson LME**, Heine T, Verge VMK, Sundler F, Danielsen N. PACAP mRNA is expressed in rat spinal cord neurons. *J Comp Neurol* 468 (*in press*), 2004.

IV **Pettersson LME**, Dahlin LB, Danielsen N. Changes in expression of pituitary adenylate cyclase activating polypeptide in rat sensory neurons in response to sciatic nerve compression (*in manuscript*), 2004.

V **Pettersson LME**, Geremia NM, Verge VMK, Danielsen N. Endogenous BDNF regulates the expression of PACAP in rat DRG neurons after sciatic nerve injury (*in manuscript*), 2004.

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Brain Research, Elsevier Science: Paper I; Neuroreport, Lippincott Williams & Wilkins: Paper II; The Journal of Comparative Neurology, Wiley-Liss, Inc: Paper III.

Thesis at a glance

***Paper I**: *" Expression of orphanin FQ/nociceptin and its receptor in rat peripheral ganglia and spinal cord"*

Aim: Investigate the expression of the neuropeptide orphanin FQ/nociceptin and its receptor ORL1 in the adult rat spinal cord, DRG, SCG and skin.

Methods: Immunohistochemistry, in situ hybridization, ^{125}I-[Tyr^{14}]-OFQ/N binding. *Unilateral inflammation*: Was established by subcutaneous injections of FCA at the tarso-tibial joint.

Results & conclusions:
In the intact adult rat, OFQ/N and ORL1 are expressed throughout the spinal cord gray matter and concentrated in neurons in the more superficial laminae and in ventral horn neurons. Expression and ligand binding are also observed in small (OFQ/N) and large (ORL1) diameter DRG neurons. No peripheral source of OFQ/N or ORL1 could be established in skin from the hindpaws (intact skin and 3 days after FCA injection). The expression of both OFQ/N and ORL1 in DRG and spinal cord dorsal horn, areas involved in nociceptive signalling, gives morphological support to the notion that OFQ/N has a role in nociceptive responses.

***Paper II:** *" Markedly reduced chronic nociceptive response in mice lacking the PAC_1 receptor"*

Aim: Evaluate the phenotypic behaviour of PAC_1-/- mice, and hence the importance of the PAC_1 receptor and PACAP for responses to chemical and thermal nociceptive and tactile stimulation. Investigate the neuropeptide expression in DRG in PAC_1-/- mice in response to injury of the sciatic nerve.

Methods: In situ hybridization, [^{125}I]-PACAP-27 binding.
Animals: PAC_1 -/- mice were compared with their wildtype littermates
Nerve injury: Unilateral nerve crush of the sciatic nerve in the upper hip region.
Formalin test: After a unilateral, subcutane injection of paraformaldehyde under the dorsal surface of the hindpaw, animals were observed in a glass container for 60 minutes to evaluate their nociceptive behaviour (register paw licking/biting time as estimate of nociceptive behaviour).
Thermal & mechanical stimulation: Animals (tail and hindpaw) were stimulated with a CO_2 laser for evaluation of response to thermal stimulation, and with von Frey monofilaments for evaluation of responses to mechanical stimulation. The withdrawal reflexes of the stimulated extremity were

documented with a digital videocamera and the nociceptive/tactile reflex threshold and latencies were calculated.

Results & conclusions: PAC_1-/- mice had an increased (doubled) nociceptive behaviour in the acute phase after formalin injection, whereas they showed a marked decrease (75% reduction) in nociceptive responses during the inflammatory phase of the formalin test. No differences in thermal response (tail and hindpaw), or response to mechanical stimulation (tail) was observed. A slightly reduced tactile reflex threshold was detected in PAC_1-/- mice after stimulation of the hindpaw. More pronounced injury induced changes in neuropeptide expression, especially of galanin, was observed in PAC_1-/- mice DRG after 4 or 10 days of sciatic nerve crush. [^{125}I]–PACAP-27 binding was very meagre in PAC_1-/- mice. The results clearly indicate that the PACAP specific receptor, PAC_1, is involved in modulation of nociceptive responses during chronic conditions such as inflammation. Further, the injury induced changes i neuropeptide expression in DRG were more pronounced in PAC_1-/- mice, implicating a neuroregulatory role for PAC_1.

***<u>Paper III</u>:** *"PACAP mRNA is expressed in rat spinal cord neurons"*

Aim: Investigate the expression of PACAP in intact adult rat spinal cord and after proximal or distal sciatic nerve transection, respectively.

Methods: Immunohistochemistry, and quantitative in situ hybridization.
Sciatic spinal (proximal) nerve injury: Transection of the sciatic nerve at its origin from the L4 and L5 spinal nerves.
Distal nerve injury: Transection of the sciatic nerve at mid-thigh level.

Results & conclusions: PACAP expression was detected in neurons primarily in the superficial layers of the intact spinal cord, but also in neurons in deeper laminae of the dorsal horn, as well as in ventral horn neurons. Both after proximal and distal sciatic nerve injury, the expression of PACAP was induced in ventral horn neurons coexpressing VAChT. No change in expression was observed in the dorsal horn neurons after injury. A more pronounced increase in ventral horn PACAP expression was found after proximal injury than after distal nerve injury. The findings gives a morphological basis which supports a role for PACAP in sensory signalling (expression in superficial laminae of the spinal cord) as well as in repair/regeneration in motor neurons after nerve injury (induction of expression of PACAP in VAChT positive ventral horn neurons).

***Paper IV:** *"Changes in expression of pituitary adenylate cyclase activating polypeptide in rat sensory neurons in response to sciatic nerve compression"*

Aim: Investigate the effects of sciatic nerve compression on PACAP and PAC_1 expression in adult rat sciatic nerve, DRG and spinal cord, and compare it with the changes induced by nerve transection or peripheral inflammation.

Methods: Immunohistochemistry, quantitative in situ hybridization, [^{125}I]-PACAP-27. *Compression injury:* Unilateral compression of a 1cm long segment of the sciatic nerve at mid-thigh level. The nerves were compressed with a silicone tube for 3, 7, 14 or 28 days.

Results & conclusions: PACAP expression was upregulated in DRG neurons (both small and larger) after 3, 7, 14 and 28 days of nerve compression. No obvious changes in PACAP or PAC_1 expression in spinal cord, and no induction of PAC_1 expression in DRG neurons were detected after compression. The pronounced upregulation in PACAP expression in the DRG neurons indicates that PACAP is involved in the response also to nerve compression injury, in addition to its suggested role during nerve transection and inflammation.

***Paper V:** *"Endogenous BDNF regulates the expression of PACAP in rat DRG neurons after sciatic nerve injury"*

Aim: Evaluate the effects of endogenous BDNF on the regulation of PACAP expression during sciatic spinal nerve transection.

Methods: Quantitative in situ hybridization.
Sciatic spinal nerve injury: Transection of the sciatic nerve at its origin from the L4 and L5 spinal nerves in conjuntion with i) intrathecal infusion of anti-BDNF via mini-osmotic pumps, or ii) intraperitoneal injections of anti-BDNF.

Results & conclusions: The upregulation in PACAP expression observed in response to nerve transection was drastically mitigated by intrathecal administration of anti-BDNF. The effect was seen in both small and large diameter DRG neurons after intrathecal administration of anti-BDNF, and both the number of neurons and the expression density in individual neurons was decreased with intrathecal antibody treatment. Further, the anti-BDNF administration affected the PACAP expression in neurons positive for trkB mRNA, and also in neurons not obviously expressing trkB. Intraperitoneal injections of anti-BDNF did not have any evident effect on the PACAP expression. The findings indicate a role for endogenous BDNF in modulation of the peptidergic phenotype in DRG neurons in conjunction with nerve injury.

Abstract

Neuropeptide expression in the nervous system is abundant and plastic, and an altered expression after injury is an example of a functional response, helping the neuron to cope with adverse changes, and involves effects on differentiation, synthesis, repair/regeneration, survival, and modulation of signal transmission. I have investigated the distribution/expression of the neuropeptides, orphanin FQ/nociceptin (OFQ/N) and pituitary adenylate cyclase activating polypeptide (PACAP), under normal conditions, in response to nerve injuries, and also studied sensory responses in mice deficient for the PAC_1 receptor (PAC_1-/-).

Expression of OFQ/N and its receptor is demonstrated in neurons in spinal cord dorsal and ventral horns, DRG and SCG. Expression in specific neurons in these tissues gives a morphological basis supporting their suggested role as modulators of sensory, especially nociceptive, transmission. Further modulatory roles are suggested by expression in motor neurons and SCG.

Expression of PACAP in spinal cord dorsal and ventral horn neurons, and induced expression in motor neurons in response to sciatic nerve injury, is demonstrated. Further, PACAP expression is induced in DRG neurons in response to nerve transection, and compression injury. Intrathecal anti-BDNF infusion mitigates this injury induced expression, suggesting that endogenous BDNF can regulate PACAP expression. The injury induced PACAP expression, in DRG and motor neurons, indicates a possible role for PACAP in repair/regeneration and modulation of the sensory/nociceptive and motor transmission. More direct evidence for this is the finding of a pronounced decrease in pain behaviour in PAC_1–/– mice, strongly suggesting a role for PACAP in modulation of inflammatory pain.

List of abbreviations

BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CGRP	calcitonin gene-related peptide
DRG	dorsal root ganglion
FCA	Freund´s complete adjuvant
GAP-43	growth associated protein 43
IgG	immunoglobulin G
L4, L5	lumbar level 4, lumbar level 5
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NT-3	neurotrophin 3
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	phosphate buffered saline
PKC	protein kinase C
OFQ/N	orphanin FQ/nociceptin
OligoDNA	oligodeoxyribonucleotide DNA
ORL1	opioid receptor like receptor
Trk (A, B, C)	tyrosin kinase A, B, C
SCG	superior cervical ganglion
SSC	saline sodium citrate
VAChT	vesicular acetylcholine transporter

Introduction

Neuropeptides have important functions as transmitters in the nervous system, as well as being modulators affecting the signal efficacy of other transmitters. This gives them a role in signalling in the normal, intact condition, although they might have their most important actions during conditions when the nervous system is stressed or challenged e.g. during a nerve injury. In spite of the extensive research around the distribution and importance of neuropeptides in sensory neurons, much is still unknown. I have focused on the expression, distribution, function and regulation of PACAP and its receptor PAC_1 in particular, but also to some extent the expression and distribution of OFQ/N and its receptor ORL1 in sensory neurons and their projection areas, in intact animals and after different types of injuries. The work has been undertaken to further elucidate neuropeptide expression and what takes place during different injury conditions. An increased understanding of the expression and regulation of neuropeptides opens up the possibility for pharmacological interventions and drug development.

A description of the morphological structures which have been studied in this work is presented below, as well as a summary of the processes involved in nerve injuries. An overview of neuropeptides and neurotrophins and their functions and regulation is also presented.

The peripheral nerves and sensory receptors

Peripheral nerves are composed of myelinated and unmyelinated axons, blood vessels, and the surrounding connective tissues, endoneurium, perineurium and epineurium. The axons in the peripheral nerves originate from sensory neurons, as well as somatic and autonomic motor neurons, i.e. both afferent and efferent neurons convey information in the same nerve trunk. The rat sciatic nerve, the nerve which was used for experimentation in this thesis, is composed of ~27 000 axons at midthigh level; 6% myelinated motor axons, 23% myelinated and 48% unmyelinated sensory axons, and 23% unmyelinated sympathetic axons (Schmalbruch, 1986).

The sensory nerve fibers are grouped into Aα and Aβ fibers, the largest myelinated fibers with conducting velocities of 30-100 m/s, Aδ small myelinated fibers which conduct at 4-30 m/s, and small, unmyelinated C fibers with conduction velocities of less than 2.5 m/s. In general there is a relation between the cell body size of the DRG neurons and their axonal size, small neurons give rise to small axons, and large neurons being the origin of large axons. Some evidence of this is the finding that both unmyelinated axons and small DRG neurons are lost after neonatal capsaicin treatment (Willis and Coggeshall, 1991, chapter 2-3).

The axons of the somatic motorneurons are myelinated and these neurons are divided into Aα (alpha motor neuron) and Aγ (gamma motor

neuron) fiber types. Aα fibers project to extrafusal muscle fibers and and Aγ fibers to the intrafusal fibers of the muscle spindle thereby controlling the muscle contraction (Brodal, 2004, chapter 11).

Sensory receptors located in the skin and subcutaneous connective tissue include *nociceptors*, *mechanoreceptors* and *thermoreceptors*. Viscera also contain mechanoreceptors and in some organs nociceptors.

The *nociceptors*, are of two main categories, Aδ mechanical nociceptors and C polymodal nociceptors, and are named after the size of the afferent fibers originating from them, and the type of stimuli activating them. Morphologically, nociceptors are bare nerve endings. Some other cutaneous nociceptors that respond to combinations of intense mechanical, thermal and chemical stimuli include Aδ mechanoheat nociceptors, Aδ and C cold nociceptors, and C mechanical nociceptors. The nociceptors have thresholds of varying levels but are best excited by noxious stimuli (Kandell et al., 2000, chapter 22, 24; Willis and Coggeshall 1991, chapter 2; Brodal, 2004, chapters 5-6).

- *Aδ mechanical nociceptors* are excited by mechanical stimuli that damage the skin. Their terminals are ensheated by Schwann cells in the epidermis. These nociceptors do not respond to noxious heat, intense cold or algesic chemicals, although, repetetive noxious heat stimuli can sensitize them.

- *C polymodal nociceptors* are responding to noxious mechanical, thermal and chemical stimuli. The effective thermal stimuli are noxious heat (>45°C) and sometimes intense cold, and chemical stimulation with injection of algesic chemicals or topical application of acid leads to activation. Some cutaneous nerve terminals probably belonging to nociceptors have been found to show immunoreactivity for calcitonin gene-related peptide (CGRP), substance P (SP) and other peptides, which may reflect a synthesis of these peptides for transport and release both centrally as neurotransmitters/modulators, and peripherally with a role e.g. in neurogenic inflammation.

- *Silent nociceptors*, nociceptors purely sensitive to chemical substances released during inflammation. These receptors require stimulation of 10-20 minutes to become activated, after which they can fire for hours.

Mechanoreceptors mediate their signals via Aβ fibers, and respond to mechanical stimulation and in some cases thermal changes, they can have a slow or fast rate of adaptation, and the sensory thresholds vary between the different types. Meissner´s and Pacinian corpuscles are two examples of rapidly adapting receptors, and Merkel receptors and Ruffini corpuscles are examples

of slowly adapting receptors, all in glabrous (nonhairy) skin. Hairy skin, covering most of the body, also contains Pacinian and Ruffini corpuscles, Merkel receptors and hair receptors.

Mechanoreceptors such as muscle spindles and Golgi tendon organs register information about posture and movement of the limbs, so called proprioreception, and in concert with mechanoreceptors in the connective tissue capsule at the joints, on the soles of the feet, the vestibular organ in the inner ear, and the photoreceptors in the retina, this information helps us to maintain balance as well as keeping track of the position of different parts of the body (Kandell et al., 2000, chapter 22 ; Willis and Coggeshall 1991, chapter 2; Brodal, 2004, chapters 5-6, 12).

There are two kinds of *thermoreceptors* in the skin responding to innocuous changes in temperature, cold and warm receptors. These receptors respond very poorly, if all, to mechanical stimuli (Kandell et al., 2000, chapter 22; Willis and Coggeshall 1991, chapter 2; Brodal, 2004, chapters 5-6).

- *Cold receptors* are innervated both by myelinated Aδ and unmyelinated C afferent fibers, and have a background discharge rate at normal skin temperature. Cold receptors increase their firing rate with falling skin temperatures and are activated by very small temperature changes. Noxious heat (> 45°C) can also evoke a response in this type of receptors, giving rise to a curious sensory illusion of a cold stimulus, the paradoxical cold response.

- *Warm receptors*, presumably free nerve endings, are responding to innocuous heat, i.e. <45°C and the signal is mediated via unmyelinated C fibers, whereas myelinated Aδ fibers, mediate noxious heat (see above).

The dorsal root ganglion

The cell bodies of the afferent sensory neurons reside in the dorsal root ganglia (DRG), and their cranial nerve equivalents. The ganglia are named and numbered according to the level of the spinal cord vertebra where they are positioned. These primary afferent sensory neurons are pseudounipolar cells (Tandrup, 1995) with peripheral process/processes which receive information from their respective sensory receptor in the periphery and a central process/processes that transmit information to the central nervous system. The central process of the neuron travel via the dorsal root into the spinal cord dorsal horn, and are therefore also referred to as dorsal root axons. The neuronal cell bodies in the DRG are a heterogenous population with different diameters, peptide expression, physiological characteristics etc (Willis and Coggeshall, 1991, chapter 3). The cell bodies are divided primarily into two categories with respect to size (Giuffrida and Rustioni, 1992), i.e small diameter (<35 μm) and large diameter (>35 μm) neurons. Neurons have also

been characterized according to their conduction velocities and their correlation with the size of the soma and axon, as well as the type of information that is transmitted in the neuron. Simplified, small cell bodies with unmyelinated small diameter axons usually conduct at lower velocities and are involved in nociceptive transmission, whereas larger cell bodies with myelinated large axons conduct at greater velocities and primarily transmit information about mechanical stimulation. In addition to these properties, the cytology of the DRG neurons is also considered and cells have been characterised as light (clear) cells (cell bodies of various diameters, although originally primarily large cells) and dark cells (always small diameter cells) (Willis and Coggeshall, 1991, chapter 3).

The presence of different morphological markers, such as peptides, receptors and enzymes is one useful way to characterize DRG neurons, altough it is still not fully known what the functional significance of many of these markers is (Fig. 1). Further, the expression of some peptides is quite plastic and varies in response to injuries and inflammation, (Hökfelt et al., 2000; and see; Neuropeptides). Small DRG neurons can be divided into two subpopulations, a peptidergic ~30-35%, and a non-peptidergic ~30% of the total neuronal population in the DRG, respectively. Glutamate is abundantly distributed and functions as a transmitter in most, if not all, of the primary sensory afferents (Broman et al., 2000). In addition, the peptidergic neurons also express e.g. calcitonin gene-related peptide (CGRP), substance P (SP), and the high affinity receptor for NGF; tyrosine kinase A (trkA), (Willis and Coggeshall, 1991, chapter 3; McMahon et al., 1997). Non-peptidergic neurons, bind the lectin IB4, are positive for thiamine monophosphatase, and express the tyrosine kinase receptor for glial cell line-derived neurotrophic factor, Ret (Molliver et al., 1997; Bennett et al., 1998). Large cells constitute ~30-40% of the total neuronal population in the DRG, and show immunoreactivity for RT97, an antibody recognizing neurofilament 200 (200kDa), and the calcium binding proteins S100 and parvalbumin (Lawson et al., 1984; Ichikawa et al., 1997). Further, large neurons express the high affinity receptors for BDNF and NT-3; trkB and trkC respectively, in the normal state (McMahon et al., 1997).

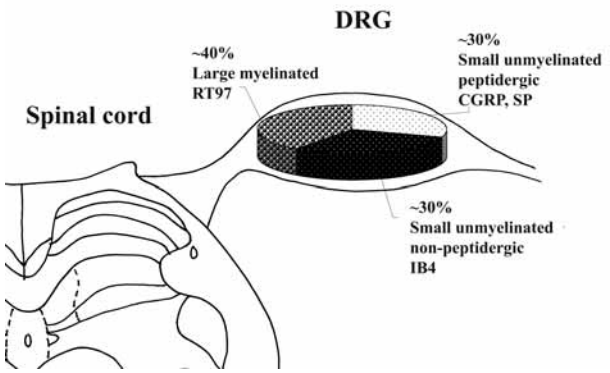

Fig. 1. Characterization of DRG neurons based on the presence of different morphological markers. DRG neurons can be divided into three groups (see above), which have only minimal overlap (based on McMahon et al., 1997).

The spinal cord

The spinal cord, which is part of the central nervous system, is divided into white and gray matter. The white matter, surrounding the central gray matter, consists of mostly myelinated afferent and efferent axons connecting neurons at different levels with each other. The gray matter contains sensory and motor neurons, as well as interneurons, and it is divided into a dorsal and a ventral part. The neurons in the dorsal horn (laminae I-VI) are involved in sensory processing, and neurons in the ventral horn are involved in transmission of somatic motor signals (laminae VIII, IX). The area in between the dorsal and the ventral horn is called the intermediate zone (lamina VII) and contains mainly interneurons projecting primarily to other laminae at the same segmental level, but cell bodies for autonomic preganglionic fibers are also present in this lamina. The sympathetic preganglionic cell bodies are located laterally in the intermediolateral column (IML) at the T1-L3 level (in the rat), whereas parasympathetic cell bodies, at the sacral levels, reside in an area corresponding to the IML. Lamina X is the area surrounding the central canal and contains neurons responding to noxious cutaneous stimulation and possibly also cells receiving input from visceral afferents (Molander et al., 1984; Willis and Coggeshall, 1999, chapter 4, 5; Brodal, 2004, chapters 3, 6, 11, 18).

The cell bodies of the primary afferent sensory neurons reside in the DRG and their axons transmit sensory information from the periphery, via the dorsal root, to the spinal cord dorsal horn where they release transmitter substances. It has also been shown that stimulation of peripheral nerves can lead to a release of neuropeptides in the periphery. Such a release can in some cases lead to induction of neurogenic inflammation. Unmyelinated, small diameter fibers mediating pain and temperature terminate in laminae I-II, although the majority of the C fibers project to lamina II. Small myelinated

(Aδ) fibers terminate in laminae I, II and V, and large myelinated (Aβ) fibers terminate in laminae III-VI. The thickest myelinated, Aα, fibers from muscle spindles and tendon organs project to laminae VI, VII and IX (Brodal, 2004) (Fig. 2). Sensory primary afferents either synapse on interneurons in the different laminae, or directly on motor neurons e.g. as in the monosynaptic stretch reflex, and/or project collaterals centrally. The major ascending projections transmitting sensory information to the cortex are the spinothalamic tract and the dorsal column/medial lemniscus pathway. Information about pain, temperature and crude touch run in the anterolateral spinothalamic tract. The primary afferents synapse on secondary neurons which decussate at the spinal cord level, a couple of segments below or above their entry level. Nerve fibers from the trigeminothalamic tract (pain and temperature information) converge with the spinothalamic tract at the level of the brainstem. The secondary neurons synapse in the thalamus, from where tertiary neurons project to the somatosensory and parietal cortex. Some collaterals diverge and terminate in the brain stem reticular formation or midbrain tectal region. Information about tactile stimulation and proprioception is conveyed via the dorsal column/medial lemniscus pathway, which runs ipsilateral in the dorsal column in the spinal cord, synapse to a secondary neuron in the gracile or cuneate nuclei and cross over to the other side at the level of the medulla. After the decussation, the fibers run in the medial lemniscus to the thalamus, where the synapse again, and the tertiary neurons project to the somatosensory, and parietal cortex (Kandell et al., 2000, chapter 22, 24; Brodal, 2004, chapter 6).

The somatic motor neurons, in lamina IX of the ventral horn, exit the spinal cord via the ventral roots, and enter a peripheral nerve on the way to their target tissues, skin, skeletal muscles (Aα fibers), and to the intrafusal fibers of the muscle spindles (Aγ fibers) (Nicolopoulos-Stournaras and Iles, 1983; Molander et al., 1984; Guyton and Hall, 2000 chapter 54; Brodal, 2004, chapter 11). The autonomic motor neuron cell bodies reside in the more lateral parts of the spinal cord, the parasympathetic at the cranio-sacral level and the sympathetic at the thoraco-lumbar levels of the spinal cord. Their preganglionic fibers exit the spinal cord via the ventral roots and project to the viscera and some muscles and glands in the head-neck region. The sympathetic neurons leave the spinal nerves via the white communicating rami and enter the sympathetic trunk where many of them synapse in the paravertebral ganglia. The postganglionic fibers re-enter the spinal nerve via gray communicating rami and project to their target tissues. Some preganglionic fibers pass directly through the ganglia and form separate nerves, splanchnic nerves, that project to prevertebral sympathetic ganglia, from where postganglionic neurons project to the final destination. Parasympathetic preganglionic neurons exit via the ventral root, follow the spinal nerves for a short distance, and leave them as separate, small splanchnic nerves which synapse in ganglia close to their target tissues (Molander et al., 1984; Brodal, 2004, chapter 18).

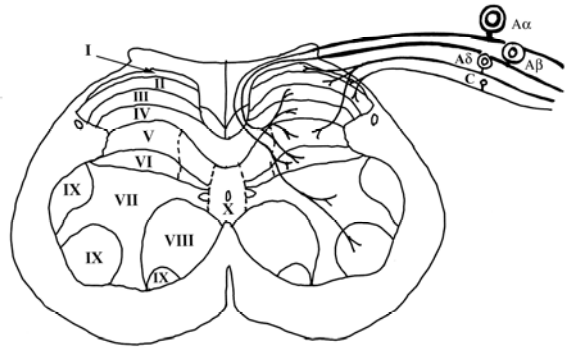

Fig. 2. Sensory input to the spinal cord. Projections of unmyelinated and myelinated small and large neurons to the spinal cord. Small, unmyelinated C fibers terminate in laminae I and II, whereas small myelinated Aδ fibers project to laminae I, II and V. The large myelinated fibers, Aβ and Aα terminate in laminae III-VI and laminae VI, VII and IX, respectively (modified after Molander et al., 1984 and Brodal, 2004).

Nerve injuries

Nerve injuries can be classified into five groups depending on the degree of injury. The definition used in the following text was described by Sunderland (1978, chapter 9).

- First degree injury: Conduction block, preserved axonal continuity and no Wallerian degeneration.
- Second degree injury: Transection of the axon and Wallerian degeneration, but preserved endoneurium.
- Third degree injury: Disruption and degeneration of the axon and endoneurium, although the perineurium is only subject to minor changes, and the general arrangement of the nerve trunk is preserved.
- Fourth degree injury: Disruption of the fascicles, continuity of the nerve trunk is maintaied by the epineurial tissue.
- Fifth degree injury: Complete loss of continuity of the nerve trunk.

When a peripheral nerve is subjected to trauma (e.g. nerve transection) the nerve can either regenerate or a permanent interruption of the neural pathway occurs, or in the worst case scenario cell death may be induced (see below), depending on the severity, location of the injury, and treatment. If the transection is complete it is considered a fifth degree injury. In the proximal

stump of the transected nerve, axons degenerate for some distance, leaving an empty endoneurial tube behind. The axons then produce a large number of sprouts that advance distally. The mechanisms inducing sprouting are not fully understood, but sprouting is probably modulated by local factors in the immediate environment. At the tip of each sprout there is a growth cone. The sprouts migrate in large numbers, surrounded by a Schwann cell (regenerating units), over the injury zone and towards the distal nerve stump. Upon reaching the distal target, there is a loss in the number of sprouts originating from the same regenerating axon. This is the same phenomenon as is seen during development of the nervous system, and results in a pruning of redundant sprouts that have not yet reached the distal target and made connections (Sunderland, 1978, chapter 7, 8; Lundborg,1988, chapter 5; Lundborg and Danielsen, 1991; Lundborg, 2000).

The distal part of the transected nerve lose contact with the cell soma and the transport of molecules essential for maintenance of the axon is interrupted. The result is that the distal part of the axon undergo Wallerian degeneration, the axon breaks down and degenerates, within the first week after transection. There is evidence that this enzymatic degeneration is calcium dependent. The Schwann cells surrounding the nerve are also affected and their myelin part degenerates and is phagocytized by both Schwann cells and invading macrophages. The Schwann cells are enlarged, undergo mitosis and form Schwann cell columns (bands of Büngner) along the region once occupied by the axon. The bands of Büngner are important as guidelines for the regenerating axon sprouts from the proximal neve stump on their way to the periphery, as well as a being a probable source of trophic molecules. Remyelination of the axons proceeds centrifugally down the fiber, and advances with the elongation of the axon sprout (Sunderland, 1978, chapter 7, 8; Lundborg,1988, chapter 5; Lundborg and Danielsen, 1991; Lundborg, 2000; Stoll et al., 2002).

The various kinds of trauma to the peripheral nerve have effects not only in the peripheral nerve fiber, but also in the cell body, which in the case of a sensory nerve, is located in the DRG. The major cell body changes that might take place in neurons after severe injury are chromatolysis (disintegration of Nissl substance, i.e. an alteration in the arrangement and concentration of the rough endoplasmic reticulum (rER), leading to a loss of the granular texture given by the Nissl substance upon staining with basic dyes), changes in the perikaryal volume, and eccentricity of the nucleus (Lieberman, 1971). These morphological changes are a superficial manifestation of major alterations in the neuronal metabolism, e.g. an increased/decreased RNA synthesis. Nerve injuries induce structural and functional changes in the cell body, redirecting the synthesis from production of transmitters, to synthesis of molecules that are important for survival, repair and regeneration (Karlström and Dahlström, 1973; Frizell and Sjöstrand, 1974). The exact signals inducing these changes are unknown, but the interruption of retrograde signalling from the target tissues may be one factor, as well as the production and release of different substances, such as cytokines and neurotrophins, at the site of injury. Nerve

crush or nerve compression also affect the peripheral nerve and the nerve cell soma, inducing similar events as those described above, although the injuries in these cases might be somewhat less severe than after a complete transection of the nerve. Nerve crush usually equals a second degree injury, and nerve compression injuries can be of various degrees, although experimentally induced compression injuries tend to be a mixture of at least a first and a second degree injury.

After severe injuries, neuronal cell death has been observed in DRG, although the studies have shown high variability in the results. Factors such as type and location of the injury, type of injured neuron, species and age differences, as well as technical factors related to estimating the size and numbers of neurons, may contribute to the variation in the results (Lieberman et al., 1974). The possibility that species and age differences influence the effect of injury on neuronal cell loss has been highlighted in several studies. Different proportions of neurons were lost after nerve transection in mice (Shi et al., 2001), kittens and cats, (Aldskogius and Risling, 1983; Risling et al., 1983), macaque monkeys (Liss et al., 1996), and rats (Tandrup et al., 2000). Further, the cell body losses were more pronounced e.g. in kittens than in cats (Aldskogius and Risling, 1983; Risling et al., 1983), reflecting the importance of age on cell survival. Further, different neuronal populations e.g. vagal and hypoglossal neurons, showed a varying susceptibility to nerve transection in rat (Aldskogius et al., 1980). Considering the consequences of the injury location, Ygge (1989) showed that there is a more pronounced cell loss in spinal primary sensory neurons after a proximal than after a distal nerve transection in rat. With the use of stereological methods, more recent studies have further investigated the effects of both proximal and distal nerve transection. Proximal nerve transection of the sciatic nerve close to the rat L5 DRG induced cell death in 22% of the neurons 15 days after transection (Vestergaard et al., 1997). A more distal transection, at mid-thigh level, induced cell death in 14% of the rat DRG neurons, and this was significant only after 8 weeks of transection (Tandrup et al., 2000). Differences in the susceptibility of neurons of different sizes were observed only after 45 days of proximal injury or 8 weeks of distal injury, when the cell loss proved to be greater in small neurons (Vestergaard et al., 1997; Tandrup et al., 2000). In addition, using stereological methods,Tandrup and coworkers (2000) observed no significant changes in mean perikaryal volumes until 4 weeks after distal nerve injury (large cells) or 8 weeks (small cells), when a decrease in cell volume was detected in DRG neurons on the operated side, whereas Vestergaard (1997) and Degn and coworkers (1999) observed declines in cell volume 4 days or 15 days after proximal nerve transection or nerve crush, respectively. Previous reports concerning changes in perikaryal volume after axotomy have shown inconsistencies, with findings of increase, decrease or no alteration at all in cell volume, and few of the reports were based on sound quantitative data as already discussed by Lieberman (1971). As mentioned above, species differences exist with regard to the effect of nerve injury on cell loss (see above) and e.g. 24% of the DRG neurons were lost in mice 7 days after distal

nerve transection (Shi et al., 2001), which is a much more pronounced loss than what was observed in rats where a smaller although significant reduction (14%), was not found until 8 weeks after transection (Tandrup et al., 2000). In addition to the changes seen in neurons, effects on glial cells have also been demonstrated with e.g. altered protein synthesis and sometimes induced cell death as the response (Ekström, 1995; Aldskogius and Kozlova, 1998).

Pain

Pain is a difficult parameter to measure in animals, and defining the quality of experimentally induced pain is not possible. However, measurement of pain is aimed at in different pain models, where an estimate of the nociceptive behaviour is made. Animal pain models are generally classified as somatic or visceral pain models. Models of visceral pain are developed in an attempt to resemble the clinical conditions of irritable bowel or irritable urinary bladder syndromes, and their pathological mechanisms might differ from those in somatic pain. Somatic pain models are divided into (Walker et al., 1999):

- ***acute nociceptive models*** (e.g. hot plate test, tail-flick test): measure the nocifensive responses, such as withdrawal reflexes, to noxious stimuli (e.g. heat) in naive animals. However, many common analgetics such as non-steroidal anti-inflammatory drugs (NSAIDs) are ineffective in these models since they affect mechanisms that develop during pathological conditions. Relying only on acute models might prevent the discovery of important events taking place in clinical (pathological) conditions.

- ***pathological pain models*:**
  - ⇒ *persistent central pain*, induced by formalin or capsaicin.
  - ⇒ *chronic inflammatory pain*, induced by carrageenan, turpentine, UV radiation or Freund´s complete adjuvant (FCA).
  - ⇒ *chronic neuropathic pain*, induced by damage or disturbance of the peripheral nerve.

Persistent stimulation of nociceptors results in sensitization in the CNS. Sensitization is generally held to be induced via facilitation of N-methyl-D-aspartate (NMDA) receptor activation, and leads to hyperalgesia and possibly allodynia (Woolf, 1996b). In the presence of allodynia a slight pressure (non painful) stimulation can result in a withdrawal reflex. This can be measured with von Frey filaments, inflicting a mechanical stimulation of the skin at increasing pressure levels. It is important to bear in mind that pain models, as well as injury models in general, should provide an accurate basis for the understanding of the clinical conditions and mechanisms studied.

Neuropeptides

Neuropeptides are widely distributed in both the central and the peripheral nervous system, as well as in endocrine tissues. Their expression is very plastic and often changes in response to perturbations in their environment, e.g. when the nervous system is subjected to stress or injury. Since neuropeptides are almost always coexpressed with one or more of the classic neurotransmitters (glutamate, γ-amino butyric acid (GABA), glycine and ATP), it is suggested that they may also have a modulatory function, i.e. they may regulate the effects of transmitters released from primary afferents in the spinal cord (increasing/decreasing the action of a transmitter by affecting its release, degradation etc). In addition, other possible roles have emerged, such as exerting trophic effects and having transmitter functions on its own. The distribution pattern of each neuropeptide is unique, although simplified, they are mainly expressed in three major types of mode. Note, that the expression pattern of a specific peptide is dependent on the type of neuron it is expressed in, and that one peptide can belong to each of the groups decribed below. The examples of peptides given below are related to expression in DRG neurons (Hökfelt et al., 2000).

- Peptides that are normally expressed at high levels; indicating that they are functionally available during homeostatic conditions. Examples: SP and CGRP.

- Peptides with low or undetectable levels of expression during normal conditions, but that are upregulated when the system is challenged, e.g. in response to nerve injury. Specific stimuli are needed for these peptides to become functionally available. Examples. VIP, galanin, NPY.

- Peptides expressed early, often prenatally, during development, which are downregulated postnatally. These peptides are thought to play a role in development and some of them are also reactivated again later in the adult stage, showing a surge in expression in response to specific stimuli. Example: galanin…

The change in neuropeptide expression in conjunction with a nerve injury gives an indication that the specific peptide is involved in the modulation of the specific nerve response. The regulation of expression of the different neuropeptides, during normal circumstances and in response to injury, is at least in part regulated by various neurotrophins (see; Neurotrophins).

Orphanin FQ/nociceptin (OFQ/N) and opioid receptor like receptor (ORL1)

The opioid receptor-like receptor (ORL1) was discovered in 1994 by several investigators (Bunzow et al., 1994; Mollereau et al., 1994; Wang et al., 1994), and shows a rather high sequence homology with the opioid receptors (Reinschied et al., 2000). Activation of the G-protein coupled ORL1 receptor results in inhibition of voltage-sensitive (NMDA) Ca^{2+} channels, inhibition of adenylate cyclase, and activation of K^{+} channels, which in turn inhibits release of several neurotransmitters (Reinscheid et al., 1995; Henderson and McKnight, 1997; Mollereau et al., 2000). The ligand for ORL1 was cloned the following year by two different groups and they named it nociceptin or orphanin FQ, respectively (Meunier et al., 1995; Reinscheid et al., 1995). OFQ/N is a 17 amino acid peptide with striking similarities to the opioid peptides. In spite of these sequence homologies, the opioid peptides do not elicit functional activity at the ORL1 receptor, nor does OFQ/N activate the opioid receptors. OFQ/N is cleaved from a larger precursor protein, and at least one other biologically active peptide product, i.e. nocistatin, is generated from the precursor. Nocistatin has been implicated in modulation of locomotion, pain perception, and it has an anti-OFQ/N activity blocking OFQ/N induced allodynia and hyperalgesia.

Many different functions of OFQ/N and its receptor have been demonstrated in the CNS, PNS, and non-neuronal tissues. Physiological functions of the system include modulation of pain and analgesia, locomotor activity, learning and memory, attenuation of stress responses, regulation of neurotransmitter and hormone release, modulation of kidney function, and potentially a role in neuronal differentiation (Reinscheid et al., 2000). The function of OFQ/N in pain responses is complex, and both nociceptive and anti-nociceptive actions have been shown, depending on the administered dose, site of administration, species, and whether acute or chronic pain is investigated (Meunier et al., 1995; Reinscheid et al., 1995; Erb et al., 1997; Henderson and McKnight, 1997; Inoue et al., 1998; Ahmadi et al., 2001; Bodnar and Hadjimarkou, 2003). OFQ/N and ORL1 have been reported to be widely distributed in the CNS (Reinscheid et al., 2000), whereas their functions and distribution in the PNS have been less studied (Wick et al., 1994; Nothacker et al., 1996; Riedl et al., 1996; Mollereau et al., 2000). We have studied the distribution of OFQ/N and ORL1 in the DRG neurons, as well as in their projection areas. The results from this study, and the previous findings of OFQ/N and ORL1 are discussed in paper I.

The neuropeptide PACAP

PACAP was first isolated from ovine hypothalamus (Miyata et al., 1989; Miyata et al. 1990), and occurs in two biologically active forms; PACAP-38 and PACAP-27, 38 or 27 amino acids long, respectively. PACAP is a member of a superfamily of structurally related peptides including secretin, glucagon, glucagon-like peptide 1, VIP and growth hormone releasing factor (Vaudry et

al., 2000). The name is based on the ability of PACAP to stimulate adenylate cyclase in cultures of rat pituitary cells. Three major receptors have been identified, the type I receptor: PAC_1 cloned by Pisegna and Wank (1993) which is the high affinity receptor for PACAP and binds PACAP with 1000 fold higher affinity as compared to VIP, and the type II receptors $VPAC_1$ (Ishihara et al., 1992) and $VPAC_2$ (Lutz et al., 1993) which bind PACAP and VIP with similar affinities. Unlike the $VPAC_1$ and $VPAC_2$ receptors, the PAC_1 receptor has a variety of splice variants characterised by the absence (short variant) or presence of either one or two of the exons of 28 (hip or hop1) or 27 (hop2) amino acids, in the third intracellular loop of the receptor cDNA (Spengler et al., 1993). A very short splice variant also exists, characterized by a N-terminal 21 amino acid deletion (Pantaloni et al., 1996). Further, discrete substitutions in the transmembrane regions II and IV of PAC_1, has been observed in an additional PACAP receptor, given the name PAC_1 TM4 (Chatterjee et al., 1996). The PAC_1 splice variants have different transduction mechanisms, and stimulate adenylyl cyclase and PLC with different potencies. All the splice variants, except PAC_1 TM4 (which stimulates calcium influx via L-type voltage sensitive calcium channels), are able to stimulate adenylyl cyclase and all except PAC_1 TM4 and the hipvariant, can stimulate PLC, resulting in calcium mobilization. PACAP-38 is a more potent stimulator of PLC than PACAP-27, whereas they are equally potent at adenylyl cyclase stimulation. Activation of the VPAC receptors stimulate cAMP production.

PACAP mRNA expression is noted early on in embryogenesis and has been observed in the developing rat brain at embryonic day 9.5 (Waschek et al., 2002) and in spinal cord at embryonic day 13 (Lindholm et al., 1998; Nielsen et al., 1998). Immunoreactivity for PACAP has been demonstrated in spinal cord and ganglia at embryonic day 16 (Nielsen et al., 1998). In the adult animal PACAP is distributed in nerve cell bodies and fibers in the CNS and PNS, as well as in endocrine cells (Vaudry et al., 2000). In DRG neurons, PACAP is expressed in primarily small size neurons during normal conditions and PACAP immunoreactivity has been demonstrated in the dorsal horn (Moller et al., 1993; Mulder et al., 1994; Zhang et al., 1995; Dun et al., 1996; Zhang et al., 1996b; Jongsma Wallin et al., 2000; Hannibal, 2002). PACAP can act as a hormone, neurohormone, neurotransmitter or trophic substance, and has effects on immune cells, circadian rhythm, appetite and feeding behaviour, motor activity, pain behaviour, cell survival and differentiation, i.e. PACAP is implicated in many different physiological functions, some of which are involving sensory processing. The presence of PACAP in small DRG neurons and in the dorsal horn has initiated several studies aimed at elucidating the role for PACAP in modulation of nociception. PACAP has been shown to either inhibit (Zhang et al., 1993b; Yamamoto and Tatsuno, 1995; Zhang et al., 1996a) or potentiate (Narita et al., 1996; Xu and Wiesenfeld-Hallin, 1996) nociceptive responses, whereas ionophoretical administration of PACAP led to excitation of spinal cord neurons (Dickinson et al., 1997). Thus the role of PACAP in nociception is yet unresolved, and needs to be further investigated. Further, a significant role for PACAP has been suggested in studies with

knockout mice, where either the receptor or the ligand gene has been deleted, e.g. mice deficient for PACAP exhibited an increased early postnatal mortality, and died in a wasted state, probably due to inadequate heat production (Gray et al., 2001; Gray et al., 2002).

Neurotrophins

Neurotrophins are a family of polypeptide neuronal growth factors, encompassing nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophins-3 and –4/5, (NT-3, NT-4/5). They initiate signal transduction by activation of receptors in the Trk family; tyrosine kinase A, B, or C. NGF being specific for trkA, BDNF and NT-4/5 for trkB, and trkC being the high affinity receptor for NT-3 (Barbacid et al., 1994). Although, NT-3 can also bind to, and activate, both trkA and trkB. The p75 receptor, is a low affinity neurotrophin receptor that can be activated by any of the neurotrophins. The trkA receptor is predominantly expressed in small diameter DRG neurons, whereas, trkB and trkC are primarily expressed in neurons of increasing sizes. However, there is considerable overlap in size among the different subpopulations, and some neurons express more than one type of trk receptor, and others none (Mu et al., 1993; Wright and Snider, 1995). According to the neurotrophin hypothesis, there is a competition for a limited amount of target derived growth factors during development of sensory neurons, leading to a massive amount of induced cell death in those neurons failing to reach this support. By contrast, in the adult neurons, the growth factors produced by the peripheral targets seem to be responsible mainly for maintenance and differentiation rather than survival, i.e neurotrophins act as target-derived survival factors and promote phenotypic differentiation in developing sensory neurons and have paracrine and/or autocrine effects on proliferating neuroblasts, whereas in the adult they function in maintaining the phenotype, and promote survival and regeneration (Lindsay, 1996; Mendell, 1999).

NGF functions as a target derived neurotrophic factor during development, promoting survival of sympathetic, and small neural crest derived sensory neurons (Levi-Montalcini, 1976). This role is shifted in the adult animal where NGF is implicated in regulation of the transmitter phenotype and possibly regenerative sprouting of neurons (Sofroniew et al., 2001). The important functions of neurotrophins evidenced by the effects of loss of different neurotrophins in mutant mice is discussed by Lindsay (1996). NT-3 is important in the development and maintenance of large fiber proprioceptive sensory neurons, and a complete lack of type Ia sensory afferents and muscle spindles has been observed in NT-3 -/- mice. Both BDNF and NT-4/5 null mutant mice showed a lack of neural placode-derived sensory neurons. Further, a large proportion of mature DRG neurons maintained in culture, express BDNF, enabling survival of the neurons in the absence of exogenous neurotrophic factors (Ernfors et al., 1990). In addition, survival of cultured neurons was adversely affected by antisense oligonucleotide treatment disrupting BDNF synthesis (Acheson et al., 1995), suggesting that BDNF

promotes survival of adult DRG neurons. BDNF also serves as a neurotransmitter or neuromodulator within the dorsal horn of the spinal cord, and sciatic axotomy or inflammatory stimulus upregulates BDNF expression in large- and small- sized DRG neurons, respectively (Cho et al., 1997b; Michael et al., 1999; Thompson et al., 1999; Zhou et al., 1999b; Karchewski et al., 2002).

Aims of the thesis

The general aim of this work was to investigate the distribution of orphanin FQ/nociceptin (OFQ/N) and pituitary adenylate cyclase activating polypeptide (PACAP) and to some extent their corresponding receptors ORL1 and PAC_1, in the primary sensory afferents and their projection areas. Further, the importance of PACAP and PAC_1 in nociceptive behaviour, their changes in expresssion in response to nerve compression or transection, and the mechanisms regulating this altered expression were studied. In detail, the studies were directed to:

- Elucidate the distribution of the two neuropeptides, orphanin FQ/nociceptin (OFQ/N) and pituitary adenylate cyclase activating polypeptide (PACAP) and their receptors, in the neuronal cell bodies of the dorsal root ganglia. The expression of OFQ/N and ORL1 was also examined in skin, in search of an endogenous peripheral source (as suggested in a study by Inoue et al., 1998, where a intraplantar injection of OFQ/N resulted in a nociceptive response). In addition to this, expression of OFQ/N, ORL1, PACAP and PAC_1, was studied in the spinal cord in adult intact animals.

- Study the possible involvement of the PAC_1 receptor in nociceptive responses which were investigated in a behavioural study with PAC_1 deficient mice. The next step was to establish how the neuropeptide expression was altered in response to nerve injuries in these receptor deficient mice.

- Investigate the PACAP expression in adult rat spinal cord, in intact animals, and after proximal or distal nerve transection.

- Study the changes in PACAP and PAC_1 expression in DRG, sciatic nerve and spinal cord in response to sciatic nerve compression.

- Investigate the mechanisms behind the regulation of the injury induced changes in PACAP expression in DRG neurons. Previously, NGF and NT-3 have been implicated in modulation of PACAP expression in response to injury, and now the possible role for BDNF was investigated.

Experimental animals and methods

In this section, an overview of the techniques used in the studies of this thesis is given. For more specific details I refer to the original papers, as the focus in this section is on the basic principles of the techniques used. The experimental models were chosen to investigate the distribution and plasticity in expression primarily of the neuropeptides OFQ and PACAP and their receptors ORL1 and PAC_1 in sensory neurons and some other tissues during normal conditions, and after injury, inflammation or compression of a peripheral nerve.

Experimental animals (papers I-V)
In papers I, III, IV and V adult Sprague-Dawley or Wistar rats were used. In paper II, adult PAC_1-/- mice and age and sex matched wild type littermates were tested. The mutant mice were generated by homologous recombination on a 129/Sv X C57BL/6J background (Jamen et al., 2000). The animals were deeply anaesthetized before surgery, and killed by overdose of anaesthetics and heart puncture after experiments. Tissues used in the different studies were, skin, sciatic nerve, DRG, spinal cord, and superior cervical ganglion (SCG). All animal procedures were approved and conducted in accordance with the local animal ethics committees.

Formalin test (paper II)
Adult male wild type (n = 8) and PAC_1 -/- (n = 10) mice were used to study the biphasic nociceptive behavioural response induced by injection of 10 µl paraformaldehyde (2%, in phosphate buffer pH; 7.2) subcutaneously under the dorsal aspect of one of the hindpaws. Immediately after injection, mice were placed in a glass container surrounded by mirrors (for optimal supervision of their behavioural responses). The observer was unaware of the phenotype of the mice.

The total time that the animals spent licking or biting the injected area on the paw was measured in 2 minute intervals, every 5 minutes during the early (0-5 minutes) and the late phase (5-60 minutes). The early phase is due to direct chemical stimulation of C-fibers (acute pain, see; Introduction), whereas nociception in the late phase is caused by inflammatory reactions in peripheral tissues and functional changes, such as sensitisation in the spinal cord dorsal horn (chronic pain, see; Introduction), initiated by the acute phase actions. The licking/biting behaviour was defined as the nociceptive response as described in Tjølsen et al., (1992). The mice were killed immediately after the one hour observation period. This test is considered to have a high clinical relevance since pain is induced by tissue damage (see; Pain).

Thermal and mechanical stimuli (paper II)
Adult male wildtype (n = 14) and PAC_1 -/- (n = 9) mice were used in these tests. The mice were handled and trained in a small hand-held tube (the restrainer), by the same person, daily for 5 days prior to the tests. Animals were accustomed to the experimental environment for approximately 2 hours in advance of the behavioural testing. The mice were placed in the restrainer, allowing for stimulation of the hind limb or tail.

To determine the thermal reflex threshold for a skin area, a CO_2 laser was used (Holmberg and Schouenborg, 1996). Threshold stimulation was defined as a stimulus duration that could elicit a withdrawal response in at least 3 out of 5 stimulations (unfocused beam, diameter 2 mm, intensity 3 W, pulse duration 6-45 ms). The duration of the laser pulses were increased in steps of 2 ms until the reflex threshold was reached. To avoid tissue damage, the maximal pulse duration used was 45 ms. The interval between each pulse was >10 ms. Laser stimulation did not elicit vocalization or avoidance behaviour other than withdrawal reflexes in the stimulated limb or tail. To estimate the nociceptive reflex latency, animals were stimulated with a pulse duration of 10 ms above their individual threshold value. The time between the stimuli application and the elicited withdrawal response was recorded with a digital video camera, and the nociceptive reflex latencies were calculated.

The mechanical reflex threshold was assessed with the use of calibrated nylon Semmes-Weinstein monofilaments (von Frey hairs). The reflex threshold was defined as the lowest force of stimulation required to elicit a visible withdrawal response in 3 or more out of the 5 consecutive tests.

Nerve compression (paper IV)
Adult female Sprague-Dawley rats were anaesthetized and the sciatic nerve was exposed unilaterally at mid-thigh level. A 10 mm long silicone tube was incised longitudinally, and closed around the nerve with the help of two 9/0 Ethilon sutures to ensure a constant diameter of compression. Animals were left with the compressed nerve for 3 (n = 3), 7 (n = 3), 14 (n = 3) or 28 (n = 4) days, after which the sciatic nerve, and lumbar DRG and spinal cord were dissected. Nerve compression injuries can be of varying degrees, although in this study it is most likely a combination of first and second degree injury according to the definition by Sunderland (see, Introduction). Evaluation of semi-thin plastic embedded sections of the sciatic nerve from the compressed area and distal to the silicone tube revealed clear signs of Wallerian degeneration, but also areas of preserved nerve morphology (Pettersson LME, unpublished findings).

Nerve crush (paper II)
Adult female wildtype (n = 10) and PAC_1 -/- (n = 10) mice underwent unilateral sciatic nerve crush in the upper hip region. Animals were anaesthetized, and the exposed sciatic nerve was crushed by application of a

pair of pliers around the nerve (2 x 15 seconds). The wound was closed and animals allowed to recover for 4 or 10 days after which animals were killed and DRG L5 dissected. Crushing the nerve results in a transection of the axon, although the continuity of the endoneurium is preserved, characterizing this as a second degree injury.

Nerve transection (papers III, V)
A complete nerve transection is an example of a fifth degree injury, resulting in a complete loss of continuity of the nerve trunk.

Distal nerve transection (paper III): Adult female Sprague-Dawley rats (n = 8), were anaesthetized and the sciatic nerve was transected unilaterally at mid-thigh level. The wound was closed and animals left to recover for 3, 7 or 14 days, respectively, when animals were killed and lumbar spinal cords were dissected.

Proximal nerve transection (papers III, V): Adult male Wistar rats were anaestethized and in addition given buprenorphine to relieve any pre- and postoperative discomfort. The sciatic nerve was transected unilaterally at its origin from the L4 and L5 spinal nerves, and a 5 mm segment was resected so as to prevent regeneration. Animals (n = 13) were allowed to survive for 3 days or 3 weeks after the surgery, when spinal lumbar spinal cords were dissected.

Proximal nerve transection and antibody administration (paper V): In this study, all animals were subjected to unilateral proximal nerve transection and in addition received either i) intraperitoneal (ip.) injections (n = 3) of either sheep anti-BDNF IgG (anti-BDNF; Chemicon International, USA) or (n = 3) sheep control IgG (control IgG; Sigma-Aldrich, USA), 1 hour before and 24 and 48 hours after injury, or ii) continuous intrathecal (ith.) infusion of either anti-BDNF (n = 4) or control IgG (n = 4) for 3 days. The intrathecal infusions were administered via mini-osmotic pumps that were inserted into the dorsal lumbar subcutaneous space immediately after transection, delivering antibodies at the level of the entry of the L5 via a silicone tubing that was attached to the pump and ran cranially along the spinal cord for ~1.5 cm. All animals were killed after 3 days and lumbar DRGs were dissected.

Tissue processing and histochemical procedures

For immunohistochemistry, the tissues were fixed in Stefanini´s fixative (2% paraformaldehyde (PF) and 15% of a saturated aqueous picric acid solution, in 0.1 M phosphate buffer, pH 7.2) over night, followed by repeated rinsing in sucrose-enriched (10%) Tyrode´s solution before embedding and freezing (papers I, II, III, IV). In paper II, tissues processed for in situ hybridization were fixed in Stefanini´s fixative as above, and sections were postfixed in 4% PF and treated with 0.25% acetic anhydide in 0.1M triethanolamine, 50 mM N-ethylmaleimide and dehydrated before hybridization. For in situ hybridization (papers I, III-V), tissue specimens were mounted in O.C.T.™ Compound and fresh-frozen in isopentane immediately after dissection (papers I, III, IV). Alternatively, animals were perfused with cold phosphate buffered saline (PBS, 0.1M), to clear away the blood, and then 4% PF for tissue fixation. The perfused tissue samples were postfixed in 4% PF (1.5 hours), repeatedly rinsed in 20% sucrose, and cryoprotected in 20% sucrose overnight before embedding and freezing (papers III, V). For ligand binding, tissues were embedded and fresh frozen immediately after dissection (papers I, II, IV). Tissue specimens were stored at -80°C until use.

Immunohistochemistry (papers I, III, IV).
OFQ/N (paper I): All dilutions were performed in PBS (0.01 M phosphate buffered saline, pH 7.3), 0.25% triton X-100 and 0.25% BSA and all steps were followed by wash in PBS, 0.25% triton X-100. Sections were incubated in moist chambers with primary antibody against synthetic OFQ/N (1:1280, Biotrend Chemikalien, Germany), for 48 hours at 4°C. Excess antibody was washed away for 15 minutes after which the sections were incubated with pig anti rabbit-FITC (1:80, DAKO, Denmark), for 1 hour at room temperature. Slides were mounted in PBS/glycerin 1:1. To establish the specificity of the antibody, additional slides were incubated for 48 hours with antibody absorbed overnight with the antigen (synthetic OFQ/N, Phoenix Pharmaceuticals, USA) in excess (100 μg/ml diluted antiserum), and processed as above.

Polyclonal antibody against PACAP 1-27 (papers III, IV): All dilutions were performed in PBS (0.01 M, pH 7.3), with 0.25% triton X-100 and 0.25% BSA, and all steps were followed by wash in PBS with 0.25% triton X-100. Sections were incubated in moist chambers with primary antibody against synthetic PACAP 1-27 (1:1280; code 88121–4, kind gift from Professor Akira Arimura, Tulane University, Hebert Center, US-Japan Biomedical Research Labs, New Orleans, LA, USA) for 18-24 hours. Excess antibody was washed away and sections were incubated with fluorescein isothiocyanate conjugated swine anti-rabbit immunoglobulin G (IgG) (DAKO, Denmark). Slides were mounted in PBS/glycerin. To establish the specificity of the immunostaining, additional slides were incubated with antibody absorbed overnight with the antigen

(synthetic PACAP 1-27, Sigma-Aldrich, St. Louis, MO, USA) in excess, and processed as above.

Monoclonal antibody against PACAP 1-38 (papers III, IV): Endogenous peroxidase activity was quenched with the treatment of 1% hydrogen peroxide in PBS, unspecific binding was blocked by treatment with 5% BSA in PBS, and slides were incubated in moist chambers with monoclonal antibody against synthetic PACAP 1-38 (1:10, code JHH 1, kind gift from Professor Jan Fahrenkrug, Bispebjerg Hospital, Copenhagen, Denmark) for 18-24 hours. The antibody has been characterized by Hannibal and coworkers (1995). Following incubation with biotinylated rabbit anti-mouse IgG (DAKO, Denmark), slides were incubated with streptavidin conjugated to horse radish peroxidase (TSA™ Biotin System, Perkin Elmer), and treated with Biotinyl Tyramide (TSA Indirect TSA™ Biotin System, Perkin Elmer). Finally, slides were incubated with avidin conjugated to Oregon Greene (Molecular Probes, USA), and mounted in PBS/glycerin. To establish the specificity of the immunostaining, additional slides were incubated with antibody absorbed overnight with the antigen (synthetic PACAP 1-38; A1439, Sigma-Aldrich, USA) in excess, and processed as above. The specificity of the secondary antibody was also tested by the omission of the primary antibody, after which slides were processed as above.

^{125} I-Ligand binding (papers I, II, IV)

^{125}I-[Tyr^{14}]- OFQ/N binding (paper I): This was used to locate binding of OFQ/N to its receptor. Sections were preincubated for 30 minutes at 20°C in a 0.05 M Tris/HCl buffer (pH 7.4), containing 2% bovine serum albumin, 5 mM $MgCl_2$ and 0.5 μg/ml bacitracin. Slides were then preincubated in the same solution with the addition of ^{125}I-[Tyr^{14}]- OFQ/N (Perkin Elmer), 600 pM, at 20°C for 1 hour. Control sections were checked for non-specific binding in the presence of non labeled OFQ/N in excess (6 μM). Slides were washed in 0.05 M Tris/HCl buffer (pH 7.4) containing 0.1% bovine serum albumin, 5 mM $MgCl_2$ and 0.5 μg/ml bacitracin, 3 x 5 minutes, at 4°C and dipped in distilled water. When slides were dry, they were covered with Hyperfilm MP (Amersham, Sweden) and stored in the dark for 15 days at 4°C, after which the film was developed as for in situ hybridization, and examined in the microscope.

[^{125}I]-PACAP-27 binding (papers II, IV): To locate binding of PACAP to its receptor, [^{125}I]-PACAP-27 was used. The procedure was the same as described above for ^{125}I-[Tyr^{14}]-OFQ/N binding, except that the slides were incubated with 150 pM [^{125}I]–PACAP-27 (Perkin Elmer) instead. Some slides were also incubated with the addition of non labeled vasoactive intestinal polypeptide

(VIP; 1.5 μM; Sigma-Aldrich) in excess. Control sections were incubated with the addition of non labeled synthetic PACAP 1-27 (1.5 μM ; Sigma-Aldrich) in excess (paper II). The slides were stored ~1 week before development of the film.

In situ hybridization (papers I-V)

Radioactively (^{35}S) labeled oligodeoxyribonucleotide (oligoDNA) probes (30-48 mers long) complementary and selective for BDNF, CGRP, galanin, GAP-43, NPY, OFQ/N, ORL1, PACAP, PAC_1, trkB and VAChT were used for detection of their corresponding mRNAs. All cDNA regions were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm.nih.gov), and no greater than 60% homology was found to sequences other than the cognate transcript, except for the probe complementary to VAChT mRNA, which showed higher sequence similarities to the vesicular monoamine transporters (VMATs). The homology between the cDNA sequences for VAChT and the VMATs is rather high (Masson et al., 1999), complicating the procedure of designing a probe for VAChT that does not run the risk of crossreacting with the VMATs. Since there are no indications of VMAT immunoreactivity or expresssion of VMAT mRNA in the spinal cord ventral horn neurons (Peter et al., 1995; Weihe and Eiden, 2000), the problem with a possible risk for crossreactivity was overridden when studying the spinal cord ventral horn (paper III). Using terminal transferase, probes were 3´endtailed with ^{35}S, and purified through columns by centrifugation. The specific activities obtained were ~1–2 x 10^9 cpm/μg. Sections were hybridized with probes at a final concentration of ~10^7 cpm/ml in a hybridization buffer containing 30-50% formamide. The sections were incubated for 18-24 h in sealed moist chambers at 37-43°C. After hybridization, slides were washed in baths with SSC, dehydrated in increasing ethanols and air dried. Finally, slides were immersed in photoemulsion and stored with desiccant at 4°C in the dark for appropriate time intervals, after which they were developed and counterstained before mounting.

Quantification and image analysis (papers III-V)

To evaluate changes in mRNA expression after different injuries and treatments, all slides were first analyzed qualitatively, and relative changes in hybridization signal were noted between the ipsilateral and contralateral sides. To further analyze the effects, the mRNA signal in injured neurons was measured quantitatively, on representative sections, and compared with the signal in the corresponding neurons on the contralateral side. All sections that were to be compared were processed at the same time under identical conditions to avoid variance in the overall signal between the different slides. Relative changes in hybridization signal between neurons on both sides were determined using an Olympus DP-50 digital camera, and computer assisted

image analysis. The percentage of the cytoplasmic area covered by silver grains were measured in all the neurons, using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health, and available from the internet by anonymous FTP from zippy.nimh.nih.gov). The density threshold was adjusted interactively for each image, so that the area per grain was constant for all neurons analyzed on a given slide, and the mRNA signal was correlated to the background labeling. The background silver grain density was measured for defined areas of the spinal cord or DRG devoid of positively labeled cell bodies.

The effect of nerve transection on PACAP mRNA expression in spinal cord neurons was investigated in paper III; the hybridization signal was examined and quantified in injured ventral horn neurons and corresponding neurons on the contralateral sides. In brief, representative sections from rats with 3 days proximal nerve transection (containing similar numbers of motor neurons on each side, and reflecting the overall labeling for motor neurons exposed to the same conditions), were quantified. The PACAP mRNA expression density was examined in all ventral horn neurons expressing GAP-43 (transected motor neurons), as well as in motor neurons in the corresponding area on the contralateral side. Relative changes in hybridization signal between neurons on both sides were determined and the percentage of the cytoplasmic area covered by silver grains were measured in all the motor neurons. Neurons were considered labeled for PACAP mRNA if they contained more than four times background labeling of silver grains.

To evaluate the effect of nerve compression on the expression of PACAP mRNA in DRG neurons (paper IV), changes in hybridization signal were were analyzed on representative slides (each slide containing double, adjacent pairs of left and right DRGs). Each individual neuron was examined and the relative changes in hybridization signal were determined, for the ipsi- and contralateral DRGs and for the different time periods, using computer assisted image analysis. Image analysis was performed on adjacent DRG sections processed for in situ hybridization to detect PACAP mRNA (n=4 animals, 1 rat per time point analyzed, 8 DRG sections where approximately 1900 neuronal DRG L5 profiles were examined). Montages of photomicrographs (500 X) were prepared from eight pairs of adjacent sections of left or right DRGs from animals with 3, 7, 14 or 28 days of unilateral sciatic nerve compression. Individual neurons with a visible nucleus in at least one of the two adjacent sections were identified and numbered (approximately 240 neurons/montage). Light microscopy images (60X) were collected and cross sectional areas of each neuronal profile and the percentage of the cytoplasmic area that was covered by silver grains were measured in all the ganglia. Individual cell diameters were calculated from the cross sectional areas on the assumption that the neurons were spherical. Neurons were defined as small when the diameter was < 35 μm, and large when the diameter was > 35 μm (Giuffrida and

Rustioni, 1992). Neurons were considered labeled for PACAP mRNA if they contained more than five times background labeling of silver grains.

After the quantification of DRG L5 neurons a somewhat less extensive examination of the DRG L4 neurons (which also support the sciatic nerve), was performed to compare the PACAP mRNA distribution with the ganglia at the L5 level. All sections were examined and selected according to the criteria above, and this time four new animals were quantified. Montages were made (n=4 animals, 1 animal per time point analyzed, 8 DRG sections and approximately 2300 DRG L4 profiles), neurons defined and numbered, whereafter each neuron (approximately 290 neurons/montage) was examined directly in the microscope (60X). The total percentage of PACAP mRNA labeled neurons was determined on the compressed and intact sides, respectively, but no measurements of neuronal diameter was made, i.e. no distinction of the distribution in relation to neuronal size was made for the DRG L4 neurons, and changes in silver grain density/neuron were not calculated.

To evaluate the effect of endogenous BDNF on the expression of PACAP mRNA in DRG neurons after axotomy (paper V), qualitative examination of all slides was done. Further, quantitaive examination of changes in hybridization signal on representative slides (where all DRG sections contained similar numbers of neurons, and the sections reflected the overall labeling for DRGs exposed to the same conditions) was performed. In the first analysis, animals were examined after intrathecal infusions of antibodies (n = 4, 2 rats per treatment analyzed), and after i.p. injections (n = 2, 1 rat per treatment analyzed). Montages of photomicrographs were made and neuronal profiles, were identified and numbered. Thereafter, each neuron (approximately 410 neurons/montage, intrathecal infusions, and 495 neurons/ montage, i.p. injections) was examined directly in the microscope (60X). The total percentage of PACAP mRNA labeled neurons was determined on the ipsilatral side after the different treatments, but no measurements of neuronal diameter was made, i.e. no distinction of the distribution in relation to neuronal size, or changes in silver grain density/neuron was calculated in these early examinations. These first and somewhat less extensive analyses were performed to establish the effects of BDNF on PACAP mRNA expression in DRG neurons, and the results indicated that a change in expression took place after intrathecal anti-BDNF infusion, but not after intraperitoneal injections.

Therefore further extensive analyses of slides from additional animals (intrathecal infusions of antibodies) were performed. Slides were first examined qualitatively and then representative slides were selected for quantitative analysis according to the criteria above. Montages were made, neurons were identified and numbered, and each individual neuron was examined. The relative changes in hybridization signal were determined with the use of computer assisted image analysis on adjacent sections processed for in situ hybridization to detect PACAP mRNA (n = 4 animals, 2 rats per treatment analyzed, 4 DRG sections where approximately 900 neuronal DRG

L5 profiles were examined), and trkB mRNA (sections adjacent to the ones analyzed for PACAP (n = 2 animals, 2 DRG sections, approximately 460 neuronal DRG L5 profiles). Montages were prepared from pairs of adjacent sections of ipsilateral DRGs hybridized for detection of PACAP and trkB mRNA, respectively. This was made both for ganglia from animals infused with anti-BDNF and ganglia infused with control IgG. Neuronal profiles were identified and numbered (approximately 240 neurons/montage), and light microscopy images (60X) were collected from each individual neuron. Cross sectional areas of every single neuronal profile and the percentage of the cytoplasmic area covered by silver grains were measured for all the neurons in all the ganglia. Individual cell diameters were calculated. Neurons were considered labeled for PACAP and trkB mRNA if they contained more than six times background labeling of silver grains.

Statistics (papers II, IV, V)

In paper III, changes in nociceptive behaviour between PAC_1-/- mice and wildtype mice during the formalin test were compared using the unpaired t-test. Changes in response to thermal and mechanical stimulation, in the same animals, were compared with the Mann Whitney U-test. In paper IV, differences in the numbers of neurons expressing PACAP mRNA in DRG after nerve compression were analyzed using the chi-square test comparing the expression in the ipsi- and contralateral neurons 3, 7, 14 and 28 days after compression. In paper V, differences in the number of PACAP or trkB mRNA expressing neurons between DRGs treated with control IgG or anti-BDNF in conjunction with 3 days of nerve transection were analyzed using the chi-square test. $p<0.05$ was considered significant in all the tests.

Results and comments

Expression of OFQ/N and its receptor ORL1 in adult rat (paper I)
In paper I the distribution and expression of OFQ/N and ORL1 was investigated in spinal cord, DRG and some other peripheral tissues such as skin and SCG. OFQ/N mRNA was found to be expressed in many of the laminae of the spinal cord, mostly concentrated to neurons in lamina II and somewhat less in laminae I, X and in a few medium and large sized ventral horn neurons. In the ventral horn OFQ/N was coexpressed with VAChT in some of the larger neurons. OFQ/N immunoreactivity was also found in neurons and fibers in the superficial layers of the spinal cord, as well as staining in fibers in some deeper dorsal horn layers, and in medium and large sized neurons in the ventral horn. This confirms the findings of mRNA expression in these areas and supports the findings of OFQ/N mRNA expression in small DRG neurons and a possible export of the peptide to the central terminals of the primary afferent neurons (see; General discussion). ORL1 mRNA was found to have a scattered expression throughout the spinal cord gray matter, with its greatest labeling density in the ventral horn, where it was coexpressed with VAChT in many of the large neurons. The receptor expression was somewhat more dense in the superficial laminae of the dorsal horn and in lamina X as compared to the rest of the gray matter, except for the ventral horn expression. Binding of radioactive OFQ/N ligand to its receptor was observed to some extent in all the spinal cord laminae except for lamina II, which seemed almost devoid of receptor. The greatest receptor density was identified in laminae I-VI, and X. Loss of ORL1 in these areas may contribute to the increased nociceptive behaviour seen in ORL1 deficient mice in the late inflammatory phase of the formalin test (Ahmadi et al., 2001), since a direct inhibition of the excitatory glutamatergic dorsal horn neurons by OFQ/N is thwarted. These findings are in good agreement with some of the earlier findings of OFQ/N and ORL1 in spinal cord (Riedl et al., 1996; Schultz et al., 1996; Neal et al., 1999a; Neal et al., 1999b; Houtani et al., 2000;). The colocalisation of ORL1, and to some extent OFQ/N, with VAChT in ventral horn neurons, as well as the size and location of these neurons, suggest that they are motorneurons. In addition, there is expression of OFQ/N in some medium sized neurons (presumably interneurons) in this area. Our findings strengthen the theory suggested by Ikeda and coworkers (1998) that interneurons might release OFQ/N to modulate the neuronal and synaptic activities of adjacent neurons, in this case motorneurons.

Previous reports regarding the expression of OFQ/N and ORL1 in the DRG (Wick et al., 1994; Andoh et al., 1997; Neal et al., 1999a; Neal et al., 1999b; Itoh et al., 2001) have shown some inconsistencies. With the use of a sensitive in situ hybridization protocol we were able to observe expression of OFQ/N in a few small, and ORL1 in many large DRG neurons. The expression of ORL1 was confirmed to some extent by the findings of ^{125}I-[Tyr^{14}]-OFQ/N binding to large neurons. Binding of ^{125}I-[Tyr^{14}]-OFQ/N to DRG neurons has not been shown earlier. Binding of radioactive ligand to lamina I in the spinal

cord gives further support to expression and axonal transport of ORL1 in medium and large DRG neurons. The discrete OFQ/N expression in small DRG neurons could not be confirmed by immunohistochemistry, which could be due to a rapid export of peptide from the DRG. In a previous study, intraplantar injection of OFQ/N into the hindlimb of mice elicited a nociceptive response, but no peripheral endogenous source of either ligand or receptor was investigated (Inoue et al., 1998). Since we did not detect expression of OFQ/N or ORL1 or immunoreactivity for OFQ/N in skin, this suggests a different endogenous peripheral source of OFQ/N and ORL1 (see; General discussion).

In SCG, low but consistent expression of OFQ/N was observed in some nerve cell bodies, whereas ORL1 was expressed in many nerve cell bodies. Giuliani and coworkers (2000) suggested a role for OFQ/N in modulation of noradrenaline release from nerve terminals innervating the resistance vessels, via action on a receptor located prejunctionally on postganglionic sympathetic fibers.

PAC_1 -/- mice –sensory physiology and neuropeptide/receptor expression (paper II)

Sensory physiology

Despite a vast knowledge around PACAP and its receptor PAC_1, their physiological importance is still not clarified. In this study we investigated the response to formalin injection, thermal laser and mechanical von Frey stimulation in PAC_1 -/- mice, and compared them with the responses in their wild type littermates. PAC_1 -/- mice showed a pronounced decrease (75% decrease) in pain behaviour in the inflammatory phase of the formalin test as compared to wild type mice (Fig. 3). In the acute tests, the PAC_1 deficient mice showed an increased pain behaviour (doubled) in the early phase of the formalin test, but did not exhibit any changes in pain threshold or latency after thermal laser stimulation of either tail or hindpaw. Changes in tactile reflex threshold was only observed after stimulation of hindpaw (reduced threshold in PAC_1 mice), but not tail. These findings indicate that PACAP, acting via PAC_1 (see below), is involved in the modulation of nociceptive responses and that it has a nociceptive effect at least during chronic pain states such as inflammation. A nociceptive role for PACAP during inflammation is further supported by the findings that PACAP expression is upregulated, primarily in small size DRG neurons, in response to peripheral inflammation (Zhang et al., 1998; Jongsma Wallin et al., 2003). In a study by Ohsawa and coworkers (2002), administration of PACAP receptor antagonist or PACAP antiserum were effective in attenuating the inflammatory, but not the acute phase, of nociceptive responses to formalin. In addition, PAC_1 -/- mice exhibited a drastic reduction in abdominal writhing in the acetic acid writhing test (Martin et al., 2003), further supporting our suggestion of PACAP as a modulator of nociception during inflammation. In the study by Ohsawa and coworkers (2002), intrathecal PACAP administration was found to reduce the latency of the thermal (hot plate) withdrawal response, whereas we did not se any

differences in the withdrawal response to laser stimulation when PAC_1 -/- mice and wild type littermates were compared. In a preliminary study, also using the hot plate as means of thermal stimulation, PAC_1 -/- mice did not show any differences in pain thresholds when compared to wild type mice (van Gaalen et al., 2000). Martin and coworkers (2003) have also studied the acute somatic pain perception in PAC_1 -/- mice with the use of the tail immersion, tail pressure and hot plate tests. In none of these paradigms did the PAC_1 -/- mice display any significant change in somatic pain perception. These inconsistencies in response to thermally induced pain may be a result of the different methods of stimulation; in the hot plate test, receptors over a large area of the skin of the hindpaw are stimulated, whereas the laser stimulation is limited to a defined area of the paw. Further, in the hot plate test the stimulation temperature of the heat stimulus is affected by parameters such as temperature of the paw and cooling by circulating blood etc, whereas the laser stimulation results in a more penetrating and constant heat stimuli. Finally, the slightly increased tactile susceptibility in PAC_1 -/- mice, which could only be observed after stimulation of hindpaw and not tail, needs to be further investigated in order to draw any conclusions about the role for PACAP in this type of stimuli which can not be considered in ordinary terms of pain response.

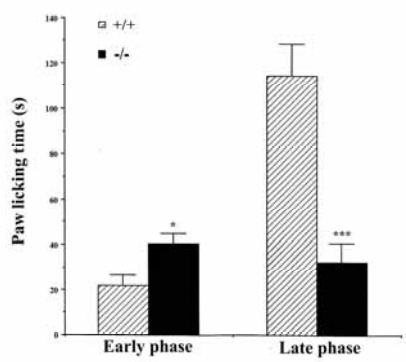

Fig. 3. Early and late phase responses of PAC_1 -/- and wildtype mice to formalin injection. In the early phase, the PAC_1 -/- mice spent approximately the doubled time licking and/or biting the injected paw, as compared to the wild type mice. In contrast, the mice lacking the PAC_1 receptor showed ~75% decrease in nociceptive behaviour in the late phase of the test. Striped bars indicate paw licking time for wild type mice, and black bars indicate PAC_1 -/- mice (from paper II).

Neuropeptide and receptor expression

The injury induced changes in neuropeptide expression in DRG neurons were more pronounced in PAC_1 -/- mice than in wild type mice, after 4 or 10 days or nerve crush. This was most evident for galanin expression, that was greatly induced, but the PACAP and NPY expression was also further increased. The CGRP expression, on the other hand, was decreased. This effect on

neuropeptide expression suggests a role for PACAP in regulation of neuropeptide expression via PAC_1.

Binding of radioactive PACAP ligand to its receptors was studied in spinal cord. Massive binding was observed throughout the spinal cord gray matter in wildtype mice, especially concentrated in the dorsal horn area. This binding was not visibly changed in the presence of high concentrations of VIP, indicating that it is almost exclusively the PACAP specific receptor PAC_1, that is present in the spinal cord. This is further supported by the fact that PAC_1 receptors do not respond to physiological concentrations of VIP (Harmar et al., 1998). In contrast, spinal cords from PAC_1 –/– mice were almost devoid of radioactive labeling, confirming the receptor deficiency in these mice, and further supporting the notion that PACAP binds primarily to PAC_1 in spinal cord, and not to the $VPAC_1$ and $VPAC_2$ receptors, which also bind VIP.

PACAP expression in spinal cord neurons –normally and in response to nerve transection (paper III)

In normal adult rat, PACAP mRNA expression was detected in the spinal cord, preferentially in neurons in lamina II, but also in lamina I, some deeper dorsal horn laminae, and in lamina X. In addition, PACAP mRNA was found to be expressed in a few medium sized ventral horn neurons (not obviously coexpressed with VAChT mRNA). PACAP immunoreactivity was observed in fibers, but not neurons, in the superficial laminae of the dorsal horn, around the central canal and to a lesser degree in the ventral horn, in uninjured animals.

After nerve transection, an induction of PACAP mRNA expression was detected in large neurons in the dorso-lateral part of lamina IX coexpressed with VAChT mRNA (3, 7, or 14 days of distal nerve injury), as well as in a more ventrally located population of lamina IX neurons (3 days or 3 weeks of proximal nerve transection). The induction of PACAP mRNA expression in neurons also expressing VAChT, as well as the size and location of these neurons, suggest that they are motorneurons. Induction of PACAP mRNA in these neurons was further supported by immunohistochemical findings of immunoreactivity for PACAP in the ipsilateral ventral horn neurons after nerve injury. The increased PACAP mRNA expression was correlated to GAP-43 mRNA expression (an injury marker) in the ventral horn neurons, and PACAP mRNA was found to be expressed in 42% of the injured ventral horn neurons, as compared to only in one or two neurons in the corresponding area on the contralateral side. No change in expression in the dorsal horn neurons was observed in response to the injury, indicating that the effect of nerve transection on PACAP mRNA expression is limited to the primary afferent neurons in the DRG (Zhang et al., 95; Zhang et al., 1996b; Jongsma Wallin et al., 2001).

Our immunohistochemical findings confirm previous reports on the distribution of PACAP in the spinal cord (Moller et al., 1993; Dun et al., 1996; Vaudry et al., 2000; Hannibal, 2002), and further illuminate the changes in distribution in response to nerve injury. However, findings of PACAP mRNA expression in the areas described above have not been reported earlier. The

only reported findings of PACAP mRNA expression in intact rat spinal cord were observed in neurons in the IML by Hannibal (2002), who also was able to detect immunoreactivity for PACAP in neurons in this area.

PACAP expression–changes in response to compression injury (paper IV)

PACAP mRNA expression was increased in lumbar DRG neurons in response to nerve compression (3, 7, 14, or 28 days). The increase ranged from ~2-4 times the number of neurons expressing PACAP mRNA on the contralateral side, i.e. 40-55% of the DRG neurons expressed PACAP mRNA on the ipsilateral side as compared to 14-21% on the contralateral side. Both the number of neurons, and the expression density/neuron were increased in response to compression injury, and PACAP mRNA expression was induced in both small and large diameter neurons. Other studies have also reported on an elevated level of neuropeptides after compression injury (Widerberg et al., 2001; Dahlin et al., 2003). Findings of an increased immunoreactivity for PACAP in DRG neurons after compression support the in situ hybridization data. No induction of PAC_1 mRNA expression or binding of radioactively labeled PACAP was observed in the DRG neurons in response to compression, indicating that there is no induction of receptor synthesis in these neurons. Ligand binding to receptor in the spinal cord was virtually the same as in previous studies of intact animals (Jongsma et al., 2000), suggesting that the increased levels of PACAP in the primary afferent neurons exercise their effects on receptors already present in the spinal cord. An elevation of the normally very weak immunostaining for PACAP in the sciatic nerve took place in fibers in the nerve segment exposed to compression. This elevation may be due to an inhibition of anterogradely transported PACAP and/or an increased transport of peptide from the DRG to the periphery. Impediment or inhibition of axonal transport is commonly observed in conjunction with compression injuries (Dahlin and McLean, 1986; Dahlin et al., 1986). Further, an increased immunoreactivity for PACAP has also been observed in the sciatic nerve in response to transection (Zhang et al., 1995; Zhang et al., 1996b). Although, the role for PACAP in the peripheral nerve has yet to be clarified, it may function as a survival factor for injured neurons or in exerting an anti-inflammatory effect regulating the production of e.g. inflammatory agents in macrophages; inhibiting the production of inflammatory agents and/or stimulating the synthesis of anti-inflammatory cytokines (Martinez et al., 1998; Delgado et al., 1999a; Delgado et al., 1999b; Waschek, 2002).

Regulatory mechanisms affecting PACAP expression during nerve transection (paper V)

PACAP mRNA expression was increased in both large and small DRG neurons in response to 3 days of sciatic spinal nerve transection. An upregulation in PACAP mRNA expression in DRG neurons has also been observed in other studies, after distal or proximal nerve transection (Zhang et al., 1995; Zhang et

al., 1996b; Jongsma et al., 2000; Jongsma Wallin et al., 2001), after peripheral inflammation (Jongsma Wallin et al., 2003), and after nerve compression (paper IV). Intrathecal infusions of sheep anti-BDNF IgG (anti-BDNF), in conjunction with sciatic spinal nerve transection resulted in a mitigation of the injury induced upregulation in PACAP mRNA expression in DRG neurons. Around 63% of the DRG neurons expressed PACAP mRNA after nerve transection, whereas only ~23% of the neurons expressed PACAP mRNA when administered anti-BDNF intrathecally immediately after the injury. PACAP mRNA expression is estimated to take place in roughly one fifth of the DRG neurons, primarily small diameter neurons, on the intact side (Mulder et al., 1994; Zhang et al., 1996b; Jongsma Wallin et al., 2001; paper IV). The reduced upregulation was reflected in the number of small and large diameter neurons, as well as in the overall expression density. The reduction in PACAP mRNA expression was also investigated with regard to colocalisation with trkB mRNA expression, and revealed that the mitigation of PACAP mRNA expression caused by neutralization of endogenous BDNF occurred in both trkB and non trkB mRNA expressing neurons. Intraperitoneal injections of anti-BDNF in conjunction with sciatic spinal nerve transection did not result in any changes in PACAP mRNA expression (see; Tissue processing and histochemical procedures).

Our findings suggest a role for endogenous BDNF in the induction of PACAP mRNA expression in response to nerve transection. However, since we were not able to detect any changes in expression after intraperitoneal injections of anti-BDNF, this may indicate that i) the systemic dose given was not sufficient to neutralize the endogenous BDNF, or ii) that with the intraperitoneal injections we were not able to target the central source of BDNF that influences sensory nerve function after injury as effectively as with the intrathecal infusions. This might suggest that the primary source of endogenous BDNF regulating PACAP mRNA expression after injury is central, from the spinal cord, or more likely from the DRG neurons themselves, where upregulation in BDNF mRNA expression has been reported in response to injury (Tonra et al., 1998; Michael et al., 1999; Zhou et al., 1999b).

General discussion

OFQ/N and PACAP

Both OFQ/N and PACAP have been found to be widely distributed in the nervous system. Further, a vast amount of functions have been suggested for these two neuropeptides, many affecting sensory, and especially nociceptive behaviour. Their effects on nociceptive behaviour are complex, and depend on factors such as the dose of administration, route of administration, whether acute or chronic pain is studied, and also what species are investigated. Many studies aimed at elucidating the roles of these peptides in nociception have reported opposite findings (see below).

OFQ/N

Paper I was directed at elucidating the distribution and expression of OFQ/N in the periphery, DRG and spinal cord areas, and to get an overall picture of of the recent findings in this area. With the use of in situ hybridization, we were able to detect expression of OFQ/N mRNA in discrete DRG neurons in the adult intact animal. Previous data concerning the expression of OFQ/N in the DRG were inconsistent, one study reporting presence of few, centrally located cells (Neal et al., 1999b), whereas others reported slight/no expression (Andoh et al., 1997; Itoh et al., 2001). However, an induction of expression was observed in small or medium diameter DRG neurons shortly after the induction of peripheral inflammation caused by carrageenan injection (Andoh et al., 1997; Itoh et al., 2001). The findings of expression of OFQ/N in laminae I and II, and ORL1 in laminae I and V, as well as in DRG neurons (small and medium sized, respectively) give a morphological basis that support a role for OFQ/N in the modulation of sensory and especially nociceptive transmission. OFQ/N mRNA expression, but not OFQ/N immunoreactive signal, was present in discrete DRG neurons, and binding of ^{125}I-[Tyr^{14}]-OFQ/N to the ORL1 receptor was only seen in some of the DRG neurons, whereas ORL mRNA expression was scattered in many medium-large diameter neurons throughout the ganglia. This suggests that an export of the peptide products might take place; peripherally and/or centrally. If exported peripherally, this might represent an endogenous peripheral source of OFQ/N that could explain the induction of a nociceptive flexor reflex after exogenous intraplantar injection of OFQ/N in rats (Inoue et al., 1998), and exported receptor might constitute the peripheral binding site for this OFQ/N. Another possible source of OFQ/N in the periphery could be circulating immune cells. However, it is notable that we were not able to detect OFQ/N in skin (intact or inflamed). If exported centrally to the primary afferent terminals in the spinal cord, this could be supported by the OFQ/N immunoreactive fibers and ligand binding we detect in the dorsal horn. OFQ/N and ORL1, might have influence on the signalling in the dorsal horn, affecting the release of other neuropeptides. Inoue and coworkers (1999), showed that OFQ/N affected SP release and signalling, when administered either centrally or peripherally. These findings support the prescence of ORL1 at both central

and peripheral terminals of primary afferent neurons. Further, an increased sensitivity to SP in the nociceptive flexor test was observed in OFQ/N deficient mice (Chen et al., 1999). A possibility for OFQ/N to modulate sensory signalling at sites outside the brain, i.e. in the periphery, as well as at the spinal cord level, may help explain the complex effects it has on e.g. nociceptive behaviours.

It is hard to speculate which are the true functional roles of OFQ/N since it seems to be dependent on many factors, and a comparison of the studies is complicated since different doses, routes of administration and also different species have been employed in the various models of nociceptive tests (see; Introduction). An antinociceptive effect at the spinal cord level is supported by the findings that OFQ/N is able to suppress the excitatory glutamatergic synaptic transmission in the dorsal horn neurons (Zeilhofer et al., 2000; Ahmadi et al., 2001). However, it is important to keep in mind that a low dose of OFQ/N resulted in a decreased pain behaviour in the formalin test in one study (Ahmadi et al., 2001), and increased nociceptive behaviour in another, where a higher dose decreased the nociceptive behaviour (Muratani et al., 2002). In the latter study, only the low dose effect could be blocked by an OFQ/N antagonist, and Muratani and coworkers (2002) therefore suggested that the antinociceptive effect at higher doses might be mediated by a receptor other than ORL1. This is contradicted by the findings by Ahmadi and coworkers (2001) who observed an antinociceptive effect of OFQ/N in the formalin test only in wild type mice, and not in ORL1 deficient mice, which would suggest that the antinociceptive effect of OFQ/N in this case is mediated via the ORL1 receptor.

PACAP

In papers II-V the focus was primarily set on the distribution/expression of PACAP, its physiological importance, its regulation in conjunction with different types of injuries and the mechanisms controlling that regulation. The different aspects are discussed below under the appropriate section.

Implications of PACAP and PAC_1 in nociception

In paper II, a behavioural characterization of the PAC_1 -/- mice was performed for the first time. In the study we were able to conclude that, in the spinal cord, PACAP exercise its actions primarily via binding to the PACAP specific receptor, PAC_1, indicating that changes observed in the PAC_1 -/- mice, but not in their wild type littermates, were likely to be actions of PACAP. We found an increased nociceptive behaviour in the PAC_1 -/- mice in the first phase of the formalin test (induced by direct chemical stimulation of C-fibers), whereas the chronic/inflammatory pain in the late phase was markedly reduced in the PAC_1 -/- mice. The decreased nociceptive behaviour in the second phase may be explained by actions on the NMDA receptor (Fig. 4). Normally, PAC_1 can stimulate protein kinase C (PKC), (Spengler et al., 1993; Vaudry et al., 2000),

and involvement of PKC is implicated in changes in pain perception. Activation of PKC is exercised via the binding of SP to its receptor NK1, which in turn induces a cleavage of PIP_2 (bis-phoshphate) to IP_3 (inositol tris phosphate) and DAG (diacylglycerol), mediated by PLC. IP_3 induces a release of Ca^{2+} from intracellular stores, and the increased levels of Ca^{2+} activates PKC. PKC is also activated directly by DAG. Activated PKC phosphorylates the NMDA receptor leading to the release of the Mg^{2+} blockage of its ion-channel, and resulting in an augmented Ca^{2+} flow into the cell with ensuing depolarisation upon glutamate activation. This renders the cell more easily excitable, and may lead to central sensitization, i.e. a hypersensitive cell. To sum up our hypothesis, inhibition of PKC by administration of a selective PKC-inhibitor, leads to a decrease in pain behaviour in the inflammatory phase of the formalin test, as shown by Wajima and coworkers (2000). We suggest that PAC_1, just as the NK1 receptor, may initiate a sensitization upon binding of PACAP, via activation of PKC. The decreased nociceptive response observed in the PAC_1 -/- mice in the inflammatory phase, may be due to an attenuated activation of PKC, since an additional activation via PAC_1 can not occur in these receptor deficient mice.

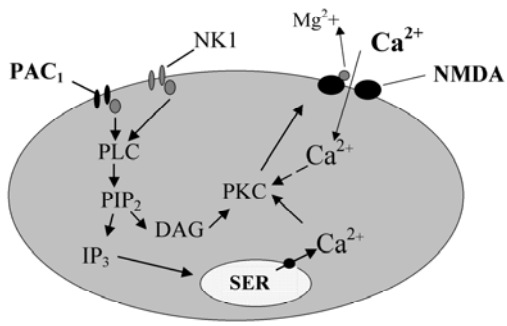

Fig. 4. A possible pathway leading to additional activation of PKC, upon binding and activation of PACAP to PAC_1 (see text above). This activation can not occur in PAC_1 -/- mice, which might explain the decreased nociceptive behaviour observed in PAC -/- mice in the inflammatory phase of the formalin test.

Other studies giving support to the notion that PACAP has a nociceptive effect in chronic pain states have been published. Ohsawa and coworkers (2002) were able to attenuate the pain behaviour in the late inflammatory, but not the early, phase of the formalin test by administration of PACAP receptor antagonist or PACAP antiserum. PAC_1 -/- mice have also been found to exhibit markedly reduced abdominal writhing behaviour in the acetic acid writhing test (Martin et al., 2003). The finding of an enhanced nociceptive response to intrathecal NMDA treatment, after intrathecal pretreatment with PACAP (Ohsawa et al., 2002), further support our hypothesis of a possible activation of PKC by PAC_1 resulting in a sensitization of dorsal horn neurons to glutamate. This has

previously been reported for SP, which produces nociceptive responses and sensitizes the dorsal horn neuron NMDA receptors to glutamate (Randic et al., 1990). To my knowledge, so far there are no reports of colocalization of PACAP and glutamate in DRG neurons. A colocalization would be very likely considering the large amount of small diameter neurons that contain glutamate (Broman et al., 2000), as well as the considerable proportion of these neurons that have been shown to express PACAP (Zhang et al., 95; Zhang et al., 1996b; Jongsma Wallin et al., 2001). Furthermore, in this context it is worth mentioning that PACAP has been observed to colocalize with glutamate in a subset of retinal ganglion cells (Hannibal et al., 2000). A colocalization and possible co-release of PACAP and glutamate would further suggest a role for PACAP in modulation of the response to glutamate in dorsal horn neurons. If PACAP does play a role in sensitization of dorsal horn neurons, then an attenuation of the nociceptive responses would take place e.g. in the PAC_1 -/- mice, where activation of this additional pathway mediated by activation of PAC_1 can not occur. However, it can not be completely ruled out that the changes observed in PAC_1 -/- mice are due to compensatory mechanisms, or an altered neuronal organisation of the DRG and spinal cord as PACAP has been shown to have neurotrophic effects (Mulder et al., 1999; Vaudry et al., 2002).

The inconsistencies in the response to acute pain or tactile stimulation need to be further investigated to be able to conclude the role for PACAP during these types of stimulation; the parameters have differed, e.g. different types of stimulation (hot plate, laser, tail immersion etc), and different types of tests (thermal pain, chemical pain, and mechanical pain etc). In addition, we have chosen to interpret our findings of an increased sensitivity to mechanical stimulation, with caution, since changes were observed in the PAC_1 -/- mice only after stimulation of hindpaw, and not tail. Further, the difference was rather slight and the range of the results were quite large. Also some findings opposing the suggested role for PACAP as a nociceptive agent contributing to inflammatory pain has been reported (Zhang et al., 1993; Yamamoto and Tatsuno, 1995; Zhang et al., 1996a), although, the rather high concentrations of PACAP used in these studies may account for the observed differences (compare with OFQ/N above).

PACAP expression in the spinal cord

In previous studies, no PACAP mRNA expression has been detected in the spinal cord, apart from in neurons in the IML (Hannibal, 2002). In paper III we have modified this picture with the observations of PACAP mRNA expression in neurons in the spinal cord, especially in laminae I, II, IX and X. We have established that the expression in the ventral horn neurons is induced in response to injury, as has previously been shown also in DRG neurons in conjunction with nerve injury or inflammation (Zhang et al., 1995; Zhang et al., 1996b; Zhang et al., 1998; Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003). Immunohistochemical observations of an induction of PACAP immunoreactivity in the ventral horn neurons after transection, supported the

findings. Reports of PACAP mRNA expression in the developing rat spinal cord (Lindholm et al., 1998; Skoglösa et al., 1999; Jaworski and Proctor, 2000) give a morphological base supporting a reactivation and induction of expression in the adult animal, especially in response to perturbations of the environment, e.g. nerve injury, which has been described for other neuropeptides (Hökfelt et al., 2000). The increase in PACAP expression in motor neurons in reponse to nerve transection suggests a role for PACAP in modulation of injury responses in motor neurons as well as in sensory (Zhang et al., 1995; Zhang et al 1996b) and sympathetic (Moller et al., 1997) neurons. A role for PACAP in modulation of injury responses in motor neurons is also supported by an increased PACAP expression in the rat facial motor nucleus after axotomy of the facial nerve (Zhou et al., 1999a) and its effect in induction of events promoting regeneration of the facial nerve in guinea pigs after transection (Kimura et al., 2003). It has been hypothesized that PACAP can act as a survival factor for axotomized neurons (Waschek, 2002), and that it has neurotrophic and neuroprotective effects (Vaudry et al., 2000; Vaudry et al., 2002), as well as being able to trigger cell cycle withdrawal, inducing transition from proliferation to neuronal differentiation (Lu and DiCicco-Bloom, 1997).

Previously, PACAP was believed only to be expressed in the primary sensory neurons terminating in the dorsal horn, and not in the spinal cord itself. Our findings of PACAP mRNA expression in the dorsal horn neurons, may have consequences on the interpretation of the involvement of PACAP in modulation of sensory, and especially nociceptive processing. In summary, the findings of PACAP expression in neurons in the superficial laminae of the dorsal horn strengthens its suggested role as a modulator of nociceptive transmission. Further, the increased expression in motor neurons in the ventral horn indicates a role for PACAP in repair/regeneration of motor neurons. The ability to detect PACAP mRNA in spinal cord neurons is probably a result of our improved in situ hybridization protocol (including relatively high hybridisation temperatures and high concentration of DTT (Dagerlind et al., 1992) yielding a high signal to noise ratio, and in conjunction with that the possibility to expose the film emulsion to the hybridized tissue for a longer period of time without any distracting increase in background labeling

Changes in PACAP expression in response to nerve compression or transection

Nerve compression is a fairly common condition in human patients, far more common than nerve transection injuries. Therefore it is of great importance to investigate the effect of experimentally induced compression. Carpal tunnel syndrome is an example of a compression injury, at wrist level, that affects the median nerve on its way through the narrow carpal tunnel between the carpal bones and the relatively rigid flexor retinaculum. The prevalence of carpal tunnel syndrome have been estimated to ~4% of the adult Swedish population, and affects slightly more women than men. The etiology of compression injuries is diverse and in most cases unknown, although factors such as

overweight or obesity, certain occupations (especially those associated with prolonged periods of wrist extension/flexion, use of excessive force with the hand or use of vibrating tools), seem to increase the susceptibility for carpal tunnel syndrome (Atroshi et al., 1999). The condition is characterized by a tingling/burning sensation and numbness in the innervation area, and can in severe cases lead to e.g. permanent loss of sensation and atrophy of the muscles, if left untreated (Rempel et al., 1999; Mackinnon, 2002). Pain may be a prominent feature especially in the severe compression injuries, and some patients experience pain/allodynia during compression and/or after decompression of the nerve. Since PACAP has been suggested to modulate nociceptive responses we wanted to monitor its expression during compression injury. In paper IV, we used an experimental model which mimics the clinical situation, to investigate the changes of PACAP and PAC_1 expression in DRG, spinal cord and the sciatic nerve. The expression after nerve compression is compared to the expression induced by nerve transection and inflammation.

PACAP expression is increased in DRG neurons in response to nerve transection, peripheral inflammation, and nerve compression. The increase takes place in slightly different populations depending on the type of injury. After nerve transection, an upregulation is detected primarily in the medium-large diameter neurons, but in some studies an induction has also been detected in some small diameter neurons (Zhang et al., 95; Zhang et al., 1996b; Jongsma Wallin et al., 2001; paper V). Peripheral inflammation results in an induced PACAP mRNA expression in primarily small diameter neurons (Zhang et al., 1998; Jongsma Wallin et al., 2003), whereas nerve compression gives rise to an upregulated expression in both small and larger diameter neurons (paper IV). The dual effect on the neuron in response to compression might reflect the complex conditions the nerve is exposed to during this type of injury. During a compression injury, the nerve is subject to ischaemia as well as a certain degree of mechanical injury. In our experimental model, an inflammatory component was also present (degeneration in the distal nerve segment and implantation of the silicone tube elicits an inflammatory reaction), which might promote the PACAP expression in smaller neurons. The consequences of compression are in relation to the duration and magnitude of the pressure applied to the nerve (Rempel et al., 1999).

Compared to the changes observed after nerve transection, compression injury induces a smaller upregulation in PACAP mRNA expression, which was expected since it is a less severe injury (first-second degree injury) than a complete nerve transection (fifth degree injury) according to the Sunderland definitions (see; Introduction). However, it is important to note that the quantification of PACAP mRNA expression after nerve transection (Jongsma Wallin et al., 2001; paper V), and compression injury (paper IV) are relative numbers and not absolute values, so a definitive comparison can not be made between the different studies. Nevertheless, we have had strong indications that the values are rather consistent, when comparing the results from quantifications of the PACAP mRNA hybridization in DRGs from the control side in different studies, even when the quantifications have been performed by

different investigators. Therefore, a comparison between the PACAP mRNA expression after nerve compression (paper IV) and transection (paper V) could be adequate, since the quantifications in these studies were performed by the same person (the author). No obvious changes in PACAP mRNA expression were observed in spinal cord after nerve compression (paper IV), whereas expression was induced after both distal and proximal nerve transection (paper III). This might also reflect that compression injury is a less severe injury than nerve transection, and therefore the effects are less extensive in the spinal cord. However, it is possible that there were some minor effects on spinal cord expression, although too small to be able to detect, and if left for longer durations, also the compression injury might have induced a visible change in PACAP expression in spinal cord neurons. It is not unlikely that the increased PACAP expression in response to nerve compression and nerve transection may affect nociceptive transmission, especially in the case of compression were the level of PAC_1 in spinal cord was virtually unchanged, as opposed to a slightly reduced ligand binding observed in the dorsal horn in response to nerve transection (Jongsma et al., 2000).

An increased immunoreactivity for PACAP was observed in the compressed sciatic nerve indicating a block of axonal transport and/or increased export of PACAP to the periphery (see; Results and comments). Inhibition of axonal transport in the peripheral branch of the axon would result in a blockage of the retrograde axonal transport of e.g. trophic factors from the target tissues to the cell soma. Impediment of retrograde transport, in conjunction with nerve compression, has been observed to induce alterations in the production in neuronal cell bodies (Dahlin and McLean, 1986; Dahlin et al., 1986). In addition, PACAP mRNA expression in the DRG is regulated by the neurotrophins, NGF, NT-3 and BDNF (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003; Pettersson et al., 2003/paper V). Complete nerve transection obviously results in inhibition of retrograde axonal transport as well. Taken toghether, this suggests that an interruption in retrogradely transported NT-3 from cells in the periphery, may be partly responsible for the increased PACAP mRNA expression that we observed in large DRG neurons after nerve transection or compression injury (papers IV, V). Further, a locally increased production of NGF and/or BDNF in response to inflammation (Donnerer et al., 1992; Cho et al., 1997) may boost the increased PACAP mRNA expression seen in small DRG neurons after compression injury (paper IV), but also to some extent after nerve transection (paper V).

Methodological considerations: As described in the introduction, cell death, changes in cell body size, and eccentricity of the nucleus in DRG neurons have been reported in conjunction with nerve injuries (see; Introduction). These events have to be considered since they would affect the proportions of labeled neurons (total number, as well as the proportions of small versus large neurons) after the different quantifications (papers IV, V). As discussed in the introduction, cell death in rat DRG neurons in response to e.g. proximal nerve transection, is rather insignificant at durations shorter than 15 days as showed

with stereological methods (Vestergaard et al., 1997). The level of transection seems to be a crucial factor, and a more distal transection (mid-thigh) did not induce any significant cell death until after 8 weeks of transection (Tandrup et al., 2000). Further, differences in susceptibility to induced cell death in neurons of different sizes were observed at the earliest 45 days after proximal nerve transection (Vestergaard et al., 1997). However, significant cell death has been observed after nerve transection at earlier stages (7 days) in mice. We studied the PACAP mRNA expression in rat DRG neurons after 3 days of proximal nerve transection (paper V), and after 3, 7, 14, or 28 days of nerve compression (at mid-thigh level; paper IV). The effects of distal or proximal nerve transection were also studied in spinal cord after 3 days or 3 weeks respectively (paper III). After 3 weeks of proximal nerve transection, cell death might theoretically have taken place in the DRG neurons, but since we studied alterations in expression in spinal cord neurons and not in the DRG, such changes would not interfere with the performed quantifications, unless motor neuronal cell death is induced. However, in the adult rat, no or only a few motorneurons are lost after mid-thigh sciatic nerve transection as discussed by Lowrie and Vrobova (1992), and after transection 10 mm distal to the cervical DRG no major cell loss occurred in the ventral horn until 6 weeks after injury (Ma et al., 2001). Transection of the peripheral process of DRG neurons may induce changes in the dorsal horn area where the central processes of these primary sensory afferents terminate (Aldskogius and Kozlova, 1998). In our study, we did not observe any obvious changes in PACAP mRNA expression in the dorsal horn after injury, although this expression was not quantified.

Changes in mean perikaryal volume was seen at the earliest 4 days after proximal nerve transection (using stereological methods), and 15 days after nerve crush (Vestergaard et al., 1997; Degn et al., 1999). Since we performed the quantifications already after 3 days of nerve transection, this should not interfere with our data. However, the effects of nerve compression were studied after different time periods, ranging from 3-28 days, but the decrease in cell volume observed after proximal nerve crush (Degn et al., 1999), was limited to <2% of cells with a diameter range around the border of 35 μm (which we use as a cut off to separate small and large cells). This indicates that cell volume shrinkage does not influence our results to any great extent. Besides, we used a less severe injury model (compression injury), and furthermore, the compression injury was inflicted at a more distal level (mid-thigh) than in the study by Degn and coworkers (1999).

Eccentricity of the nucleus would interfere with the proportions of small versus large neurons in our quantifications, since they are based on the criteria that neurons with a visible nucleus in at least one of two adjacent sections is quantified, and the diameter of the neuronal profile is then estimated on the assumption that the cell body is spherical. If eccentricity of the nucleus was to take place, this would make us underestimate the size of some of the neurons if sectioned at the edge of the neuron, but still showing a visible (eccentric) nucleus. Since we did not see any obvious signs of eccentricity of the nuclei in the DRG neurons after nerve compression (3, 7, 14 and 28 days; paper IV),

after proximal nerve injury (3 days; paper V), or on semi-thin plastic embedded sections after compression (Pettersson LME, unpublished findings), this was not regarded as a major issue .

Changes in PACAP expression –regulation by endogenous BDNF

Neurotrophins have been shown to affect the regulation of various neuropeptides (Verge et al., 1995; Woolf, 1996a), and we have previously shown that both NGF and NT-3 have effects on the regulation of PACAP mRNA expression in DRG neurons. NGF induces an upregulation in PACAP mRNA expression in intact, as well as in inflamed or transected small DRG neurons, whereas NT-3 downregulates PACAP mRNA expression in intact neurons, and mitigates the injury induced upregulation seen primarily in large neurons in response to nerve transection (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003). BDNF, which is another member of the neurotrophin gene family of neurotrophic factors (see; Introduction), is normally expressed in ~30-50% (levels vary between the different reports), mostly small, trkA positive DRG neurons (Ernfors et al., 1990; Wetmore and Olson, 1995; Apfel et al., 1996; Michael et al., 1999). BDNF is upregulated in sensory neurons in response to nerve injury, implying a possibility for BDNF in the modulation of the peptidergic phenotype in DRG neurons after nerve injury. The pattern of BDNF mRNA expression in response to nerve injury and inflammation is similar to that of PACAP. An upregulation in BDNF mRNA expression is reported in response to i) nerve transection; upregulation in primarily medium-large diameter/trkB and trkC positive neurons and a time dependent downregulation in a subpopulation of small neurons (Michael et al., 1999, Zhou et al., 1999b; Karchewski et al., 2002), ii) inflammation; upregulation in primarily small trkA expressing DRG neurons (Cho et al., 1997b) or iii) NGF treatment, inducing an upregulation in both trkA and non-trkA expressing neurons (Apfel et al., 1996). These similarities in injury induced responses between BDNF and PACAP further indicate that BDNF might be involved in the modulation of PACAP expression.

Transection of the sciatic spinal nerve resulted in an upregulation in PACAP mRNA expression in both small and large DRG neurons (paper V). We found that intrathecal infusion, but not intraperitoneal injections of sheep anti-BDNF IgG, were effective in mitigation of the injury increased PACAP mRNA expression in DRG neurons after proximal nerve transction. The reduction in expression mediated by intrathecal infusion is found in both small and large diameter neurons, in neurons expressing trkB as well as in neurons not obviously expressing trkB. This suggests that the effect of endogenous BDNF on PACAP mRNA regulation in neurons after axotomy is not exclusively a direct effect on neurons expressing detectable levels of trkB (the high affinity receptor for BDNF). The mitigation might also be an effect of BDNF signalling via trkB expressing neurons and/or non-neuronal cells, which in turn release e.g. cytokines and growth factors and regulate the PACAP mRNA expression in non-trkB neurons. Another possibility would be that

BDNF acts on the common neurotrophin receptor, p75, which is expressed in a majority of the DRG neurons, and also in other cell types (Karchewski et al., 1999), thereby effecting a more global response. Finally, some caution when interpreting the data is needed, only one pair of DRGs were analyzed quantitatively and the expression of trkB mRNA in transected neurons infused with control IgG was very low when compared with the trkB immunoreactivity after axotomy in a previous report (Foster et al., 1994). This might indicate that the slides were underdeveloped and that in some of the neurons expressing trkB at low levels, the levels were too low to detect. In this case, trkB expression would be underestimated also after transection, both on the control infused and anti-BDNF treated animals, resulting in an underestimation of neurons showing coexpression of PACAP and trkB. In order to resolve this problem additional experiments will be performed.

After axotomy of the sciatic nerve, there is a reduction in the retrograde supply of NT-3 to the DRG from the periphery, which might be partly responsible for the upregulation in BDNF expression observed in these neurons (Tonra et al., 1998; Michael et al., 1999, Thompson et al., 1999; Zhou et al., 1999b). Support for this theory is given by the findings that NT-3 infusion has a negative effect on BDNF expression in intact and injured DRG neurons (Karchewski et al., 2002). NGF has also been shown to produce a dramatic increase in BDNF mRNA expression in DRG neurons (Apfel et al., 1996), as well as having effects on BDNF expression in conjunction with both nerve transection (Verge et al., 1996; Shadiack et al., 2001; Karchewski et al., 2002) and inflammation (Cho et al., 1997b; Thompson et al., 1999). A speculation would be that the effects of NGF and NT-3 on the regulation of PACAP mRNA expression are mediated at least partly via BDNF. PACAP has in turn been observed to induce the expression of BDNF in hippocampal neurons (Yaka et al., 2003), and could possibly also modulate actions/expression of BDNF, NGF and NT-3 in other neurons. The findings that the injury induced changes after nerve crush were more pronounced in PAC_1-/- mice than in wild type mice (paper II), indicate a neuroregulatory effect also for PACAP and suggests a connection between different neuropeptides in regulating each other.

Taken together, our findings indicate a role for endogenous BDNF in modulation of the peptidergic phenotype and regulation/induction of PACAP mRNA expression in DRG neurons in response to injury.

Conclusions and plans for the future…

- OFQ/N and ORL1 are expressed in spinal cord gray matter, OFQ/N especially in superficial laminae of the dorsal horn, and in ventral horn motor neurons (where ORL1 is also observed) and interneurons. In addition both the ligand and the receptor are expressed in small and large diameter DRG neurons, respectively. Taken together, these findings suggest a possibility for OFQ/N to modulate sensory signalling at sites outside the brain, i.e. in the periphery, as well as at the spinal cord level, and may help explain the complex effects it has on e.g. nociceptive behaviours. Further, the presence of OFQ/N and ORL1 in interneurons and motor neurons and also in the SCG suggests further possibilities for modulatory functions in the somatic and autonomic motor systems.

- PACAP is in involved in the modulation of inflammatory nociceptive behaviour, and the effect is mediated via the PAC_1 receptor. A possible effect on acute chemically induced pain is also suggested.

- PACAP is expressed in adult rat spinal cord neurons, both in the superficial laminae of the dorsal horn and in ventral horn neurons. Increased expression in ventral horn motor neurons is induced in conjuction with nerve injury, and the increase is more pronounced after a proximal than a distal nerve transection. This implicates that the possibilities for modulation of sensory/nociceptive transmission is more complex than previously expected, and further suggests a role for PACAP in repair and regeneration of motor neurons.

- A pronounced increase in PACAP expression is observed in DRG neurons, both in response to nerve transection, and nerve compression. Complete nerve transection is a more severe injury than compression (although not as common in the clinic), and this is reflected in the response; a somewhat greater surge in PACAP expression. These alterations in PACAP expression indicate a role for PACAP in modulation of the injury response and possible involvement in pain modulation as well as in the repair/regenerative processes.

- Our previous studies have shown that NGF and NT-3 are involved in the regulation of PACAP expression, and in this work, we have been able to show that endogenous BDNF is involved in controlling PACAP expression (in response to nerve transection).

Future plans: Additional in situ hybridization analysis of trkB and PACAP mRNA expression and colocalisation after injury, and in conjunction with anti-BDNF infusions will be performed. A second study, examining if the effects of endogenous BDNF on PACAP mRNA regulation is limited to nerve transection, or if an effect is also executed in conjunction with peripheral inflammation. Further, studies investigating the tactile responses, and possible development of allodynia, during experimentally induced compression, and after decompression of the nerve. Such effects are sometimes observed in the clinical conditions, and after decompressing surgery. The effects on neuropeptide expression will also be observed.

Svensk sammanfattning (Summary in Swedish)

Information om vår omvärld förmedlas med hjälp av elektriska och kemiska signaler i kroppens olika nervbanor till hjärnan där vi tolkar signalerna. Dessa s.k. sensoriska signaler förmedlar information om så skilda saker som hur maten smakar till hur varmt det är, hur ett föremål ser ut, känns osv, till smärtinformation som hjälper oss att undvika farliga situationer. I vissa situationer då nervsystemet är retat eller skadat kan stimuli som normalt inte utlöser smärta, uppfattas som smärtsamt. Neuropeptider är en grupp av små äggviteämnen (peptider) som dels kan fungera som transmittorer (förmedlar vidare den elektriska signalen mellan olika nervceller), men också modulera styrkan hos de signaler som skickas i nervsystemet. Neuropeptider är därför viktiga komponenter bl. a. vid fortledning av sensoriska och motoriska (kontrollerar muskelfunktion) signaler, men även i andra situationer. Förändringar i neuropeptiduttryck i samband med t. ex. en nervavskärning, ger en indikation om att den specifika peptiden är inblandad i, och kan modulera det specifika nervsvaret.

I mitt avhandlingsarbete har jag studerat två olika neuropeptider; orphanin FQ/nocicpetin (OFQ/N) och pituitary adenylate cyclase activating polypeptide (PACAP), för att ta reda på var de bildas normalt, hur deras uttryck regleras i olika skadesituationer (nervavskärning och nervinklämning), samt även vilken roll de spelar för upplevelse av smärta. Detta har studerats hos mus och framför allt hos råtta. Båda dessa neuropeptider har tillskrivits en mängd olika funktioner, bl.a. har de föreslagits kunna modulera sensoriska signaler, och framför allt smärtsignaler.

Vi fann att både OFQ/N och dess receptor (den molekyl som medierar effekten då en specifik neuropeptid binder in till och aktiverar ”sin” receptor) uttrycks både i nervceller i ryggmärgens bakre horn samt i sensoriska dorsalrotsganglier i det perifera nervsystemet, dvs de strukturer som är inblandade i förmedlingen av olika sensoriska signaler. Dessa fynd styrker OFQ/N:s roll som transmittor av dessa signaler och dess möjlighet att modulera känselsignaler. OFQ/N och dess receptor uttrycktes också i motoriska nervcellkroppar (i ryggmärgens främre horn), som kontrollerar kroppens muskler, vilket indikerar att OFQ/N kan ha en funktion också vid modulering av motoriska signaler.

Uttryck av den andra neuropeptiden, PACAP detekterades i nervcellkroppar i dorsalrotsganglier och i de områden i ryggmärgen som är relaterade till fortledning av känsel och smärtinformation, samt i ett fåtal nervcellkroppar i de områden i ryggmärgen som är involverade i motorisk signalering. Dessutom ökade uttrycket av PACAP i ryggmärgens motoriska neuron efter nervskada, vilket indikerar att PACAP är inblandat i och modulerar signalering i detta system. PACAP skulle därmed kunna ha en roll vid reparation och utväxt av de skadade nervcellsfibrerna. En dramatisk ökning av PACAP uttrycket skedde också i dorsalrotsganglierna både efter nervavskärning, och efter nervinklämning. Att en sådan förändring sker i

känselnervceller som svar på skada talar för att PACAP kan fungera som modulator för smärtsignaler.

Vi har redan i tidigare studier visat att vissa tillväxtfaktorer för nervceller kan påverka PACAP uttrycket vid skada och för att vidare studera de mekanismer som styr regleringen av PACAP vid skada, tillsatte vi en antikropp som binder upp och inaktiverar en specifik tillväxtfaktor, BDNF (brain derived neurotrophic factor), som normalt finns i nervsystemet. BDNF uppregleras också efter nervskada, och detta sker framför allt i samma populationer av känselnervceller som uppreglerar PACAP. Den uppreglering av PACAP som normalt sker efter nervskada var klart minskad efter tillförsel av antikropp mot BDNF, vilket talar för att BDNF kan reglera PACAP uttryck vid skada.

Efter nervinklämning fann vi även en ökad förekomst av PACAP i den perifera inklämda nerven. Detta innebär att det kan ha skett en en ökning av transporten av PACAP till nervens perifera ändar och/eller att en uppdämning av en sådan transport skett i den inklämda nerven. PACAP har föreslagits ha en anti-inflammatorisk roll, då det kan stimulera inflammatoriska celler (makrofager) till en ökad frisättning av anti-inflammatoriska substanser, samt hämma deras produktion av inflammationsinducerande ämnen. En del andra neuropeptider har istället en inflammationsinducerande effekt och man har funnit att frisättning av dessa peptider perifert kan leda till s.k. neurogen inflammation (d.v.s. inflammation som orsakas då immunförsvaret stimuleras via sensoriska nerver).

Slutligen undersöktes smärtsvaren hos genmanipulerade möss som saknade högaffinitetsreceptorn för PACAP, dvs PACAP kan inte binda in till denna receptor och aktivera den signalväg som det normalt gör hos djur vilka har receptorn. Resultaten av denna studie var att de möss som saknade en signalväg för PACAP uppvisade ett starkt nedsatt smärtbeteende som svar på lokal inflammation jämfört med normala möss. Detta pekar på att PACAP har en smärtdrivande effekt vid inflammation. Dessutom uppvisade de möss som saknade receptorn ett högre uttryck av en del andra neuropeptider, vilket skulle kunna förklaras av att PACAP även påverkar reglering av andra neuropeptider efter skada. Sammanfattningsvis verkar PACAP ha en roll vid modulering av både smärtsignaler och motoriska signaler, samt kan bidra till reparation och utväxt vid nervskada. En tredje funktion skulle kunna var en påverkan på det inflammatoriska svar som uppträder efter skada.

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APPENDIX
(Papers I–V)

Paper I

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Research report

Expression of orphanin FQ/nociceptin and its receptor in rat peripheral ganglia and spinal cord

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Abstract

Expression of the neuropeptide orphanin FQ/nociceptin (OFQ/N) and its receptor, the opioid receptor-like receptor (ORL1), have been found to have a wide distribution in the central nervous system, and in brain areas involved in sensory perception in particular. The effects of OFQ/N on, e.g., sensory transmission are very complex, and a modulatory effect on pain perception has been suggested. We therefore wanted to investigate the distribution of OFQ/N and ORL1 in the spinal cord and DRG, and also in SCG and some other peripheral tissues. The methods used were in situ hybridization, immunohistochemistry and ligand binding. We found that OFQ/N and ORL1 mRNA are expressed in DRG; primarily in small and large neurons, respectively. In spinal cord, mRNA for OFQ/N and ORL1 is expressed in neurons in laminae I, II and X, and in ventral horn neurons. Further, immunoreactivity for OFQ/N is observed in fibers and neurons in the superficial laminae of the dorsal horn and around the central canal, and also in neurons in the ventral horn of the spinal cord. Receptor ligand binding to the spinal cord grey matter is demonstrated, primarily concentrated to the dorsal horn and around the central canal, and also to medium and large size DRG neurons. These findings on the morphological distribution pattern of OFQ/N and ORL1 at the cellular level may support the notion that OFQ/N is involved in modulating pain transmission. Further, expression of OFQ/N and ORL1 mRNA was also found in SCG, whereas expression was undetectable in skin.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: Regional localization of receptors and transmitters

Keywords: Dorsal horn; Dorsal root ganglia; Neuropeptide; Opioid; Sensory neurons; Superior cervical ganglia

1. Introduction

The neuropeptide orphanin FQ/nociceptin (OFQ/N) and its receptor, opioid receptor-like receptor (ORL1), are suggested to modulate a wide range of behaviours and sensory perception in particular. The functional effects of OFQ/N are complex, and it has both nociceptive and antinociceptive actions depending on the administered dose, route of administration, species investigated and also whether acute or chronic nociceptive behaviour is examined [1,5,11,14,21] and reviewed in Ref. [8]. The diverse effects of OFQ/N are consistent with its supposed neuromodulatory function, exercised via ORL1. The receptor is a G_i/G_0-protein coupled receptor whose activation results in inhibition of voltage-sensitive (NMDA) Ca^{2+} channels, inhibition of adenylate cyclase and activation of K^+ channels, which in turn inhibits release of several neurotransmitters [21] (reviewed in Refs. [8,15]).

OFQ/N and ORL1 have been reported to have a wide distribution in the central nervous system of the rat and mouse (reviewed in Ref. [20]), whereas the functions and distribution in the periphery have been less studied [16,19,22,26]. In a study by Inoue et al. [11], an intraplantar injection of OFQ/N into the hind limb of mice elicited a nociceptive response, probably via the release of substance P (SP), but no endogenous peripheral source of, or binding site for OFQ/N was established.

We have focused on the expression of ORL1 and its ligand in spinal cord and dorsal root ganglia (DRG) and also in some other peripheral tissues such as skin and

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superior cervical ganglia (SCG). This, since we wanted to establish if OFQ/N and its receptor are located in primary sensory neurons as well as in spinal cord areas related to sensory pathways, including nociceptive pathways. SCG was included in the study in order to compare the expression in a functionally different peripheral ganglion. The methods used were in situ hybridization, immunohistochemistry and ligand binding. Findings of a peripheral location of OFQ/N and ORL1 synthesis could imply a role for the OFQ/N system in the peripheral components of the nociceptive pathways and perhaps also in other sensory functions.

2. Materials and methods

2.1. Animals and tissue processing

This study was approved by the Animal Ethics Committee at Lund University. Spinal cord (cervical, thoracal and lumbar segments), DRG (corresponding), SCG and hypothalamus were dissected from adult male ($n=8$) and female ($n=15$), Sprague–Dawley rats (170–200 g; Møllegaard, Denmark). To allow for some comparison, corresponding tissues from 129/Sv mice (20–25 g, B&K Universal, Sweden), male ($n=5$) and female ($n=7$) were included in the analysis. The results from the mice studies are only reported in the text, since they were similar to the results obtained from the rats.

Normal and inflamed skin was obtained from rats ($n=3$, 3 days inflammation; and $n=3$, control animals) in which Freund s complete adjuvant (2×75 μl) had been injected under the skin around the tarso-tibial joint on the right side. Animals were killed after 3 days and skin from the affected joint was dissected (for further details see Ref. [27]). All animals were killed by heart puncture under pentobarbital (60 mg ml^{-1}, i.p.) anaesthesia. Tissue specimens were mounted in O.C.T. Compound (Tissue-Tek®, Histolab Products, Göteborg, Sweden), fresh-frozen in isopentane at −30 °C immediately after dissection, and stored at −80 °C for in situ hybridization and ligand binding. For immunohistochemistry, the tissues were fixed in Stefanini s fixative (2% paraformaldehyde and 15% of a saturated aqueous picric acid solution, in a 0.1 M phosphate buffer, pH 7.2) overnight, followed by repeated rinsing in sucrose-enriched (10%) Tyrode s solution. Specimens were mounted in Tissue-Tek®, frozen on dry ice and stored at −80 °C. Tissue specimens were sectioned (6 μm, adjacent sections for colocalisation, and 10 μm for immunohistochemistry and in situ hybridization, and 20 μm for ligand binding) in a cryostat and thaw-mounted on to Super Frost® Plus slides (Menzel-Gläser, Germany).

Hypothalamus was used as a positive control in immunohistochemistry and ligand binding. Spinal cord dorsal horn laminae I and II were defined in the microscope by using darkfield imaging, allowing these two superficial laminae to be distinguished from the other laminae.

2.2. In situ hybridization

Radioactively ^{35}S-labeled oligodeoxyribonucleotide (oligoDNA) probes for detection of OFQ/N, ORL1 and the vesicular acetylcholine transporter (VAChT) mRNAs were used for in situ hybridization. OligoDNA probes complementary to and selective for OFQ/N mRNA nucleotides 432–461 [14], ORL1 mRNA nucleotides 729–758 [6] and VAChT mRNA nucleotides 683–715 [24] were synthesised and purified at the Biomedical Resource Facility, Lund University, Sweden. VAChT is expressed in all cholinergic neurons [24], and colocalisation of VAChT with either of the other two probes was studied to check for expression of OFQ/N or ORL1 mRNA in motorneurons. All used cDNA regions were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm.nih.gov, May 2001); no greater than 60% homology was found to sequences other than the cognate transcript. All stages prior to hybridization were carried out under sterile conditions and all dilutions were performed in autoclaved double-distilled water to prevent degradation of RNA by external RNAses. Probes, at a concentration of 1.3 ng/μl were incubated at 37 °C for 1 h with 4.9% terminal transferase enzyme (Perkin-Elmer, Zaventem, Belgium) to ligate the [^{35}S]dATP, 15% (37 MBq, Perkin-Elmer) to the 3′ end, in a terminal transferase buffer (cacodylic acid sodium salt 98 mM, manganese chloride tetrahydrate 0.99 mM, magnesium chloride hexahydrate 0.98 mM and dithiothreitol (DTT) 89 μM). The reaction was stalled by lowering the temperature and adding the following substances to a final concentration of 0.01 M EDTA, 0.10 μg/μl yeast tRNA (Sigma, St. Louis, MO, USA), and 5.6 mM Tris-based buffer including 0.44 mM EDTA. After the labeling, probes were purified by centrifugation in Chroma spin-10 columns (Clontech, Intermedica, Stockholm, Sweden). Thereafter the activity, approximately 1.6×10^{9} cpm/μg, was measured in a liquid scintillator. In order to minimize background labeling as a result of unspecific binding between the phosphorothioate moiety of the probe and disulfides in the tissue, *N*-ethylmaleimide (NEM) (Sigma) in saline sodium citrate (SSC, 1×SSC=0.15 M NaCl and 0.015 M sodium citrate) was added [28] to a final concentration of 30 mM, and probes were stored at −18 °C.

Tissue sections were dehydrated in graded ethanol concentrations (70, 95 and 99%), delipidated in chloroform and air dried. Hybridization was conducted with ^{35}S-labelled probe to a final activity of 10 000 cpm (0.68 and 1.3 pmol/ml) and 20 000 cpm (1.3 pmol/ml) for OFQ/N, VAChT and ORL1 probe, respectively. The hybridization solution was prepared by adding the probe to a solution of 50% formamide, 4×SSC, 1×Denhardt s solution (0.02% bovine serum albumin (BSA), 0.02% Ficoll and 0.02%

polyvinylpyrrolidone), 10% dextran sulphate, 0.24 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, 1% sarcosyl and 0.2 M DTT. Hybridization was conducted overnight with 50 μl hybridization solution/slide under parafilm, in air-sealed moist chambers at 43 °C. Control slides were incubated with an addition of unlabeled probe in 100-fold molar excess. Following hybridization, the slides were dipped in 1×SSC (10 min, room temperature), and the parafilm was removed. The slides were then bathed in 0.5×SSC (4×15 min, 55 °C), and 1×SSC (30 min, room temperature). The sections were dehydrated in ethanol (70 and 95%) with 0.3 M ammonium acetate (3 min, respectively) and 99% ethanol (5 min), and air dried. Finally, slides were immersed in Ilfords K5 photoemulsion (diluted 1:1 in distilled water) and stored at 4 °C. After 4–5 weeks the slides were developed in Kodak D19 (5 min), rinsed in tap water, fixed in Kodak polymax fix (10 min) and rinsed (10 min). Counterstaining was performed with Htx (60 s) and excess dye was washed away with tap water. Slides were mounted in Kaiser s medium (glycerol–gelatin, Merck, Darmstadt, Germany).

2.3. Immunohistochemistry

All dilutions were performed in PBS (0.01 M phosphate-buffered saline, pH 7.3), 0.25% Triton X-100 and 0.25% BSA and all steps were followed by wash in PBS, 0.25% Triton X-100. Sections were incubated in moist chambers with primary antibody against synthetic OFQ/N (1:1280, Biotrend Chemikalien, Köln, Germany), for 48 h at 4 °C. Excess antibody was washed away for 15 min after which the sections were incubated with pig anti-rabbit FITC (1:80, Dako, Copenhagen, Denmark), for 1 h at room temperature. Slides were mounted in PBS/glycerin (1:1). To establish the specificity of the antibody, additional slides were incubated for 48 h with antibody absorbed overnight with the antigen (synthetic OFQ/N, Phoenix Pharmaceuticals, Belmont, CA, USA) in excess (100 μg/ml diluted antiserum), and processed as above.

2.4. ^{125}I-[Tyr^{14}]-OFQ/N binding

^{125}I-[Tyr^{14}]-OFQ/N binding was used to locate binding of OFQ/N to its receptor. Sections were preincubated for 30 min at 20 °C in a 0.05 M Tris–HCl buffer (pH 7.4), containing 2% bovine serum albumin, 5 mM $MgCl_2$ and 0.5 μg/ml bacitracin. Slides were then preincubated in the same solution with the addition of ^{125}I-[Tyr^{14}]-OFQ/N (Perkin-Elmer), 600 pM, at 20 °C for 1 h. Control sections were checked for non-specific binding in the presence of 6 μM OFQ/N. Slides were washed in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1% bovine serum albumin, 5 mM $MgCl_2$ and 0.5 μg/ml bacitracin, 3×5 min, at 4 °C and dipped in distilled water. When slides were dry, they were covered with Hyperfilm MP (Amersham, Solna, Sweden) and stored in the dark for 15 days at 4 °C, after which the film was developed as above for in situ hybridization.

3. Results

3.1. In situ hybridization

Expression of OFQ/N mRNA was found predominantly in neurons in lamina II, but also in lamina I in spinal cord dorsal horn. Further, OFQ/N mRNA was expressed in neurons dorsolaterally to the central canal, in some medium and a few large sized neurons in the ventral horn and also in a small number of cells scattered throughout the grey matter (Fig. 1). Colocalisation of mRNA for OFQ/N and VAChT in the ventral horn was seen in a few neurons. In addition, OFQ/N mRNA was expressed in smaller neurons negative for VAChT mRNA, and VAChT mRNA was also expressed in larger ventral horn neurons negative for OFQ/N mRNA (not shown). Cells expressing ORL1 mRNA were scattered throughout the grey matter with the greatest labeling density in large ventral horn neurons, but also in neurons dorsolaterally to the central canal and to some extent in the dorsal horn (Fig. 2). Many large ventral horn neurons did coexpress mRNA for ORL1 and VAChT (Fig. 3), although some neurons positive for ORL1 mRNA and negative for VAChT mRNA and vice versa, were also found. In DRG, distinct OFQ/N mRNA expression was seen in a subpopulation of small to medium sized neurons, whereas labeling for ORL1 mRNA was present in many large and some medium sized neurons (Fig. 4). In SCG, a low but consistent OFQ/N mRNA expression was evident in nerve cell bodies in rat, but questionable in mouse, whereas ORL1 mRNA labeling was present in many nerve cell bodies of both species (Fig. 5). No expression of OFQ/N or receptor mRNA was found in normal or inflamed skin (not shown). No labeling was found in the controls hybridized in the presence of the corresponding unlabeled probe in excess. Apart from the inconsistency in OFQ/N mRNA expression in SCG between rats and mice, there was no overt difference between females and males and rats and mice in the distribution pattern or labeling intensity for either OFQ/N or ORL1 mRNA in DRG or spinal cord.

3.2. Immunohistochemistry

OFQ/N immunoreactive fibers and neurons were seen in the superficial layers of spinal cord (lumbar level), and occasionally also in deeper laminae of the dorsal horn (Fig. 6a). Many large and medium sized ventral horn neurons showed intense immunoreactivity for OFQ/N (Fig. 6b). In the hypothalamus, fibers and some cell bodies were immunoreactive for OFQ/N, whereas in DRG, SCG or skin (normal or inflamed) no specific immunoreactivity could be detected (not shown). There was no overt

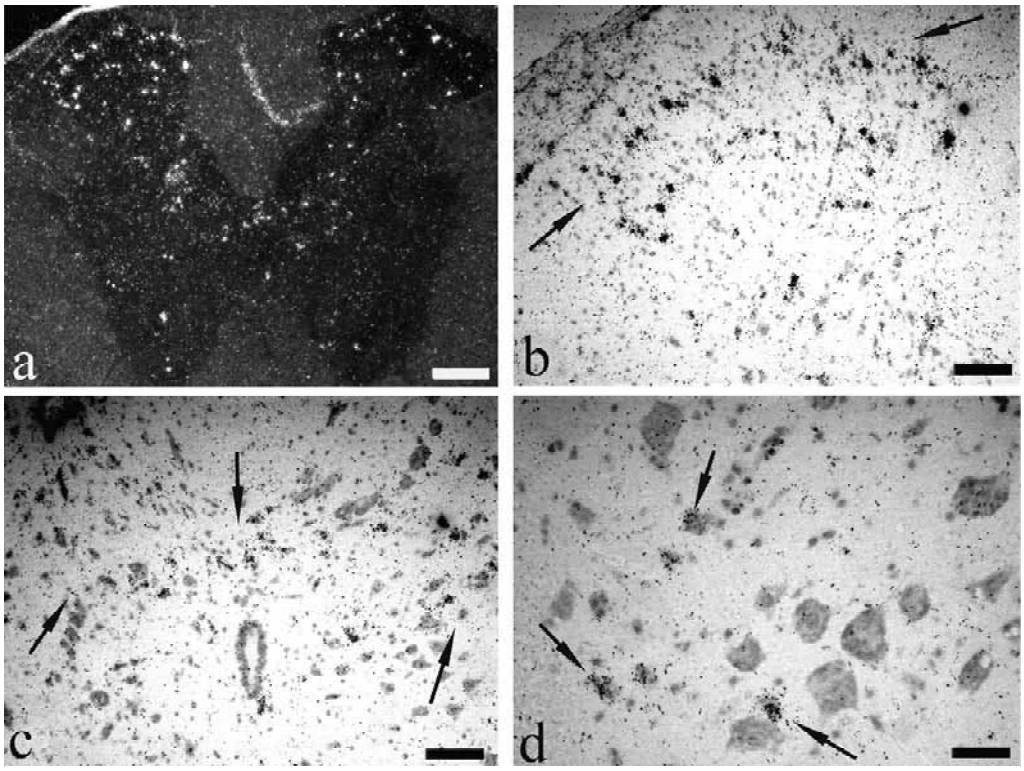

Fig. 1. In situ hybridization for OFQ/N mRNA in rat spinal cord. Darkfield image showing labeling of OFQ/N mRNA (a). Brightfield images showing OFQ/N mRNA labeling in dorsal horn neurons (b), in neurons dorsolaterally (area marked with arrows) to the central canal (c), and in some ventral horn neurons, presumably interneurons (d). Dorsal horns upward in figure. Arrows in (b) indicate the outer edge of lamina I, and exemplify labeled neurons in (d). Scale bars: (a) 200 μm; (b,c) 100 μm; (d) 50 μm.

difference between females and males in the intensity or distribution pattern of the immunoreactivity for OFQ/N in the tissues examined. Further, no obvious differences in distribution pattern were found in immunoreactivity of spinal cord, DRG, SCG, skin or hypothalamus, when rats and mice were compared.

3.3. ^{125}I-[Tyr^{14}]-OFQ/N binding

Binding of the radioactive ligand (female rat, lumbar level studied) was detected throughout spinal cord grey matter, with the greatest receptor density in the dorsal horn laminae I–VI, with the exception of lamina II which was almost devoid of receptor signal. Also, lamina X was intensely labeled (Fig. 7a). Further, large and medium sized DRG neurons showed signal for ^{125}I-[Tyr^{14}]-OFQ/N binding (Fig. 7c). No ligand binding was found in the control sections (Fig. 7b,d). Receptor binding was also evident in hypothalamus (not shown).

4. Discussion

In the present study, we have used several techniques to identify the sites of expression of OFQ/N and ORL1 in spinal cord, DRG, SCG and skin from rat and mouse. We performed the study in order to get an overall picture of the distribution of this putative pain modulating peptide and its receptor in spinal cord and peripheral ganglia such as DRG. Due to previous inconsistent data concerning the mRNA distribution of OFQ/N in DRG [2,13,18], we had to modify the hybridization protocol to enable detection of low levels of mRNA. We optimized the reaction conditions by using high concentrations of DTT which strongly suppresses unspecific labeling, and unfixed tissue which gives a higher yield of specific labeling as compared to fixed tissue (Ref. [4], and our own experiences). In addition, we elevated the hybridization temperature, added NEM to the probe, and washed more harshly to improve the signal to noise ratio.

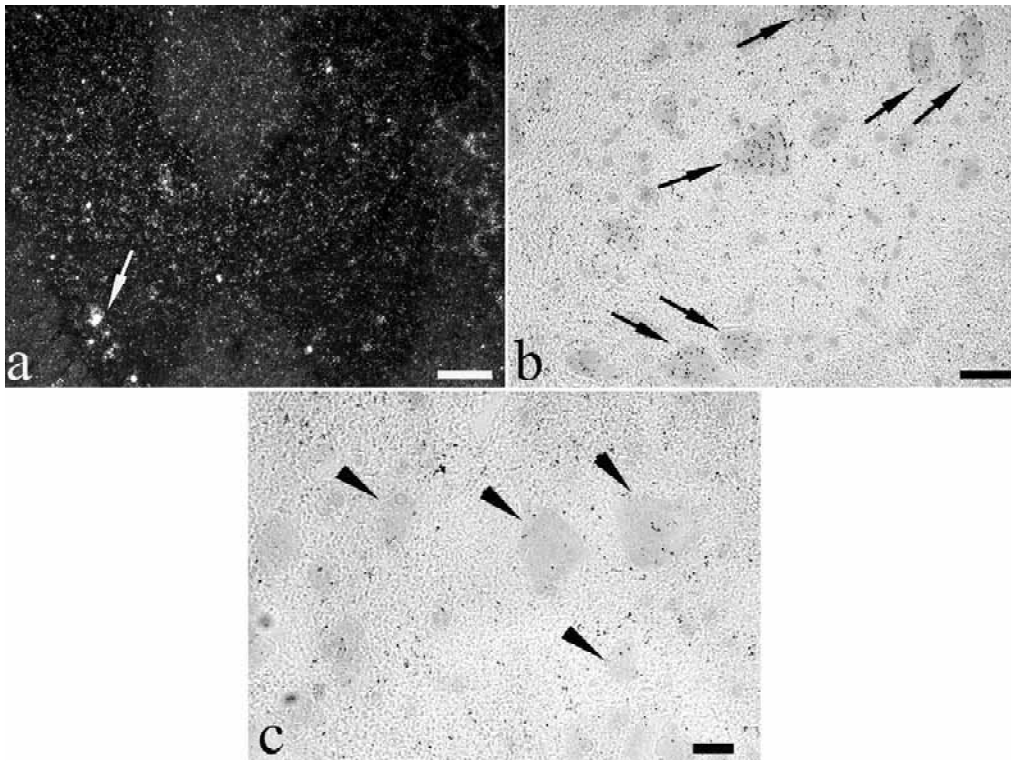

Fig. 2. In situ hybridization for ORL1 mRNA in rat spinal cord. Darkfield (a) and brightfield images of ORL1 mRNA labeling (b) and control slide hybridized with unlabeled probe in excess (c). The labeling appears scattered throughout the grey matter (a) with the greatest density in ventral horn neurons (b). No labeling of the neurons was detected in the control slides (c). Dorsal horns upward in figure. Arrows (a,b) exemplify labeled ventral horn neurons, presumably motorneurons. Arrowheads (c) exemplify unlabeled ventral horn neurons. Scale bars: (a) 200 μm; (b) 50 μm; (c) 25 μm.

Previously, Andoh et al. [2], have used RT-PCR technique and found only slight or no expression of OFQ/N mRNA in normal rat DRG, and furthermore, Itoh et al. [13] found no labeling using in situ hybridization. However, both reports observed a marked and rapid induction in OFQ/N mRNA expression after Carageenan elicited paw inflammation. Neal et al. [18] found a few centrally located cells expressing OFQ/N mRNA. In other studies, ORL1 mRNA was detected in DRG neurons, whereas no ligand binding was seen [17,26]. We now report on the localization of both OFQ/N mRNA and ORL1 mRNA to specific neurons in both genders of intact rat and mouse DRG (cervical, thoracic and lumbar levels). OFQ/N mRNA was found in a minority of primarily small sized DRG neurons, whereas a more vast expression of ORL1 mRNA was confined to medium and large sized DRG neurons. The ligand binding studies demonstrated that the ORL1 receptor was present on the cell bodies of medium and large sized DRG neurons (lumbar level). This indicates that the ORL1 receptor may also function in DRG neurons, besides being exported to the nerve terminals. That ORL1 may be exported (see also below) is supported by the presence of ligand binding in lamina I, an area where some larger DRG neurons terminate. Also, our in situ hybridization data show that larger DRG neurons express ORL1 mRNA. The difference between our findings, and the findings of Neal et al. [17] reporting a non existing ligand binding to the DRG (cervical/thoracic levels), might be due to different DRG levels studied.

In spinal cord, OFQ/N immunoreactivity was seen in some fibers and neurons in the superficial layers of the dorsal horn and to a lower extent in deeper laminae and around the central canal. These findings are in accord with previous reports [22,25]. Also large and medium sized ventral horn neurons, presumably motorneurons and interneurons, respectively, displayed immunoreactivity for OFQ/N. These findings support the findings of Neal et al. [18]. We can further confirm findings of OFQ/N and ORL1 gene expression in spinal cord [9,17,18], where we detected distinct OFQ/N mRNA expression and scattered

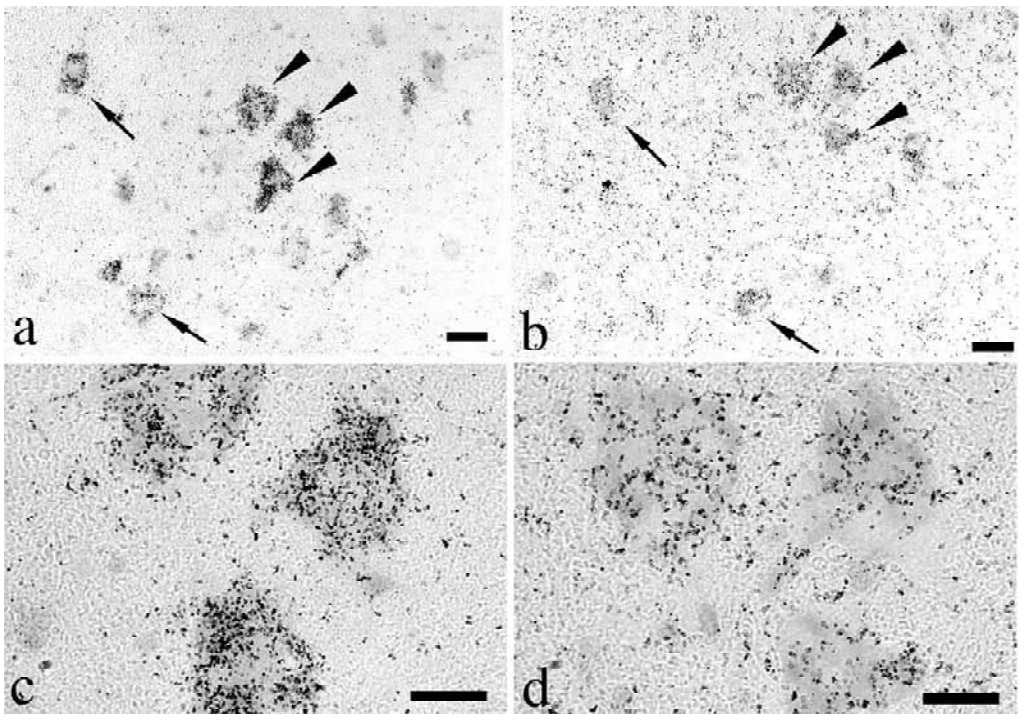

Fig. 3. Colocalisation of VAChT mRNA and ORL1 mRNA detected by in situ hybridization in rat spinal cord ventral horn. Many large ventral horn neurons that express VAChT mRNA (a,c) also express ORL1 mRNA (b,d) as seen in these adjacent sections. Arrows and arrowheads examplify neurons that are labeled for both VAChT and ORL1 mRNA. Arrowheads indicate labeled neurons present in all pictures. Scale bars: (a,b) 50 μm; (c,d) 25 μm.

ORL1 mRNA expression in both rat and mouse spinal cord, laminae I–II and lamina X, at all levels examined. It is not inconceivable that the loss of ORL1 in these areas may contribute to the increased nociceptive behaviour seen in the ORL1 deficient mice during the late inflammatory phase of the formalin test [1], where the direct inhibition of excitatory glutamatergic spinal cord dorsal horn neurons by OFQ/N is failing.

We also detected OFQ/N and ORL1 mRNA expression in ventral horn neurons in the spinal cord. Particularly ORL1 mRNA, is expressed in many of the large ventral horn neurons, most likely motorneurons, as indicated by the size, location and coexpression with VAChT in these cells. In addition, OFQ/N mRNA expression is also present, although in a somewhat lower amount of medium sized neurons (possibly interneurons) and in a few large sized neurons, in this area. The immunoreactivity for OFQ/N in motorneurons and adjacent medium sized neurons fits well with the in situ hybridization findings in the ventral horn (see above), and these findings strengthen the theory suggested by Ikeda et al. [10], that OFQ/N release from interneurons may modulate the neuronal and synaptic activities of adjacent neurons, in this case motorneurons.

Since we found OFQ/N and ORL1 mRNA expression in the DRGs, but failed to detect immunoreactivity for OFQ/N and saw ligand binding only to some of the DRG neurons, one might speculate that the protein products at least in part are exported elsewhere. Possible export locations are: (i) peripherally; which in the case of the receptor could constitute the binding site for OFQ/N and account for the nociceptive effects (probably via a SP release) seen after intraplantar administration of the ligand [11]. Exported ligand could constitute an endogenous source of OFQ/N peripherally. It is notable, however, that we could not detect any OFQ/N immunoreactivity in normal or inflamed skin. (ii) Centrally; to the central terminals of the primary afferents in the spinal cord. We did find immunoreactivity for OFQ/N, primarily in the superficial laminae of the dorsal horn, and ligand binding to the receptor throughout the grey matter mostly concentrated to the dorsal horn (laminae I and III–VI). However,

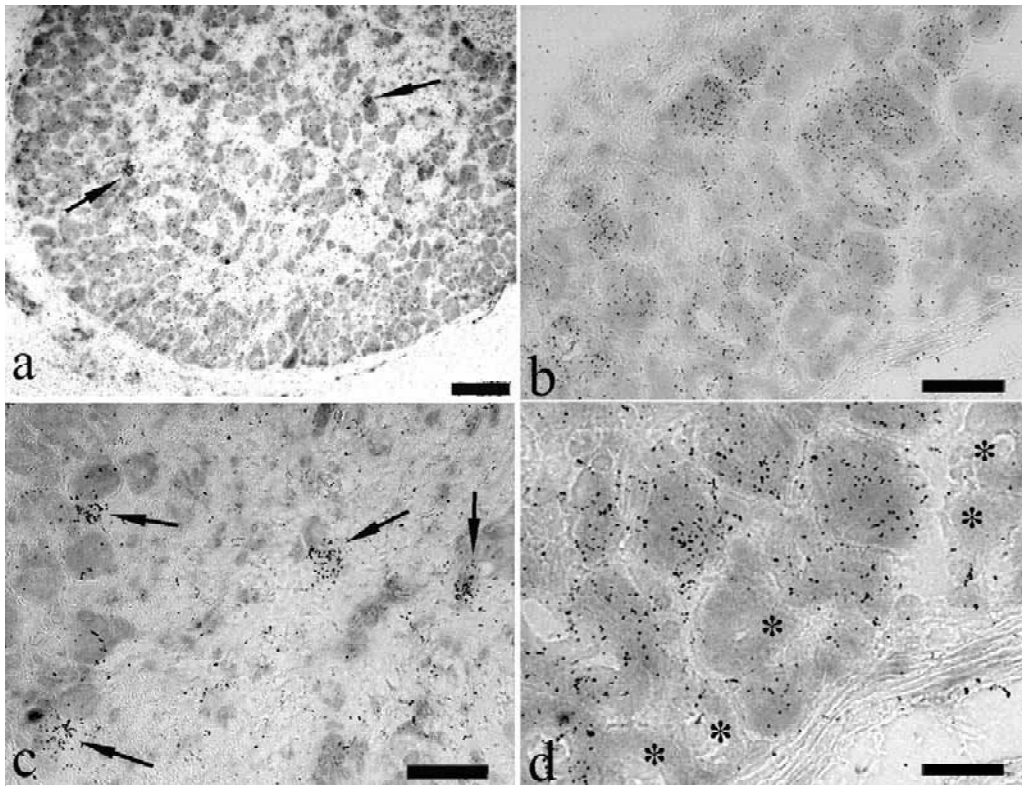

Fig. 4. In situ hybridization for OFQ/N and ORL1 mRNA in rat DRG. OFQ/N mRNA is found in a few small to medium sized neurons (a,c), whereas ORL1 expression is detected primarily in medium to large sized neurons (b,d). Arrows and asterisks examplify labeled and unlabeled neurons, respectively. Scale bars: (a) 100 μm; (b,c) 50 μm; (d) 25 μm.

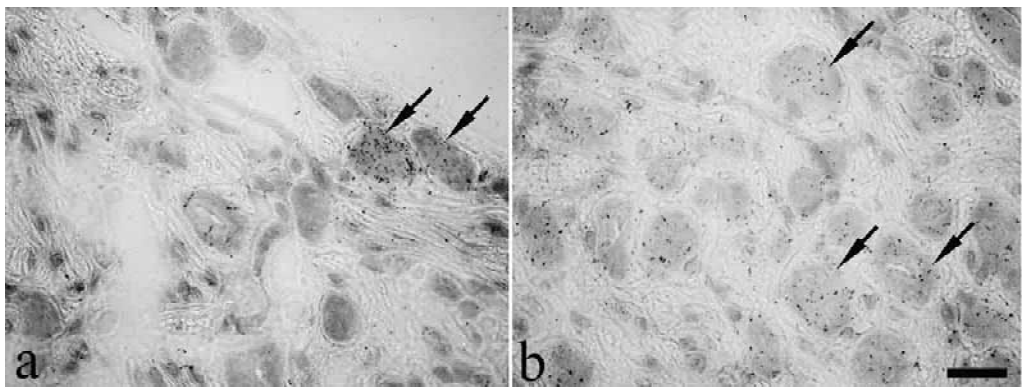

Fig. 5. In situ hybridization for OFQ/N and ORL1 mRNA in rat SCG. Distinct OFQ/N mRNA expression is visible in a few cell bodies (a), whereas ORL1 mRNA is expressed in a majority of the cell bodies (b). Arrows examplify labeled neurons. Scale bar: 25 μm.

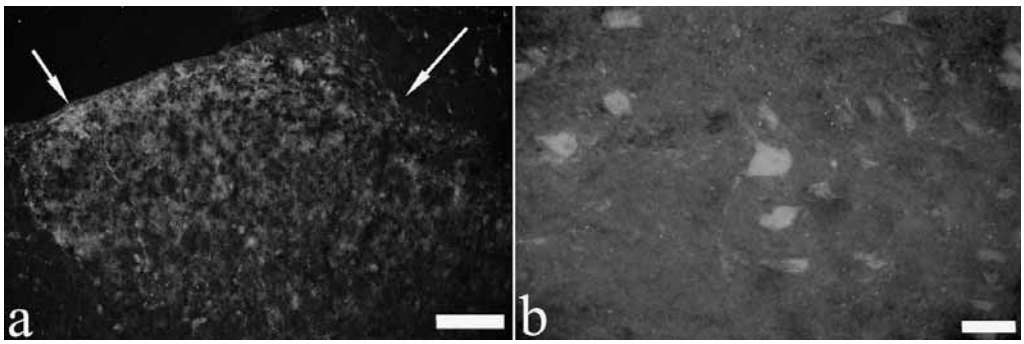

Fig. 6. OFQ/N immunoreactivity in rat spinal cord dorsal and ventral horns. OFQ/N immunoreactive fibers are seen in the superficial layers, and occasionally also in deeper layers of the dorsal horn (a). Ventral horn neurons, presumably motorneurons and interneurons, immunoreactive for OFQ/N (b). Arrows indicate the dorsal edge of lamina I. Scale bars: (a) 100 μm; (b) 50 μm.

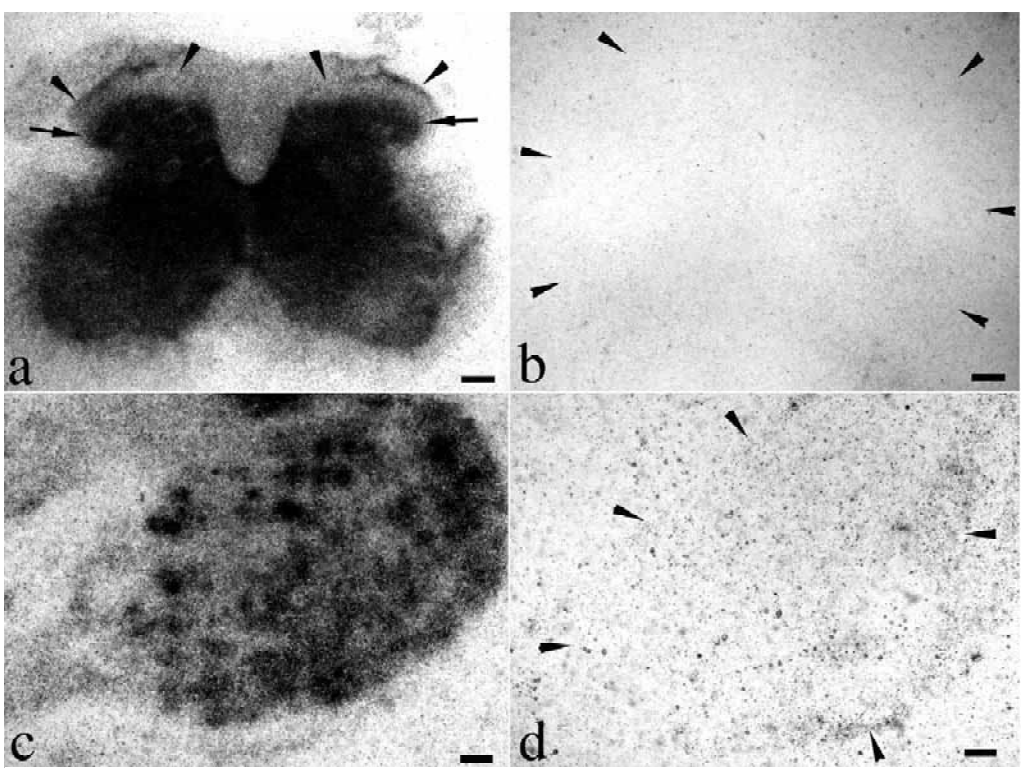

Fig. 7. Autoradiographic distribution of ^{125}I-[Tyr^{14}]-OFQ/N binding sites in rat spinal cord and DRG. Binding of radioactive ligand is scattered throughout the spinal cord grey matter with its greatest labeling density in lamina I, III–VI, and X (a). In the DRG, ligand binding is distributed primarily to medium and large size neurons (c). No ligand binding is found in the presence of high concentration of unlabeled OFQ/N (b,d). Dorsal horns upward in figure. Arrowheads delineate the outer edge of lamina I and arrows the outer edge of lamina III in (a). Arrowheads delineate the outlines of the spinal cord (b), and DRG (d), respectively. Scale bars: (a,b) 200 μm; (c,d) 100 μm.

the in situ hybridization data showed that ORL1 was also synthesized within the dorsal horn. The presence of OFQ/N and ORL1 in these areas might modify the release of other neurotransmitters. In a study by Inoue et al. [12], OFQ/N administration, centrally or peripherally, affected the SP signaling and release, both at a central and peripheral level. Their findings support the presence of ORL1 both at the central and peripheral terminals of primary afferent neurons. The loss of a functional OFQ/N system might interfere with neurotransmitter release and signaling, as indicated by an increased sensitivity in a nociceptive flexor test after peripheral or spinal SP administration in OFQ/N deficient mice [3].

In contrast to the situation in the DRGs, much of the protein product from cells expressing OFQ/N or ORL1 mRNA around the central canal and in the ventral horn seems to accumulate in the cell bodies, supported by the immunoreactivity for OFQ/N and by ligand binding in these areas. It cannot be excluded that the effects on locomotor behaviour, a decrease in locomotor activity [21] or an inhibitory effect on spontaneous locomotor behaviour in mice after i.c.v. OFQ/N injection [23], are mediated also via the ORL1 expressing ventral horn motor neurons, and not exclusively at cerebral levels.

Our finding of ORL1 mRNA expression in SCG fits very well with the suggestions by Giuliani et al. [7], of an OFQ/N receptor location prejunctionally on postganglionic sympathetic nerve fibers, and hence a role for the OFQ/N system in the modulation of noradrenaline release from nerve terminals innervating the resistance vessels. The apparent inconsistency with respect to the presence of OFQ/N mRNA in rat and mouse SCG may be due to an expression in mouse below the detection level for in situ hybridization. We found indications of a weak OFQ/N mRNA expression in mouse SCG in some experiments, but choose to interpret these findings with caution.

The presence of OFQ/N (lamina I and II) and ORL1 (lamina I and V) in spinal cord and in DRG (small and medium sized neurons), i.e., areas involved in nociceptive signalling, indicate theoretically, that regulation of pain by OFQ/N can occur at many different sites along the pathway. The variety of potential sites for pain modulation by the OFQ/N system hints to the many possibilities for the ligand to exercise its complex effects. The presence of OFQ/N and ORL1 expression in the ventral horn of the spinal cord and in parts of the sympathetic nervous system suggests further modulatory roles of OFQ/N and its receptor.

Acknowledgements

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Paper II

Markedly reduced chronic nociceptive response in mice lacking the PAC_1 receptor

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The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) has been proposed to have a role in nociception. Here we have used the formalin test, thermal laser stimulation and mechanical von Frey stimulation to investigate possible alteration of $PAC_1^{-/-}$ mice nociceptive behaviour. Our finding, that $PAC_1^{-/-}$ mice have a substantial, 75% decrease in nociceptive response during the late phase, provides clear evidence that the specific PACAP-receptor PAC_1 is involved in the mediation of nociceptive responses during chronic conditions such as inflammation. $PAC_1^{-/-}$ mice had small or no changes in the response to mechanical and thermal laser stimulation. This suggests a limited, if any, involvement of PAC_1 in nociception after short-lasting stimuli. Injury-induced changes in DRG neuropeptide expression were more pronounced in $PAC_1^{-/-}$ mice, implying neuroregulatory functions of PAC_1. *NeuroReport* 12:2215–2219 © 2001 Lippincott Williams & Wilkins.

Key words: Dorsal root ganglion; Formalin test; Inflammation; Mechanical von Frey; Neuropeptides; PACAP; Pain measurements; Sciatic nerve injury; Sensory thresholds; Thermal laser

INTRODUCTION

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) acts via three different receptors: the PAC_1 receptor is a PACAP-selective receptor; the $VPAC_1$ and $VPAC_2$ receptors bind PACAP and VIP with similar affinity. Both the PACAP receptors and PACAP itself are differently distributed throughout the peripheral and central nervous system, suggesting distinct functional roles in different systems (for review see [1]).

In the dorsal root ganglion (DRG) PACAP is expressed in small- to medium-sized neurons projecting to superficial layers of the dorsal horn of the spinal cord and known to be involved in nociception. After intrathecal application of PACAP, both potentiation [2,3] and inhibition [4–6] of nociceptive responses have been observed. Furthermore, ionophoretically administered PACAP leads to excitation of spinal dorsal horn neurons [7]. Hence, the role of PACAP in nociception is far from resolved. Furthermore, PACAP expression in rat DRG neurons is upregulated after adjuvant-induced inflammation [8] as well as after nerve transection [9,10].

Despite this detailed knowledge about PACAP and its receptors, the physiological role of PACAP is still unclear. Here we report on phenotypic alterations of $PAC_1^{-/-}$ mice [11] concerning formalin-induced pain behaviour, thermal and mechanical stimuli, PACAP binding and distribution of sensory neuropeptides after nerve injury.

MATERIALS AND METHODS

Animals: The study was approved by the Animal Ethics Committee at Lund University. Adult male and female $PAC_1^{-/-}$ mice [11] and age- and sex-matched wild type mice were used.

Formalin test: Male wild type ($n=8$) and $PAC_1^{-/-}$ ($n=10$) mice were used to study biphasic nociceptive behavioural responses by injecting 10 µl of 2% paraformaldehyde in phosphate buffer (pH 7.2) s.c. under the dorsal surface of the left hind paw [12]. Immediately after the injection the mice were placed in a glass box surrounded by mirrors to improve visibility. The observer was unaware of which strain the mice belonged to. The total time spent licking the injected paw in 2 min intervals every 5 min during the early phase (0–5 min) and the late phase (5–60 min) was determined using a stop watch and defined as nociceptive response as described [12]. The mice were killed immediately after the 60 min observation period.

Thermal and mechanical stimuli: Adult male wild type ($n=14$) and $PAC_1^{-/-}$ ($n=9$) mice were used. The mice

were handled and trained in a small hand-held tube (the restrainer) by the experimenter daily for 5 days prior to the tests. On the day of the experiment, animals were accustomed to the experimental environment for about 2 h before the behavioural tests. The mice were placed in the restrainer, allowing the hind limb or tail to be stimulated. To determine the thermal reflex threshold for a skin area, a CO_2 laser was used [13]. The threshold was defined as the stimulation duration that, in three or more of five tests (unfocused beam, diameter 2 mm, intensity 3 W, pulse duration 6–45 ms) could elicit a withdrawal response. The duration of the laser pulse was increased in steps of 2 ms until the reflex threshold was reached. The interval between each pulse was >10 ms. To avoid overt tissue damage, the maximal pulse duration used was 45 ms. Laser stimulation did not elicit vocalisation or avoidance behaviour other than withdrawal reflexes in the stimulated limb or tail. The lateral side of the midportion of the tail or the midplantar surface of the right hind paw was stimulated. To enable an estimate of nociceptive reflex latency the animals were stimulated with a pulse duration of 10 ms above each individual threshold value and the time (ms) between the stimuli onset (laser pulse) and the elicited withdrawal response was recorded. The withdrawal responses were documented with a digital video camera (50 frames per sec) and the nociceptive reflex latencies were calculated by analysing the video recordings frame by frame. A small LED indicated the onset of the laser pulse.

The mechanical reflex thresholds of tail (the side of the midportion) and hind paw (plantar skin proximal to the digits) were assessed with calibrated nylon Semmes-Weinstein monofilaments, commonly referred to as von Frey hairs (North Coast Medical, Inc., San Jose, CA, USA; tested force levels were 0.069, 0.10, 0.34, 0.74, 2.0, 2.8, 5.3, 8.5, 14, 23, 47, 61, 68 and 89 mN, calibrated on a fine balance). The reflex threshold was defined as the lowest force required to elicit a visible response in three or more of five tests.

Nerve injury: Adult female wild type ($n=10$) and $PAC_1{}^{-/-}$ ($n=10$) mice underwent unilateral sciatic nerve crush. The right sciatic nerve was exposed under pentobarbital (50 mg/kg)/diazepam (6 mg/kg)/physiological saline i.p. anaesthesia, and crushed in the upper hip region by applying a specially designed pair of pliers for 2×15 s. The wound was closed by sutures and the mice were allowed to recover in a heated cage to maintain their body temperature. Four or 10 days after surgery mice were killed and right and left L5 DRG were removed by dissection and processed for *in situ* hybridisation.

Ligand binding: Adult male wild type and $PAC_1{}^{-/-}$ mice were killed and lumbar spinal cords were dissected. The protocol used for ligand binding has been described in detail [9]. In brief, fresh-frozen 20 µm sections were preincubated in a buffer containing Tris–HCl, BSA, $MgCl_2$ and bacitracin followed by a 1 h incubation in the same buffer including either 150 pM [^{125}I]PACAP-27 (NEN; specific activity; 2200 Ci/mmol) or 150 pM [^{125}I]PACAP-27 and 1.5 µM VIP (Sigma, St.Louis, MO). The non-specific binding was determined in the presence of 1.5 µM PACAP-27 (Sigma). Finally, the slides were washed, air-dried and exposed to Hyperfilm MP (Amersham, Solna, Sweden) for 5 days.

In situ *hybridisation:* OligoDNA probes complementary to and selective for the following mRNAs were synthesised and purified (Biomedical Resource Facility, Lund University, Sweden): galanin, nucleotide sequence 131–160 [14]; PACAP, sequence 467–496 [15]; CGRP, sequence 245–274 [16] and NPY, sequence 186–221 [17]. All probes were checked against the Genbank database (NIH, March 2001) to ensure that $\leqslant 60\%$ homology was found to sequences other than the cognate transcript. Labelling of the probes and the protocol for *in situ* hybridisation has been described in detail [9]. In brief, the probes were labelled with [^{35}S]dATP and the sp. act., as measured by liquid scintillation counting, was $\sim1\times10^9$ c.p.m./µg. Dissected DRGs were fixed in Stefanini's fixative (2% paraformaldehyde, 15% picric acid in PBS), rinsed and immersed in 20% sucrose in PBS, frozen and cut at 10 µm. Prior to hybridisation, the sections were fixed in 4% paraformaldehyde and treated with 0.25% acetic anhydride in 0.1 M triethanolamine, 50 mM N-ethylmaleimide and dehydrated. Hybridisation was performed at 42°C for 18 h followed by stringent post-hybridisation washes and dehydration. The slides were covered with film emulsion and exposed for 1–3 weeks at 4°C. Control sections were treated as above, with a 100-fold molar excess of unlabelled probe added to the hybridisation solution.

RESULTS

$PAC_1{}^{-/-}$ mice have markedly reduced late phase response in the formalin test: The nociceptive behaviour response measured as time spent licking the injected paw during the early phase (0–5 min) was significantly longer (doubled) for $PAC_1{}^{-/-}$ mice than for wild type mice (Fig. 1). In contrast, during the late phase (5–60 min) $PAC_1{}^{-/-}$ mice had a significantly shorter (25%) licking time than wild type mice (Fig. 1). No difference in erythema and oedema in response to the formalin injection was detected between the two groups.

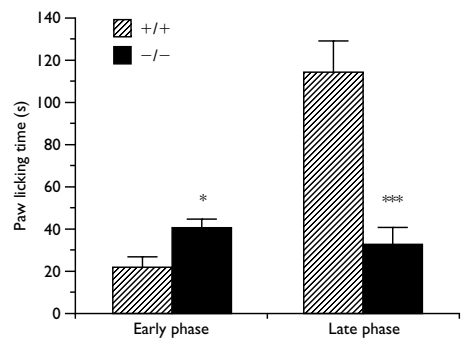

Fig. 1. Differences in time (mean ± s.e.m.) spent licking the formalin-injected hind paw between wild type (striped bars; $n=8$) and $PAC_1{}^{-/-}$ (black bars $n=10$) mice. During the late phase $PAC_1{}^{-/-}$ mice showed a dramatic and significant decrease of nociceptive behaviour response (32 ± 8 s) as compared to wild type mice (115 ± 14 s; $p<0.0001$; unpaired *t*-test). In the early phase (0–5 min), $PAC_1{}^{-/-}$ mice had a small but significantly increased licking time (46 ± 7 s) as compared to wild type mice (22 ± 5 s; $p=0.0151$; unpaired *t*-test).

PAC_1 receptor involvement in the response to thermal and mechanical stimuli $PAC_1^{-/-}$ mice did not have a significantly altered thermal reflex threshold or reflex latency, as compared to wild type mice, when the hind paw or tail was stimulated. Typically for the $PAC_1^{-/-}$ mice the thermal reflex thresholds on the hind paw varied between 14 and 18 ms and the latency varied between 80 and 320 ms. The corresponding figures for wild type mice were 10–18 ms and 80–320 ms, respectively. Similar figures were obtained from both groups for the tail.

$PAC_1^{-/-}$ mice had a small but significantly reduced tactile reflex threshold (median 0.34 mN; range 0.10–0.74 mN) compared with wild type mice (median 0.74 mN; range 0.10–8.5 mN) when the hind paw was stimulated with von Frey filaments ($p < 0.05$; Mann Whitney U-test). No difference in tactile reflex thresholds was found for the tail (median 2.0 mN; range 2.0–89 mN and median 4.0 mN; range 0.10–89 mN, in $PAC_1^{-/-}$ and wild type respectively).

Injury-triggered changes in neuropeptide expression are more extensive in $PAC_1^{-/-}$ mice: After sciatic nerve injury the expression of several neuropeptides changes: galanin, NPY and PACAP mRNA expression is increased whereas CGRP and substance P expression is decreased [18,19]. Qualitative examination of mRNA expression for these neuropeptides indicated that injury-triggered changes were more pronounced in $PAC_1^{-/-}$ mice. This was particularly evident for galanin, which in $PAC_1^{-/-}$ mice had a much higher injury-induced expression level when compared to wild type mice (Fig. 2).

[^{125}I]PACAP-27 binding is blunted in $PAC_1^{-/-}$ mice: In spinal cord from wild type mice, [^{125}I]PACAP-27 binding was seen with the highest density in the dorsal horn with a lower density throughout the rest of the grey matter (Fig. 3). The presence of a high concentration of PACAP-27 (Fig. 3) resulted in no [^{125}I]PACAP-27 binding, whereas the presence of high concentration of VIP did not visibly change the [^{125}I]PACAP-27 binding (not shown). In spinal cord sections from $PAC_1^{-/-}$ mice meagre [^{125}I]PACAP-27 binding was detected (Fig. 3).

DISCUSSION

PACAP is normally expressed mostly in small DRG neurons and the PAC_1 receptor is present in the dorsal horn [9]. To study the possible involvement of the PAC_1 receptor in nociception we used the formalin test, which is a model of nociception that in mice results in a biphasic nociceptive behavioural response. The first phase starts immediately after injection of formalin, lasts for 3–5 min, and is probably due to direct chemical stimulation of chemosensitive nociceptive fibers. The second phase is exhibited between 15 and 60 min after formalin injection and seems to result from a combination of peripheral inflammatory processes and sensitisation in the spinal cord induced by the first phase [12]. Our findings that $PAC_1^{-/-}$ mice have a profoundly decreased nociceptive response during the late phase of the formalin test indicate that the specific PACAP receptor PAC_1 is involved in chronic inflammatory nociception. However, PAC_1 does not seem to be necessary in the production of erythema and oedema

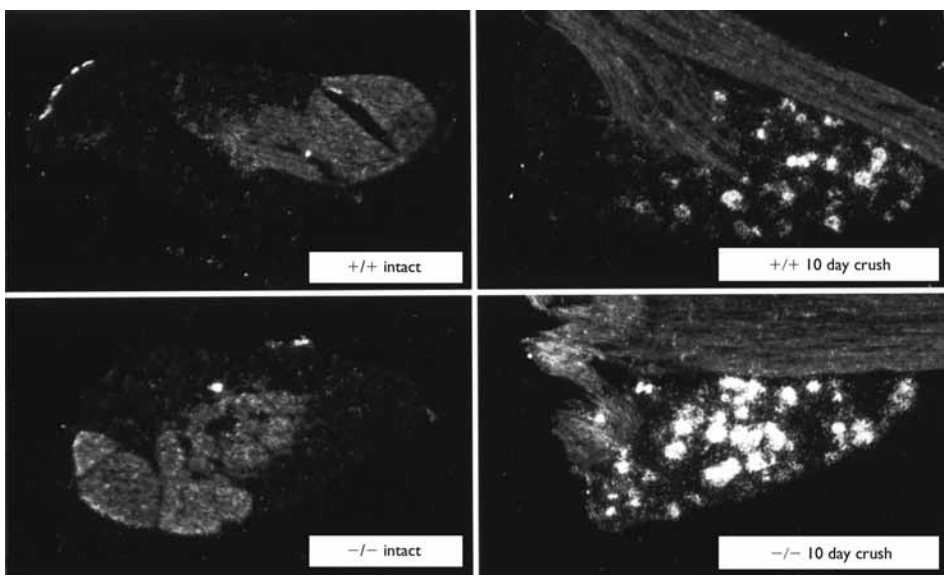

Fig. 2.. Dark-field photomicrographs (low magnification) of L5 DRG sections from wild type and $PAC_1^{-/-}$ mice following 10 days sciatic nerve crush, processed for *in situ* hybridisation to detect galanin mRNA. In intact DRG no galanin-expressing neurons are seen in either wild type or $PAC_1^{-/-}$ mice. After sciatic nerve crush, galanin expression in wild type mice is induced, but in $PAC_1^{-/-}$ mice the level of expression is much greater.

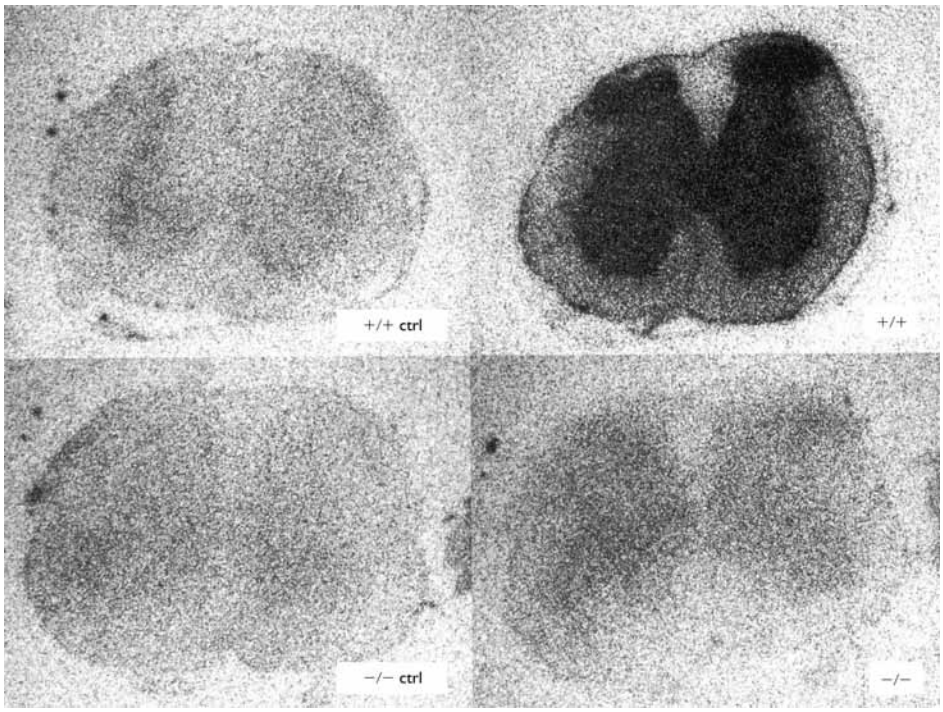

Fig. 3. Autoradiographic distribution of [^{125}I]PACAP-27 binding sites in lumbar spinal cord in wild type and $PAC_1^{-/-}$ mice. In wild type mice the highest density is seen in the dorsal horn and moderate binding is seen throughout the whole grey matter. In $PAC_1^{-/-}$ mice meagre [^{125}I]PACAP binding is seen. In the presence of high concentrations of unlabelled PACAP (ctrl) no labelled binding could be detected.

since there was no visible difference between the $PAC_1^{-/-}$ and wild type mice. That PACAP is acting via the PAC_1 receptor in the spinal cord and not via the other two receptors, $VPAC_1$ and $VPAC_2$, is supported by the finding that only scarce PACAP binding could be detected in the spinal cord from $PAC_1^{-/-}$ mice. It is likely that PACAP plays a role in the mediation of nociceptive responses during more chronic conditions such as inflammation a contention further supported by the finding that PACAP expression is upregulated after induced inflammation [8]. However, it cannot be completely ruled out that the altered behaviour in the formalin test observed for $PAC_1^{-/-}$ mice is due to a changed neuronal organisation of the DRG and spinal cord since PACAP is known to have neurotrophic effects (for review see [18]). The present data also suggest that the PAC_1 receptor may be involved in acute chemo-nociceptive behaviour as indicated by the increased licking behaviour by $PAC_1^{-/-}$ mice during the early phase of the formalin test and mechano-nociceptive behaviour indicated by the reduced threshold for the withdrawal reflex. However, $PAC_1^{-/-}$ mice and wild type mice did not differ in reflex responses to thermal stimulation. A preliminary report has also demonstrated that $PAC_1^{-/-}$ mice did not have changed nociceptive threshold in a hot plate test [20]. Taken together, these data suggest that the role of the PAC_1 receptor in acute nociceptive behaviour should be interpreted with caution, since the difference in mechanical reflex thresholds between $PAC_1^{-/-}$ mice and wild type mice was found only on the hind paw and not tail and that there were no differences in reflex responses after thermal stimulation. Furthermore, the difference observed during the early phase of the formalin test was not of the same magnitude as that found in the late phase.

Involvement of protein kinase C has been implicated in changes in pain perception. Protein kinase C is thought to be activated via the G-protein coupled tachykinin receptor NK1, which induces the production of diacylglycerol and inositol–triphosphate that in turn activates the protein kinase. Activated protein kinase C phosphorylates NMDA receptors leading to wind-up and central sensitisation (for review see [21]). It has been shown that intrathecal administration of a selective protein kinase C inhibitor attenuated the formalin-induced paw flinching, preferentially in the late phase [22]. Since the PAC_1 receptor stimulates protein kinase C (for review see [1]) it is conceivable that it is the interruption in the protein kinase C pathway that leads to the decreased nociceptive response in the late phase of the formalin test seen in $PAC1^{-/-}$ mice. Also in NK1 receptor

knock-out mice, the late phase of the formalin test was attenuated by 45% [23]. It is possible that the role of the PAC_1 receptor is very similar to that described for NK1 receptor in inflammatory pain perception. The formalin-induced nociceptive response in $PAC_1^{-/-}$ mice shows striking similarities to that described in a study of diabetic mice [24]. The diabetic mice also had an increased nociceptive response in the first phase and an almost blunted response in the second phase. Surprisingly, the changes in the second phase were reversed by a protein kinase C inhibitor, calphostin C [24].

A number of neuropeptides, e.g. galanin and NPY, are up-regulated in DRG after nerve injury whereas others, e.g. CGRP and substance P, are down-regulated [19]. Previous studies have indicated that the changed expression of neuropeptides after nerve injury depends on the altered supply of neurotrophins from the peripheral target and/or non-neuronal cells within the nerve [19]. The findings in this study, that $PAC_1^{-/-}$ mice had greater injury-induced changes, especially of galanin but also of PACAP, NPY, CGRP and substance P, suggests that the PAC_1 receptor may be involved also in the expression of other neuropeptides after nerve injury. Since we have observed in uninjured $PAC_1^{-/-}$ mice (unpublished preliminary findings) that the immunoreactivity for galanin, PACAP, CGRP, NPY and substance P is increased in dorsal horn fibers, one might speculate that the PAC_1 receptor is involved in the expression of neuropeptides also in intact mice. PAC_1 receptor mRNA or PACAP binding is not found in postnatal rat DRG neurons [9] but the presence in non-neuronal cells, such as satellite cells within the nerve and DRG, is yet to be determined. If such cells do express the PAC_1 receptor it is possible that PAC_1 is involved in the release of cytokines regulating the DRG neuronal phenotypes. In cerebral glial cells, such as astrocytes, PACAP alters the production of neurotrophic factors responsible for neuronal proliferation and/or differentiation (for review see [1]) and several studies indicate that PACAP modulates the production of cytokines in immune cells and the PAC_1 receptor has been demonstrated in macrophages [25].

CONCLUSION

The formalin test has been proposed to have greater relevance for clinical situations than tests that employ measurements of nociceptive reflexes or thresholds. The most striking observation of $PAC_1^{-/-}$ mice was the markedly reduced nociceptive response in the late inflammatory phase of the formalin test, suggesting that PACAP plays a role in mediating/modulating nociceptive responses after inflammation.

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Paper III

PACAP mRNA is Expressed in Rat Spinal Cord Neurons

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ABSTRACT

This study examines the expression of pituitary adenylate cyclase activating polypeptide (PACAP) mRNA in the rat spinal cord during normal conditions and in response to sciatic nerve transection. Previously, PACAP immunoreactivity has been found in fibers in the spinal cord dorsal horn and around the central canal and in neurons in the intermediolateral column (IML). Furthermore, in the dorsal root ganglia, PACAP immunoreactivity and PACAP mRNA expression have been observed preferentially in nerve cell bodies of smaller diameter terminating in the superficial laminae of the dorsal horn. However, neuronal expression of PACAP mRNA in adult rat spinal cord appeared limited to neurons of the IML. By using a refined in situ hybridization protocol, we now detect PACAP mRNA expression in neurons primarily in laminae I and II, but also in deeper laminae of the spinal cord dorsal horn and around the central canal. In addition, PACAP mRNA expression is observed in a few neurons in the ventral horn. PACAP expression in the ventral horn is increased in a population of large neurons, most likely motor neurons, both after distal and proximal sciatic nerve transection. The proposed role of PACAP in nociception is strengthened by our findings of PACAP mRNA-expressing neurons in the superficial laminae of the dorsal horn. Furthermore, increased expression of PACAP in ventral horn neurons, in response to nerve transection, suggests a role for PACAP in repair/regeneration of motor neurons. J. Comp. Neurol. 468:000–000, 2004.

Indexing terms: anterior horn cells; axotomy; neuropeptides; posterior horn cells

The 38- or 27-amino acid neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalamus and is named after its ability to stimulate cAMP formation in anterior pituitary cells (Miyata et al., 1989). Since then, several further functions of PACAP have been established, and the peptide can act as a hormone, neurohormone, neurotransmitter, or trophic factor. The wide variety of actions include effects on circadian rhythm, appetite and feeding behaviour, motor activity, pain behaviour, cell survival, and differentiation. PACAP has been found to be distributed in nerve cell bodies and fibers in the central and the peripheral nervous systems, as well as in endocrine cells and tissues (for an extensive review, see Vaudry et al., 2000). In the peripheral sensory nervous system, immunoreactivity and mRNA signal for PACAP have been found preferentially in nerve cell bodies of smaller diameter in the dorsal root ganglia (DRG; Moller et al., 1993; Zhang et al., 1995, 1996b; Dun et al., 1996). Under normal conditions, roughly a fifth of the DRG neurons express PACAP mRNA or show immunoreactivity for PACAP (Zhang et al., 1996b; Jongsma et al., 2000). In the spinal cord, PACAP immunoreactivity has been found in nerve fibers in the

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superficial laminae of the dorsal horn, dorsolaterally to the central canal (Moller et al., 1993; Zhang et al., 1995, 1996b; Dun et al., 1996; Jongsma et al., 2000; Vaudry et al., 2000), and also in fibers and neurons in the intermediolateral column (IML; Dun et al., 1996; Hannibal, 2002). Until now, immunoreactivity or hybridization signal for PACAP in spinal cord neurons have not been detected, except for in neurons in the IML (Hannibal, 2002).

The discovery that PACAP is expressed in small DRG neurons (Moller et al., 1993; Mulder et al., 1994; Zhang et al., 1996b; Jongsma et al., 2000) led to the belief that PACAP might modulate pain pathways, and several studies investigating this possibility have been performed in rats and mice. The application of PACAP intrathecally has been shown to either inhibit (Zhang et al., 1993b, 1996a; Yamamoto and Tatsuno, 1995) or potentiate (Narita et al., 1996; Xu and Wiesenfeld-Hallin, 1996) nociceptive responses, whereas the administration of PACAP ionophoretically led to an excitation of spinal cord dorsal horn neurons (Dickinson et al., 1997). The PACAP preferring receptor PAC_1 is expressed in dorsal horn neurons but not in DRG neurons (Jongsma et al., 2000), supporting the view that PACAP produced by DRG neurons has its targets in the spinal cord. Studies of mice deficient for PAC_1 (Jongsma et al., 2001; Martin et al., 2003) have assessed that these mice have a decreased nociceptive response to inflammatory/chemical pain, indicating a nociceptive role for PACAP during these conditions. Furthermore, PACAP mRNA expression is up-regulated in small–medium DRG neurons after inflammation (Zhang et al., 1998; Jongsma Wallin et al., 2003) and in medium-large size neurons after sciatic nerve injury (Zhang et al., 1995, 1996b; Jongsma Wallin et al., 2001).

Although extensively studied, PACAP mRNA expression in neurons of the adult rat spinal cord was thought to be limited to neurons in the IML (Hannibal, 2002). However, recent refinement of our technologic approaches has revealed that the initial assessment of PACAP expression in spinal cord neurons may have been underestimated. Thus, the aim of this study was to re-investigate PACAP expression in the spinal cord under homeostatic conditions, and further evaluate the effects after peripheral nerve injury. We now report that spinal cord neurons in dorsal and ventral horn areas and neurons around the central canal, do express PACAP mRNA and also that the expression pattern is altered in the ventral horn neurons in response to nerve injury.

MATERIALS AND METHODS

Experimental structure

The study was performed in two laboratories: the first part was undertaken in Lund, Sweden, and the second part in Saskatoon, Canada. This was done to study the PACAP mRNA expression after two types of nerve injuries. Also, the findings from the different studies, in which slightly different material and methods were used, support and confirm each other. The study was approved by the Animal Ethics Committee in Malmö/Lund, respectively, the NIH policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol 19920164).

Distal nerve injury (study performed in Lund)

Spinal cords (lumbar segments) were dissected from normal, uninjured male and female, and injured (distal sciatic nerve transection) female Sprague-Dawley rats (200–400 g). The animals were anaesthetized with pentobarbital (25 mg/kg)/diazepam (4 mg/kg)/physiological saline i.p., and the sciatic nerve was transected on one side, at mid-thigh level (distal injury), after which the wound was closed with sutures and the animals were left to recover in heated cages. At 3, 7, or 14 days after the nerve transection, the animals were killed by heart puncture after the administration of an overdose of pentobarbital/diazepam/physiological saline i.p. Tissue specimens (spinal cords, lumbar segments) from the intact and injured rats were mounted in O.C.T. Compound (Tissue-Tek, Histolab products AB, Göteborg, Sweden), fresh-frozen in isopentane immediately after dissection, and stored at −80°C for in situ hybridization. For immunohistochemistry, the tissues were fixed in Stefanini's fixative (2% paraformaldehyde [PF] and 15% of a saturated aqueous picric acid solution, in 0.1 M phosphate buffer, pH 7.2) overnight, followed by repeated rinsing in sucrose-enriched (10%) Tyrode's solution. Tissue specimens were sectioned (6 μm; adjacent sections for studies of colocalization of different mRNAs, or 10 μm for additional in situ hybridization and immunohistochemistry) in a cryostat and thaw-mounted on to Super Frost Plus slides (Menzel-Gläser, Germany). Spinal cord tissue sections were examined and dorsal horn laminae I and II were defined in the microscope by using darkfield imaging to separate these laminae from deeper laminae. In darkfield illumination, lamina II appears as a dark band, enabling separation between lamina II and deeper laminae (Pettersson et al., 2002).

In situ hybridization. Normal, uninjured male (n = 4) and female (n = 2), and injured female (n = 8), Sprague-Dawley rats were used. Radiolabeled (^{35}S) oligodeoxyribonucleotide (oligoDNA) probes for detection of PACAP and the vesicular acetylcholine transporter (VAChT) mRNAs were used for in situ hybridization. OligoDNA probes complementary to and selective for PACAP (1) mRNA (nucleotides 700–747; Hurley et al., 1995), PACAP (2) mRNA (nucleotides 1038–1067; Ogi et al., 1990), and VAChT mRNA (nucleotides 683–715; Roghani et al., 1994) were synthesized and purified (University of Calgary DNA services, Alberta, Canada, PACAP [1] or Biomedical Resource Facility, Lund University, Sweden, for PACAP [2] and VAChT). VAChT is expressed in all cholinergic neurons (Roghani et al., 1994), and colocalization of PACAP mRNA with VAChT mRNA was studied to check for PACAP expression in motor neurons. All cDNA regions used were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm.nih.gov, April 2003); no greater than 60% homology was found to sequences other than the cognate transcript, except for the probe complementary to VAChT mRNA, which showed higher sequence similarities to the vesicular monoamine transporters (VMATs). The homology between the cDNA sequences for VAChT and the VMATs is rather high (Masson et al., 1999), complicating the procedure of designing a probe for VAChT that does not run the risk of cross-reacting with the VMATs. Because there are no indications of VMAT immunoreactivity or expression of VMAT mRNA in the spinal

cord ventral horn neurons (Peter et al., 1995; Weihe and Eiden, 2000), the problem with a possible risk for cross-reactivity was overridden.

To prevent degradation of RNA by external RNAses, all steps before hybridization were performed under RNase free conditions, and all dilutions were performed in autoclaved double-distilled water. Probes (1.3 ng/μl) were incubated at 42°C for 1 hour with 4.9% terminal transferase enzyme (Perkin Elmer, Zaventem, Belgium) to ligate the [^{35}S]dATP (15%, 37 MBq, Perkin Elmer) to the 3′ end, in a terminal transferase buffer (cacaodylic acid sodium salt 98 mM, manganese chloride tetrahydrate 0.99 mM, magnesium chloride hexahydrate 0.98 mM and dithiothreitol [DTT] 89 μM). The temperature was lowered, and the following substances were added to a final concentration of 0.01 M ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA), 0.10 μg/μl yeast tRNA (Sigma, St. Louis, MO), and 5.6 mM Tris based buffer containing 0.44 mM EDTA (pH 8.0), to stall the reaction. Purification of the labeled probes was performed by centrifugation in Chroma spin-10 columns (Clontech, Intermedica, Stockholm, Sweden). Thereafter the activity, approximately $0.42–1.2 \times 10^9$ cpm/ml, was measured in a liquid scintillator, and probes were stored at −18°C.

Air-dried sections were hybridized with radiolabeled probes at a final activity of 10^7 cpm/ml. The hybridization solution contained the probe in a solution of 50% formamide, 4× saline sodium citrate (SSC), 1× Denhardt's solution (0.02% bovine serum albumin [BSA], 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 10% dextran sulphate, 0.24 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, 1% sarcosyl and 0.2 M DTT. Hybridization, with 50–100 μl of hybridization solution/slide, was conducted overnight at 42°C under parafilm in air-sealed moist chambers to prevent evaporation. Control slides were incubated with the addition of the corresponding unlabeled probe in 100-fold molar excess. After hybridization, the slides were bathed in 1× SSC (10 minutes, room temperature), the Parafilm was removed, and the slides were then washed in 1× SSC (4 × 15 minutes, 55°C, and an additional 30 minutes, room temperature). All sections were dehydrated in ethanol, 70% and 95% (3 minutes, respectively) and 99% ethanol (5 minutes), air-dried, and immersed in Kodak NTB2 photoemulsion (diluted 1:1 in distilled water). After 4.5 weeks storage with desiccant in light-sealed containers at 4°C, the slides were developed in Kodak D19 (3–5 minutes), rinsed in tap water, fixed in Kodak Polymax fix (10 minutes), and rinsed (10 minutes). Counterstaining with 0.5% toluidine blue (in 60% ethanol) was performed, and excess dye was washed away with tap water. Slides were mounted in Pertex (Histolab, Göteborg, Sweden).

Proximal nerve injury (study performed in Saskatoon)

To further investigate the distribution of PACAP mRNA in the rat spinal cord, additional experiments were performed in collaboration with the Canadian laboratory where the first author had access to a method for infliction of a more proximal and, hence, more complete nerve injury. In this part of the study, male Wistar rats (150–200 g) exposed to unilateral, proximal nerve transection were used. The sciatic nerve was transected at its origin from the L4 and L5 spinal nerves, and a 5-mm segment was resected to prevent regeneration. All animals were given buprenorphine (0.05 mg/kg) to relieve any pre- and postoperative discomfort. Treatment of animals and tissues was principally performed as in Lund, but some changes exist and major differences in the tissue fixation and in situ hybridization procedures are listed in the following.

Three days, or 3 weeks after surgery, animals were deeply anesthetized and perfused with cold phosphate buffered saline (PBS, 0.1 M), to clear away the blood, and 4% PF for tissue fixation. Dissected tissues (lumbar spinal cord) were post-fixed in 4% PF (1.5 hours), repeatedly rinsed in 20% sucrose, and cryoprotected in 20% sucrose (overnight). Tissues were placed in cryomolds and stored at −80°C.

In situ hybridization. Male Wistar rats with proximal nerve injury, 3 days (n = 4) or 3 weeks (n = 9), were examined. Probes against PACAP (1) and growth associated protein 43 (GAP-43) mRNA (nucleotides 70–117; Karns et al., 1987) were used. Probes were synthesized and purified at the University of Calgary DNA Services, Alberta, Canada. GAP-43 is a nervous tissue-specific protein that is highly expressed during development and regeneration (Karns et al., 1987) and was used as an indicator of injured neurons.

The perfused tissues were sectioned and slides were air-dried and post-fixed in 4% PF (20 minutes), washed in PBS (3 × 5 minutes), treated with proteinase K at 37°C (20 μg/ml; 7–8 minutes), rinsed in PBS (5 minutes), fixed in 4% PF (5 minutes), rinsed in PBS (2 × 5 minutes), rinsed in diethyl pyrocarbonate (0.1%) −H_2O (5 minutes), and dehydrated in increasing ethanol concentrations (70%, 90%, 100%; approximately 1 minute in each). Labeling of probes (at a concentration of 3.1 ng probe/μl), with [^{35}S]dATP (39%) and terminal transferase enzyme (11.8%) was performed in a terminal transferase buffer (sodium cacaodylate 500 mM, $CoCl_2$ 10 mM, mercaptoethanol 1 mM; pH 7.2), for 1.5–2 hours at 37°C. Probes were then purified through NENSORB-20 columns (New England Nuclear, USA). The activity was measured to approximately $0.27–0.28 \times 10^9$ cpm/ml (sections were hybridized with probes at a concentration of 10^7 cpm/ml).

Quantification and image analysis of in situ hybridization signal. To evaluate the effect of nerve transection on the expression of PACAP mRNA in spinal cord neurons, all slides were analyzed qualitatively, and relative changes in hybridization signal were noted between the ipsilateral and contralateral sides of the spinal cord. To further analyze the effect, the PACAP mRNA signal in injured ventral horn neurons and corresponding neurons on the contralateral sides was quantified as described for DRG neurons (Jongsma Wallin et al., 2003). In brief, representative sections from rats with 3 days proximal nerve transection (containing similar numbers of motor neurons on each side and reflecting the overall labeling for motor neurons exposed to the same conditions) mounted on the same slide (to avoid variance in overall signal between different slides) were selected for quantitative analysis. All neurons expressing GAP-43, i.e., transected motor neurons, were examined regarding the density of PACAP mRNA expression, as well as motor neurons in the corresponding area on the contralateral side. The relative changes in hybridization signal between the neurons on the ipsilateral and contralateral sides were determined using an Olympus DP-50 digital camera, and computer assisted image analysis (n = 2 animals). The percentage of the cytoplasmic area covered by silver grains were mea-

sured in all the motor neurons, using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health, and available from the internet by anonymous FTP from zippy.nimh.nih.gov). Neurons were considered labeled for PACAP mRNA if they contained more than four times the background labeling of silver grains. The background was determined from measurements of the silver grain density over 12 defined areas of the spinal cord devoid of positively labeled cell bodies.

Immunohistochemistry (study performed in Lund)

Normal, uninjured male (n = 1) and female (n = 2), and injured (distal nerve transection) female (n = 5) Sprague-Dawley rats were used. Immunoreactivity for PACAP was investigated by using two different antibodies.

Monoclonal antibody against PACAP 1-38. Tissue slides were washed in PBS/Triton X-100 (PBS, 0.01 M, pH 7.3, and 0.25% Triton X-100), for 1+15 minutes and blocked with 1% H_2O_2 in PBS for 10 minutes, washed again (PBS/Triton X-100), and blocked with 5% BSA in PBS for 20 minutes. Sections were incubated in moist chambers with a monoclonal antibody against PACAP 1-38 (1:10, code JHH 1, kind gift from Professor Jan Fahrenkrug, Bispebjerg Hospital, Copenhagen, Denmark) overnight at 4°C. Excess antibody was washed away (PBS/Triton X-100), after which the sections were incubated with biotin-conjugated secondary antibody (rabbit anti-mouse IgG, 1:600, DAKO, Glostrup, Denmark), for 1 hour at room temperature, and then washed again for 3 × 5 minutes (PBS/Triton X-100). To enhance the signal, sections were incubated for 30 minutes with streptavidin conjugated to horseradish peroxidase (1:500, TSA Biotin System, Perkin Elmer), after which slides were washed again for 3 × 5 minutes (PBS/Triton X-100), and incubated with Biotinyl Tyramide for 8 minutes (1:100, TSA indirect TSA Biotin System, Perkin Elmer), washed again for 3 × 5 minutes (PBS/Triton X-100), and incubated with avidin conjugated to Oregon Green for 30 minutes (1:500, Molecular Probes, Eugene, OR). Finally, slides were washed for 3 × 5 minutes (PBS/Triton X-100) and mounted in PBS/glycerin (1:1). All substances except Biotinyl Tyramide were diluted in PBS/Triton X-100 (0.25%)/BSA (0.25%). Biotinyl Tyramide was diluted in the amplification buffer provided with the TSA-Indirect kit (TSA Biotin System, Perkin Elmer). To establish the specificity of the immunostaining, additional slides were either incubated (1) with antibody absorbed overnight with the antigen (synthetic PACAP 1–38; A1439, Sigma) in excess (100 μg/ml diluted antiserum), or (2) with the omission of the primary antibody, and processed as above.

Polyclonal antibody against PACAP 1-27. All dilutions were performed in PBS (0.01 M, pH 7.3), with 0.25% Triton X-100 and 0.25% BSA, and all steps were followed by wash in PBS with 0.25% Triton X-100. Sections were incubated in moist chambers with primary polyclonal antibody against PACAP 1-27 (1:1,280; code 88121-4, kind gift from Professor Akira Arimura, Tulane University, Hebert Center, US-Japan Biomedical Research Labs, New Orleans, LA), for 18–24 hours at 4°C. Excess antibody was washed away for 15 minutes, after which the sections were incubated with fluorescein isothiocyanate conjugated swine anti-rabbit IgG (1:80, DAKO, Copenhagen, Denmark) for 1 hour at room temperature. Slides were mounted in PBS/glycerin 1:1. To establish the specificity of the immunostaining, additional slides were incubated with antibody absorbed overnight with the antigen (synthetic PACAP 1-27, Sigma) in excess (100 μg/ml diluted antiserum), and processed as above.

Photomicrographs

Sections were analyzed in an Olympus BX-60 microscope connected to an Olympus DP-50 digital camera. Photomicrographs were captured by using the Viewfinder Lite software, version 1.0 (Pixera Corporation, Berkshire, UK) and stored in TIF format. Image editing software, Adobe Photoshop 5.0 was used to adjust size, brightness, and contrast.

RESULTS

In situ hybridization

Expression of PACAP mRNA in the normal, adult rat spinal cord was localized to neurons preferentially in lamina II, but also in lamina I, deeper dorsal horn laminae, and in neurons around the central canal (Fig. 1a–c). Furthermore, PACAP mRNA expression was found in a few medium ventral horn neurons (Fig. 1d), where it was not obviously coexpressed with VAChT mRNA.

A schematic drawing (Fig. 2a), showing the localization of the spinal cord laminae at lumbar level 5 according to (Molander et al., 1984), is presented to enable comparison of the areas of mRNA expression after the two types of injury. After distal nerve transection injury, an induction of PACAP mRNA expression was observed in large neurons located in the dorsolateral part of lamina IX (Figs. 2a,b, 3a,c,e). In this population of large neurons, coexpression for VAChT mRNA was also observed (Fig. 3b,d). No PACAP mRNA expression was detected in the corresponding neurons on the contralateral side (Fig. 3f). After 3 days of proximal nerve injury, an up-regulation of PACAP mRNA expression was detected both in the dorsolateral and more ventral populations of lamina IX neurons (Figs. 2a,c, 4a,c,e). The induction of PACAP mRNA expression was correlated to GAP-43 expression (Fig. 4b,d,f) and showed an overlap in expression (Fig. 4a–f). The quantitative analysis revealed that after proximal nerve transection (3 days) PACAP mRNA was expressed in 42% of the neurons positive for GAP-43 mRNA on the injured sides, as compared with only one or two cells expressing PACAP mRNA on the contralateral sides (not colocalized with GAP-43 mRNA). The levels of expression ranged between 5.0 and 52.6 (median, 12.6) and 5.6 and 10.2 (median, 6.9) times the background expression in the ipsilateral and contralateral motor neurons respectively. The PACAP mRNA expression was still elevated in the ipsilateral ventral horn neurons 3 weeks after injury (not shown).

No obvious changes in the expression pattern were observed when the different injury durations (3 vs. 7 vs. 14 days; or 3 days vs. 3 weeks) were studied. In the dorsal horn, the number of cells or density of PACAP mRNA expression did not differ noticeably between intact and injured animals (not shown). Furthermore, no change in the density of PACAP mRNA hybridization signal was detected when the ventral horn from intact animals was compared with the same area in the contralateral ventral horn from injured animals (distal injury), and only a mi-

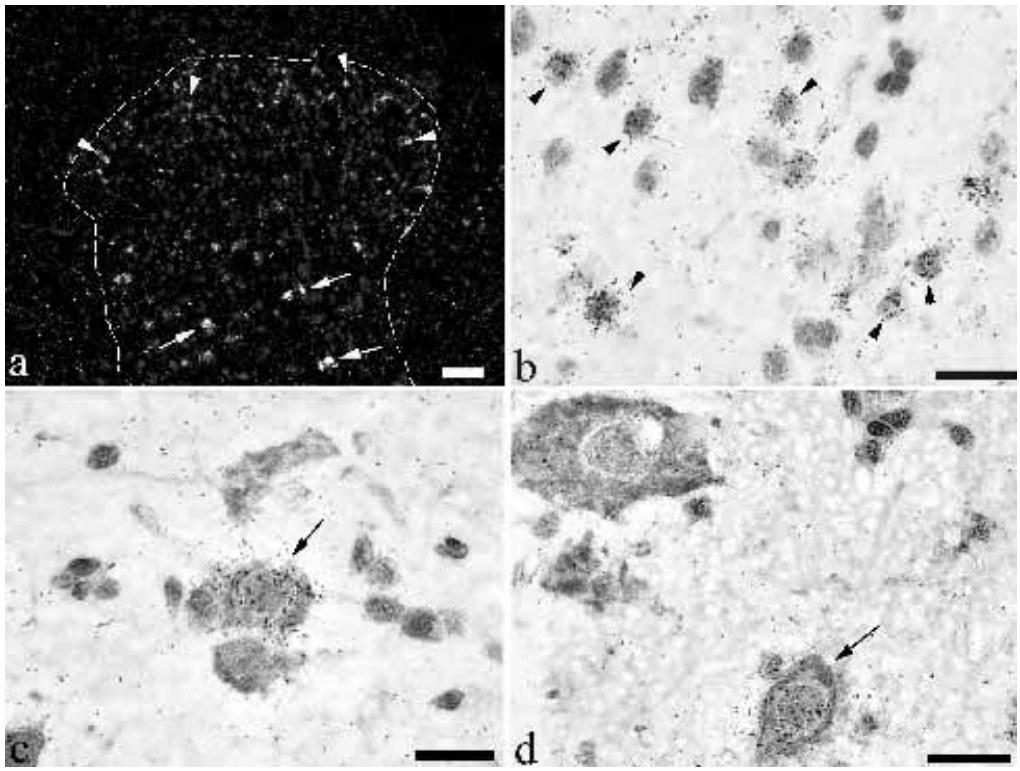


Fig. 1. **a–d:** Darkfield (a) and brightfield (b–d) images of the in situ hybridization labeling of pituitary adenylate cyclase activating polypeptide (PACAP) mRNA in adult rat spinal cord. Neurons expressing PACAP mRNA are localized preferentially in the superficial laminae of the dorsal horn (a,b) but also are scattered throughout the deeper laminae of the dorsal horn (a) and around the central canal (c). d: Furthermore, occasional ventral horn neurons show PACAP mRNA expression. a: The dashed line outlines the border of the dorsal horn. Arrowheads indicate some of the PACAP mRNA-expressing cells in laminae I–II, and arrows exemplify PACAP-positive neurons in deeper dorsal horn laminae, around the central canal and in the ventral horn. Scale bars = 100 μm in a, 25 μm in b–d.

nor increase in expression was seen in the contralateral ventral horn neurons after the proximal nerve injury. No labeling was found in the controls hybridized in the presence of the corresponding unlabeled probe in excess (not shown). There were no obvious differences in the expression pattern between the two PACAP probes, or when normal male and female rats were examined.

Immunohistochemistry

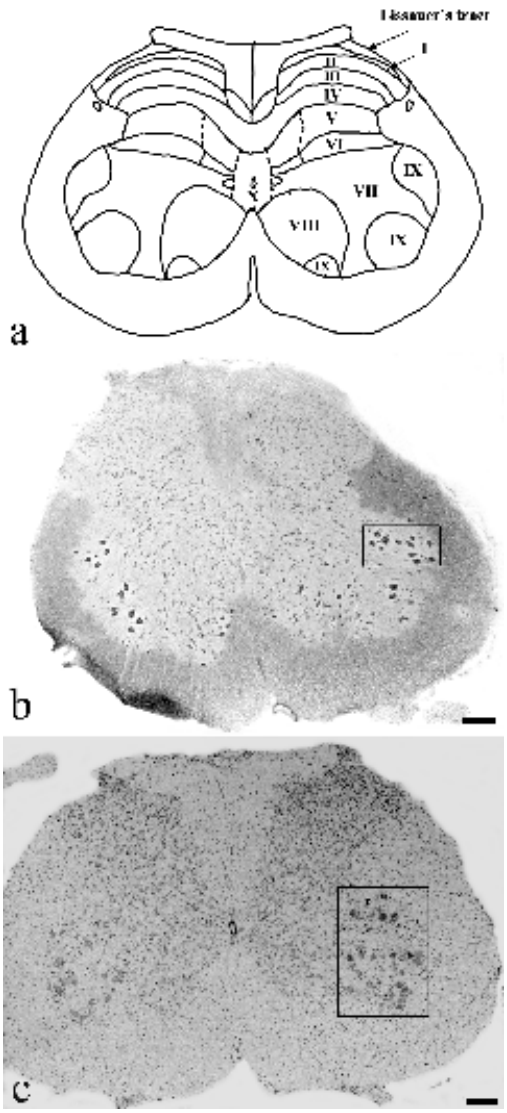
In the spinal cord, PACAP immunoreactivity was observed in fibers, primarily in the superficial laminae, around the central canal, and to a lesser extent in the ventral horn (Fig. 5a–d). These findings confirm previous reports on the distribution of PACAP peptide in the spinal cord. No immunopositive signal for PACAP could be detected in spinal cord neurons in the dorsal or ventral horn from uninjured animals (Fig. 5a,b,d). After 3, 7, or 14 days of distal nerve injury, an induction of PACAP immunoreactivity was detected in some of the large ventral horn neurons located dorsolaterally (Fig. 5c,e). These are neurons that correspond to the dorsolateral population of laminae IX neurons (Fig. 2a). There were no obvious differences in the immunoreactivity for PACAP that could be related to the duration of the injury. No PACAP immunoreactivity was seen in neurons in the contralateral ventral horn (Fig. 5f).

When examining the control slides that had been incubated with antibody preabsorbed with the synthetic peptide, or slides where the primary antibody had been omitted, no specific staining was observed (not shown). The two different PACAP antibodies gave a similar pattern of immunoreactivity in the dorsal horn, whereas only the monoclonal antibody gave a specific staining of ventral horn neurons.

DISCUSSION

We performed the present study to get a comprehensive, overall picture of the expression and distribution of the neuropeptide PACAP in the spinal cord. With the use of in

situ hybridization, we were able to identify sites of expression of PACAP mRNA in neurons in the superficial laminae of the spinal cord dorsal horn, in a few neurons in deeper laminae of the dorsal horn, around the central canal, and in ventral horn neurons. PACAP mRNA expression in these areas has not been described previously. We could detect PACAP mRNA expression in the adult spinal cord probably as a result of an optimization of the in situ hybridization protocol yielding a high signal to noise ratio, resulting in improved sensitivity and reduced unspecific background staining, which permitted longer exposure of the hybridized tissue with the film emulsion, further enhancing the detection levels. We could also confirm earlier findings (Moller et al., 1993; Dun et al., 1996; Vaudry et al., 2000; Hannibal, 2002) of immunoreactivity for PACAP in nerve fibers in the spinal cord dorsal horn and around the central canal and further illustrate the distribution pattern of PACAP in the spinal cord after nerve injury.



PACAP mRNA expression in dorsal horn neurons

The expression of PACAP in DRGs is primarily confined to small neurons (Mulder et al., 1994; Zhang et al., 1995, 1996b). No expression of PACAP has been reported previously in the spinal cord dorsal horn neurons, and the effects of PACAP in the DRG/dorsal horn, thus, have been considered to be limited to the superficial laminae of the spinal cord dorsal horn, where release of PACAP is thought to modulate nociceptive responses in a complex manner (see introduction section). Our findings of an expression of PACAP mRNA also in neurons in the spinal cord dorsal horn and especially in the superficial laminae, may have consequences on the interpretation of the involvement of PACAP in modulation of sensory, e.g., nociceptive, transmission. The level and distribution of PACAP mRNA signal in the dorsal horn neurons were not visibly affected after transection of the sciatic nerve. This finding suggests that the effect of nerve transection on PACAP mRNA expression in the sensory nervous system is limited to the primary afferent neurons in the DRG, leaving the expression in the postsynaptic dorsal horn neurons unaffected. The expression of PACAP mRNA in the dorsal horn neurons could not be confirmed with immunostaining for PACAP. Possible explanations for this inconsistency might be changes in the rate of translation, processing, and degradation of mRNA, and/or fast transport and release of PACAP peptide out of dorsal horn neurons. Furthermore, PACAP immunoreactivity in cell somas could be masked by the abundant presence of intensely PACAP-immunoreactive fibers in this area, making it hard to detect any immunoreactivity of the peptide in nerve cell bodies, which are small in this area. A third, less likely, possibility is that the PACAP mRNA is not translated into functional peptide.

Fig. 2. **a–c:** Schematic drawing of the rat spinal cord at lumbar level 5 (a) and low magnification, brightfield images of spinal cords hybridized for detection of pituitary adenylate cyclase activating polypeptide (PACAP) mRNA 3 days after distal nerve injury (b) or 3 days after proximal nerve injury (c). b,c: The boxed areas in the light photomicrographs indicate the areas magnified in Figures 3a,c,e and 4c,e. The asterisks on the injured side are included to aid in the localization of individual neurons and are inserted at the same place in corresponding figures (Figs. 3a,c,e and 4a,c,e). Neurons are visualized with toluidine blue counterstaining. b: Note that the hybridization signal for PACAP mRNA is very hard to detect at this low level of magnification. c: After proximal injury, signal for PACAP mRNA expression is discernible already at low level of magnification. a: The schematic drawing, modified from Molander et al. (1984), is included to facilitate laminae localization and distinction. Scale bars = 200 μm in b,c.

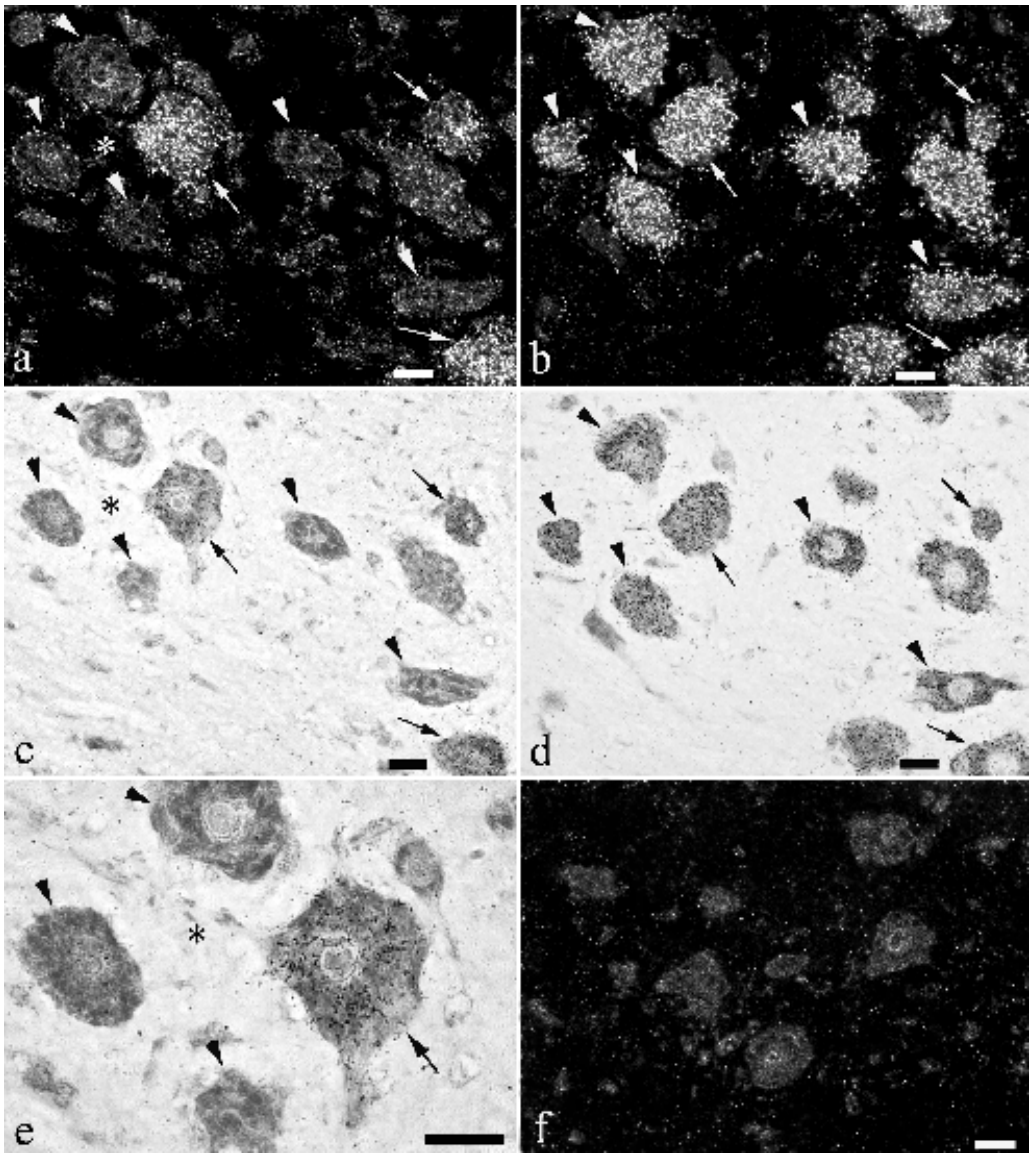


Fig. 3. **a–f:** Darkfield and brightfield images showing the pituitary adenylate cyclase activating polypeptide (PACAP; a,c,e,f) and vesicular acetylcholine transporter (VAChT; b,d) mRNA expression, in consecutive sections, in the ventral horn of the rat spinal cord 3 days after distal nerve transection. a,c,e,f: PACAP mRNA expression is induced in the dorsolateral group of lamina IX neurons ipsilateral (a,c,e), but not contralateral (f) to the injury. f: No changes in the density of PACAP mRNA labeling can be detected on the contralateral side when compared with the expression in spinal cord from uninjured rats. a–e: Neurons coexpressing PACAP and VAChT mRNA in the consecutive sections are indicated with arrows. Arrowheads in a–e indicate neurons present in the adjacent sections, that express VAChT but not PACAP mRNA. The asterisks in a,c are indicating the area enlarged in e, for an overview of the area, see Figure 2 b. Scale bars = 25 μm in a–f.

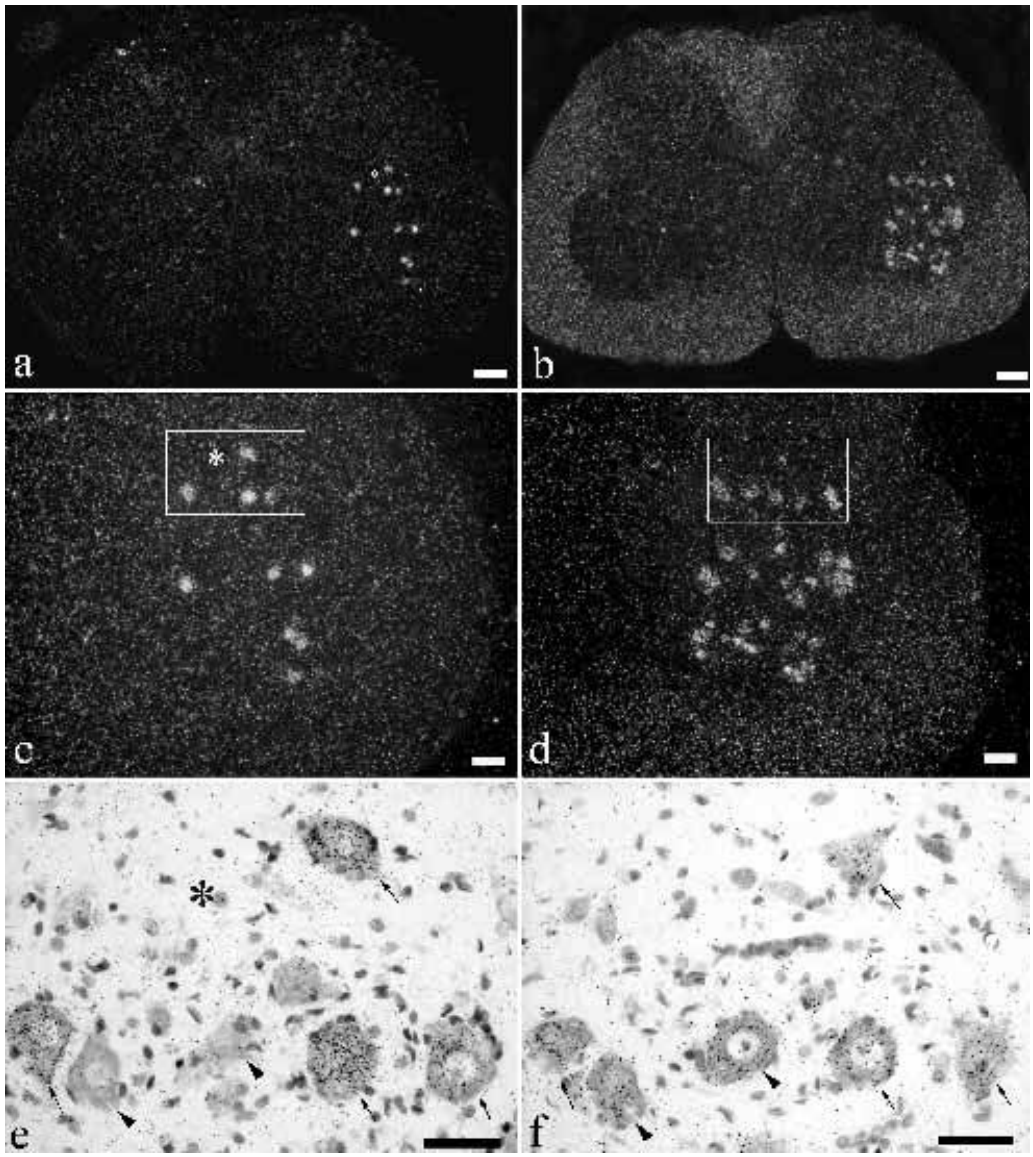


Fig. 4. **a,c,e:** Darkfield and brightfield images visualizing the induction of pituitary adenylate cyclase activating polypeptide (PACAP) mRNA expression in the ventral horn neurons also after proximal nerve injury. **b,d,f:** Injured neurons are shown hybridized to detect elevated growth associated protein 43 (GAP-43) mRNA expression, to identify which neurons are injured. The boxed area in c,d is enlarged in e,f, to visualize colocalization of PACAP and GAP-43 mRNA in nerve cell bodies. PACAP mRNA expression is induced in a subpopulation of the injured, GAP-43-expressing neurons. e,f: Arrows indicate neurons expressing both PACAP and GAP-43 mRNA, and arrowheads indicate injured neurons devoid of signal for PACAP mRNA. a,c: Note that, after proximal injury, neurons from both the dorsolateral and the ventrolateral group of lamina IX are expressing PACAP mRNA, as compared with distal nerve injury, which results in an induction of PACAP mRNA expression only in the dorsolateral group, indicating the increased severity of the proximal injury model. Asterisk in a indicates the area enlarged in c,e. Also, this is the same area shown in Figure 2c. Scale bars = 200 μm in a,b, 100 μm in c,d, 50 μm in e,f.

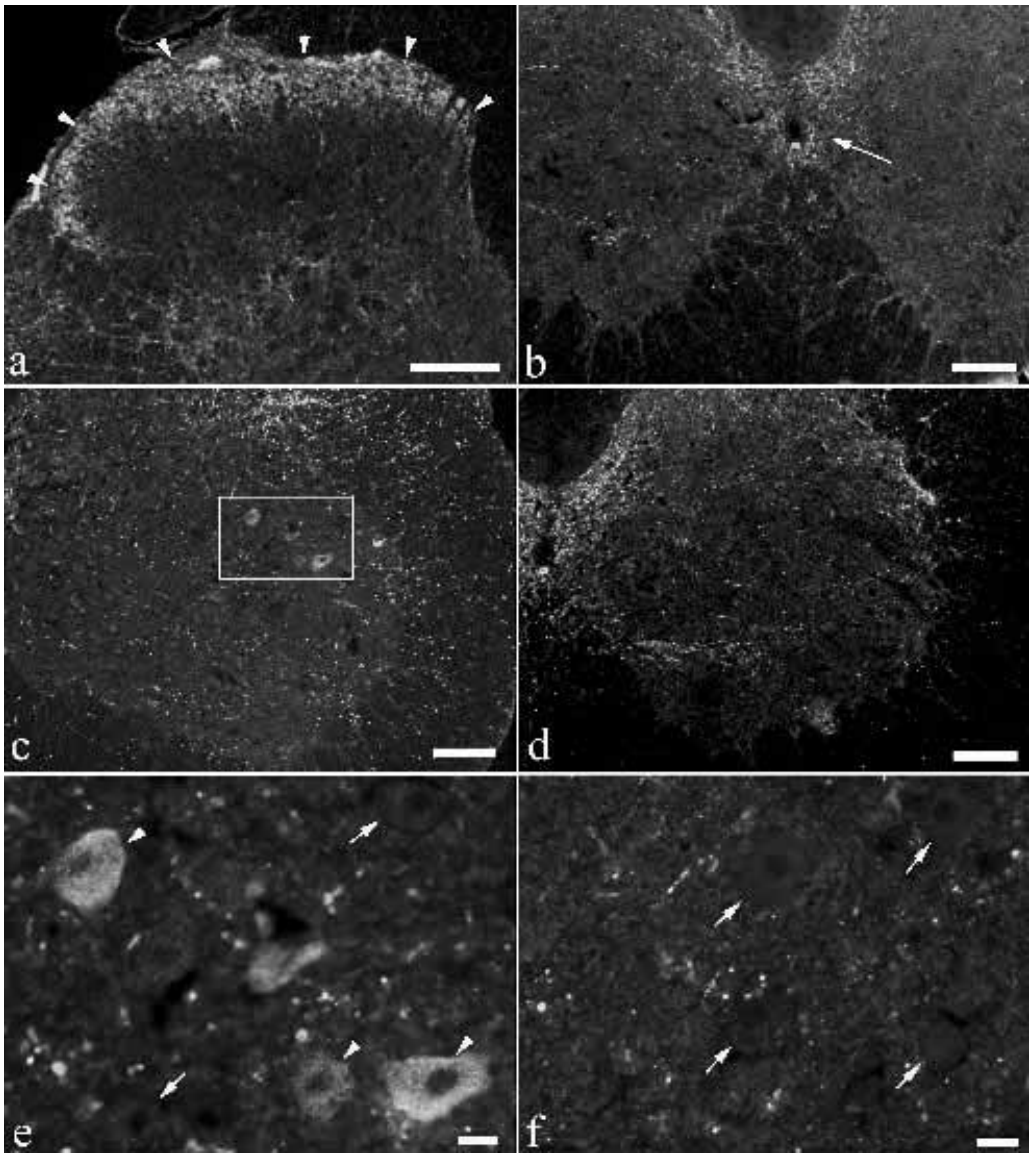


Fig. 5. **a–f:** Immunohistochemical staining for pituitary adenylate cyclase activating polypeptide (PACAP) in rat spinal cord, uninjured (a,b,d) and 3 days after distal nerve transection; ipsilateral (c,e) and contralateral (f). a: Immunoreactivity in the spinal cord from normal, uninjured animals, is seen in fibers in the superficial laminae of the dorsal horn, but no further immunoreactivity in neurons in this area can be detected. b,d: Radiating fibers showing PACAP immunoreactivity are also found around the central canal (b) and in the ventral horn (d). d,f: No sign of PACAP immunoreactivty can be detected in the ventral horn neurons in uninjured animals (d) or on the contralateral side after injury (f). c: Induction of PACAP immunoreactivity, in a few of the large neurons located in the dorsolateral group of lamina IX is detected ipsilateral to the injury. The area in c is enlarged in e, and to visualize the immunoreactivity for PACAP in the neurons, the focus is set on the fine grains in the nerve cell bodies (e). The fine grain appearance is suggestive of a vesicular peptide localization. Because the depth of focus between the grains and the background is different, the background appears slightly out of focus. Arrowheads in a indicate the dorsal edge of lamina I. Arrow in b points out the location of the central canal. Arrowheads in e indicate neurons stained for PACAP content, and arrows in e,f exemplifies neurons that are not immunoreactive for PACAP. Dorsal horns upward in all figures. The antibody used was monoclonal antibody against PACAP 1-38 (JHH 1). Scale bars = 200 μm in a–d, 25 μm in e,f.

Injury-induced changes in PACAP mRNA expression in ventral horn neurons

When we explored the mRNA expression in the ventral horn, we found a few neurons expressing PACAP mRNA in normal, uninjured animals. A more striking finding was the induction of PACAP mRNA expression after axotomy. After distal nerve transection, expression was induced in large cells located laterally in the ventral horn; dorsolateral group of lamina IX (Fig. 2a). The expression of PACAP mRNA in these neurons was colocalized with VAChT mRNA expression. We came to the conclusion that the induction of PACAP mRNA expression in the ventral horn lamina IX after injury most likely takes place in motor neurons as indicated by the size, location, and colocalization with VAChT mRNA in these neurons. The induction of PACAP mRNA in the dorsolateral ventral horn is supported by immunohistochemical findings of PACAP immunoreactivity in some of the neurons in this area after injury. Immunoreactivity for PACAP in neurons in this area has not been reported previously and was not found when spinal cords from normal, uninjured rats were examined.

The neurons located in the dorsolateral group of lamina IX are motor neurons carrying signals to the more distal muscles of the hindlimb (Nicolopoulos-Stournaras and Iles, 1983). That the dorsolateral group of lamina IX neurons supply the muscles affected by the distal nerve transection explains the up-regulation in expression and further supports an involvement of PACAP in motor injury responses (see below). No change in expression was seen in motor neurons in the more ventral group of lamina IX neurons, i.e., neurons that project to more proximal muscles of the hindlimb. The axons of these neurons travel in nerve branches that were spared from transection in the distal transection injury model. To further examine the effect of nerve transection on the expression of PACAP mRNA in different neuronal populations in the spinal cord, we used an injury model with a more proximal nerve transection. After the proximal nerve transection, an induction of PACAP mRNA expression was seen in both groups of neurons (dorsolateral and ventral groups) of lamina IX, i.e., neurons supplying both proximal and more distally located muscles of the hindleg. Thus, we could see a selective recruitment of neurons expressing PACAP mRNA in subpopulations in the ventral horn after the two types of injury, strengthening the specificity of the PACAP mRNA induction after injury and supporting its role in the response also to motor nerve injury. The coexpression of PACAP and GAP-43 mRNA in these neurons indicates that induction of PACAP mRNA expression takes place in injured neurons. PACAP mRNA expression was investigated 3, 7, and 14 days after distal, and 3 days after proximal, nerve transection. Previous studies concerning the effects of axotomy on the pattern of neuropeptide mRNA expression in motor neurons have yielded different results, depending on the injury duration and the peptide mRNA examined (Zhang et al., 1993a). To further examine the expression pattern of PACAP mRNA in motor neurons, a longer injury duration was investigated, and we found the levels of PACAP mRNA expression to be elevated still 3 weeks after proximal nerve transection. No changes were evident when the expression was compared 3 days with 3 weeks after nerve injury. When compared with uninjured rats, no changes in PACAP mRNA expression or PACAP immunoreactivity were detected on the contralateral side of the spinal cord after distal nerve transection, speaking against a contralateral effect on these neurons. A slight increase in PACAP mRNA expression was indicated contralaterally after proximal injury, suggesting that a minor contralateral effect might be present after this type of injury.

Potential effects of PACAP

The increase in PACAP mRNA expression in response to sciatic nerve axotomy suggests that PACAP plays a role also in the injury response in motor neurons, in addition to its presumed role as a mediator of injury responses in rat sensory (Zhang et al., 1995, 1996b) and sympathetic (Moller et al., 1997) neurons. A role for PACAP in modulation of the injury response in motor neurons is also supported by previous findings of an increased PACAP mRNA expression in the rat facial motor nucleus after axotomy of the facial nerve (Zhou et al., 1999) and induction of events promoting regeneration of the facial nerve in guinea pigs after transection (Kimura et al., 2003). It has been hypothesized that PACAP can act as a survival factor for axotomized neurons and that this action in some cases might differ from that of the classic growth factors (Waschek, 2002). Only a few motorneurons expressing PACAP mRNA could be detected on the contralateral sides as compared with the expression in 42% of the transected motor neurons on the ipsilateral sides, indicating a subsequent recruitment after nerve injury. Furthermore, in injured motor neurons the median level of expression was almost doubled as compared with the expression levels in the few neurons expressing PACAP mRNA on the contralateral sides. These findings lend further support to the notion that PACAP acts as a neurotrophic factor and has neuroprotective effects (for review, see Vaudry et al., 2000, 2002). PACAP exerts neurotrophic effects in the rat cerebellum; stimulating proliferation of cerebellar granule cells in culture, inhibiting programmed cell death of granule cells cultured under conditions promoting apoptosis, as well as evoking a transient increase in the number of granule cells in molecular and internal granule cell layers in vivo (Cavallaro et al., 1996; Chang et al., 1996; Vaudry et al., 1999). Furthermore, PACAP triggers cell cycle withdrawal, inducing the transition from proliferation to neuronal differentiation, in cultured rat cortical neuron precursors (Lu and DiCicco-Bloom, 1997), protects cultured cortical neurons from the cytotoxic effect of high concentration of glutamate (Morio et al., 1996), and increases the survival of septal cholinergic neurons in vitro and after injury in vivo (Takei et al., 2000).

Previously, no findings of PACAP mRNA expression in adult rat spinal cord dorsal and ventral horn neurons have been reported. Although, in the developing rat spinal cord, expression of PACAP mRNA has been found at embryonic day 13-until birth (Lindholm et al., 1998; Nielsen et al., 1998; Skoglosa et al., 1999; Jaworski and Proctor, 2000). The expression was found throughout the neuraxis, enriched in the spinal cord ventricular zone, in postmitotic cells of the spinal cord stroma, and in neurons in IML. The distribution of PACAP mRNA in embryonic spinal cord gives a morphologic basis for the expression of PACAP mRNA in this tissue in the adult animal. Furthermore, some of the developmentally active neuropeptides are down-regulated postnatally but reactivated and up-regulated again in response to perturbations in their en-

vironment, such as damage and stress to the nervous system, e.g., nerve injury (Hokfelt et al., 2000). Tentatively, the expression of PACAP mRNA in the rat spinal cord ventral horn is an example of such an expression: low, or lack of expression in adult uninjured motor neurons, and an increased expression in response to nerve injury.

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Paper IV

Changes in expression of pituitary adenylate cyclase activating polypeptide in rat sensory neurons in response to sciatic nerve compression

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Abstract

In the present study, expression of pituitary adenylate cyclase activating polypeptide (PACAP) in rat dorsal root ganglion (DRG) neurons and sciatic nerve following experimental sciatic nerve compression, was studied with the use of immunohistochemistry and quantitative in situ hybridization. Previously, we have investigated changes in PACAP expression after nerve transection, and here, the far more frequently encountered condition of nerve compression injury is examined. Nerve compression was performed unilaterally on the rat sciatic nerve, at mid-thigh level, by application of a narrow silicone tube around the nerve for 3, 7, 14 or 28 days, respectively. We detect a statistically significant upregulation in the number and density of PACAP mRNA expression, both in small and large DRG neurons, in response to nerve compression. An increased immunoreactivity for PACAP is also found in the ipsilateral DRG and in the compressed sciatic nerve segment and adjacent nerve tissue. The present findings can be compared with previous studies where we have shown that PACAP expression is upregulated in DRG; in response to peripheral inflammation (primarily in small-medium size neurons), and after axotomy (dramatic upregulation in medium-large size neurons). In view of the recent findings of an increased PACAP expression in DRG after nerve compression, as well as the previous findings of a modulation of PACAP expression in response to axotomy and inflammation, it is likely that PACAP is involved also in the modulation of the response to peripheral nerve compression.

Key words: DRG, in situ hybridization, nerve injury, neuropeptide, PACAP

List of abbreviations

BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
DRG	dorsal root ganglion
IgG	immunoglobulin G
L4, L5	lumbar level 4, lumbar level 5
NGF	nerve growth factor
NT-3	neurotrophin 3
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	phosphate buffered saline
OligoDNA	oligodeoxyribonucleotide DNA
SSC	saline sodium citrate

Introduction

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) is normally expressed in small-medium size dorsal root ganglion (DRG) neurons (Moller et al., 1993; Zhang et al., 1995; Dun et al., 1996; Zhang et al., 1996b), whereas expression of the PACAP preferring receptor, PAC_1, has not been found in DRG neurons, but rather in dorsal horn neurons (Jongsma et al., 2000; Vaudry et al., 2000). The expression of PACAP is very plastic, and is modified in response to both inflammation (Zhang et al., 1998; Jongsma Wallin et al., 2003) and nerve transection (Zhang et al., 1995; Zhang et al., 1996b; Jongsma Wallin et al., 2001), where small-medium size and medium-large size neurons respectively, are responding. Therefore, a modulatory role for PACAP is suggested during these conditions. Further, PACAP has been implicated in the modulation of pain, both inhibition (Zhang et al., 1993b; Yamamoto and Tatsuno, 1995; Zhang et al., 1996a) and potentiation (Narita et al., 1996; Xu and Wiesenfeld-Hallin, 1996; Jongsma et al., 2001).

Nerve compression is a far more common condition than nerve transection, and the prevalence of e.g. carpal tunnel syndrome (compression of the median nerve in the narrow carpal tunnel between the carpal bones and the relatively rigid flexor retinaculum, at wrist level) has been estimated to approximately 4% of an adult Swedish human population, predominantly women (Atroshi et al., 1999). The etiology of compression injuries is diverse and in most cases the cause is unknown. The condition is initially characterized by numbness, and a tingling and/or burning sensation in the area of innervation. If left untreated, the condition can lead to severe problems, e.g. permanent loss of sensation and atrophy of the innervated muscles, reviewed by (Rempel et al., 1999; Mackinnon, 2002). Pain can be a prominent feature, especially during severe compression, and we therefore wanted to investigate whether there is a change in PACAP expression also in response to nerve compression. Some patients experience the pain or allodynia during compression, and/or after decompression of the affected nerve. In the present study we used a rat model, which mimics the clinical situation, to study nerve compression (Weisl and Osborne, 1964; Dahlin and Kanje, 1992). With the use of a narrow silicone tube, which was applied around the sciatic nerve, the nerve was compressed for various time periods. Expression of PACAP was investigated with regards to PACAP mRNA content and PACAP immunoreactivity in rat sciatic nerve, DRG and spinal cord. Further, the expression and distribution of the PACAP receptor, PAC_1, in DRG and spinal cord, was examined with in situ hybridization and ligand binding.

Experimental procedures

Experimental animals and tissue processing

This study was approved by the Malmö/Lund Animal Ethics Committee on Animal Experiments, and all efforts were made to minimize the number of animals used, and their suffering. The left sciatic nerve of adult, female Sprague-Dawley rats (approximately 200 g; Møllegaard, Denmark) was exposed under pentobarbital (19 mg/kg)/ diazepam (3 mg/kg)/ physiological saline anaesthesia, (i. p.). A 10 mm long silicone tube (internal diameter 0.76 mm) was incised longitudinally and wrapped around the nerve at mid-thigh level. Thereafter, two 9/0 Ethilon sutures were applied around the silicone tube, closing it to a constant diameter (Fig. 1). The wound was sealed by sutures and the rats were allowed to recover in heated cages to maintain body temperature. Animals were left with the compressed nerve for 3 (n=3), 7 (n=3), 14 (n=3) or 28 (n=4) days respectively, and checked daily. All animals were killed by heart puncture after administration of an overdose of pentobarbital (60 mg/ml, i.p.) anaesthesia, and spinal cord (lumbar segment), DRGs lumbar level (L4-5), and sciatic nerves (a 20 mm segment of the nerve beneath, proximal and distal to the silicone tube, plus the corresponding part of the contralateral nerve) were dissected from the rats. The tissues from the contralateral side were used as controls. Tissue specimens were mounted in O.C.T. Compound (Tissue-Tek®, Histolab products AB, Göteborg, Sweden), fresh-frozen in isopentane at -30°C immediately after dissection, and stored at -80°C for in situ hybridization and ligand binding. For immunohistochemistry, the tissues were fixed in Stefanini´s fixative (2% paraformaldehyd and 15% of a saturated aqueous picric acid solution, in a 0.1 M phosphate buffer, pH 7.2) overnight, followed by repeated rinsing in sucrose-enriched (10%) Tyrode´s solution. Specimens were mounted in Tissue-Tek®, frozen on dry ice and stored at -80°C. Tissue specimens were sectioned (6 µm, adjacent sections for colocalisation and quantification of mRNA; 10 µm for immunohistochemistry; 20 µm for ligand binding) in a cryostat and thaw-mounted on to Super Frost® Plus slides (Menzel-Gläser, Germany). Sciatic nerves were sectioned longitudinally to visualize the compressed and surrounding areas in the same sections.

All sections were analyzed in an Olympus BX-60 microscope connected to an Olympus DP-50 digital camera. Photomicrographs were captured using the Viewfinder Lite software, version 1.0 (Pixera Corporation, Berkshire, United Kingdom) and stored in TIF format. Image editing software, Adobe Photoshop® 5.0 was used to adjust size, brightness and contrast.

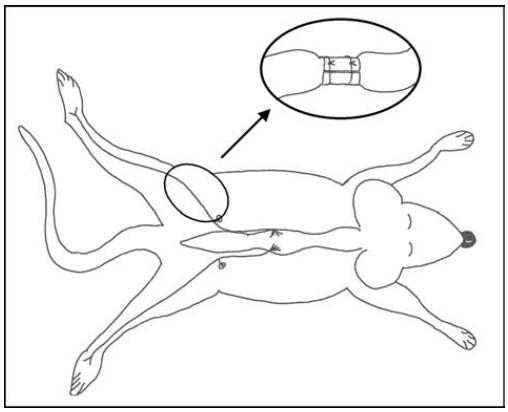

Fig. 1. Schematic drawing of the experimental compression model. A silicone tube (inner diameter 0.76 mm) was incised longitudinally, and wrapped around the left sciatic nerve at mid-thigh level. Sutures were applied around the tube closing it (see inset). The wound was sutured and animals were left with the compressed nerve for 3, 7, 14 or 28 days, respectively.

Immunohistochemistry

The protocol for immunohistochemistry has been described in detail (Pettersson et al., 2004). The procedures are described in brief in the following.

Polyclonal antibody against PACAP 1-27:

Sections were incubated in moist chambers with primary antibody against synthetic PACAP 1-27 (1:1280; code 88121–4, kind gift from Professor Akira Arimura, Tulane University, Hebert Center, US-Japan Biomedical Research Labs, New Orleans, LA, USA) for 18-24 hours. Excess antibody was washed away and sections were incubated with fluorescein isothiocyanate conjugated swine anti-rabbit immunoglobulin G (IgG) (DAKO, Copenhagen, Denmark). Slides were mounted in PBS/glycerin (1:1).

To establish the specificity of the immunostaining, additional slides were incubated with antibody absorbed overnight with the antigen (synthetic PACAP 1-27, Sigma-Aldrich, St. Louis, MO, USA) in excess, and processed as above.

Monoclonal antibody against PACAP 1-38:

Endogenous peroxidase activity was quenched with the treatment of 1% hydrogen peroxide in PBS, unspecific binding was blocked by treatment with 5% BSA in PBS, and slides were incubated in moist chambers with monoclonal antibody against synthetic PACAP 1-38 (1:10, code JHH 1, kind gift from Professor Jan Fahrenkrug, Bispebjerg Hospital, Copenhagen, Denmark) for 18-24 hours. Following incubation with biotinylated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark), slides were incubated with streptavidin conjugated to horse radish peroxidase (TSA™ Biotin System, Perkin Elmer), and treated with Biotinyl Tyramide (TSA Indirect TSA™ Biotin System, Perkin Elmer). Finally, slides were incubated with avidin conjugated to Oregon Greene (Molecular Probes, Eugene, OR, USA), and mounted in PBS/glycerin (1:1).
To establish the specificity of the immunostaining, additional slides were incubated with antibody absorbed overnight with the antigen (synthetic PACAP 1-38; A1439, Sigma-Aldrich, St. Louis, MO, USA) in excess, and processed as above.

In situ hybridization

Radiolabeled (^{35}S) labeled oligodeoxyribonucleotide (oligoDNA) probes for detection of PACAP mRNA was used for in situ hybridization. OligoDNA probes complementary to and selective for the PACAP mRNA was synthesized; PACAP –nucleotides 701-747 (Hurley et al., 1995), and PACAP receptor; PAC_1 mRNA, a mix of probes complementary to –nucleotides 380-412, 629-661, 1421-1453 and 1673-1705 (Hashimoto et al., 1993) were synthesized and purified at the University of Calgary DNA services, Alberta, Canada, respectively at the Biomedical Resource Facility, Lund University, Sweden. All used cDNA regions were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm.nih.gov, October 2003); no greater than 60% homology was found to sequences other than the cognate transcript.

Labeling of the probes and the complete protocol for in situ hybridization has been described in detail (Pettersson et al., 2002). To prevent degradation of RNA by external RNases, all steps prior to hybridization were carried out under RNase free conditions and all dilutions were performed in autoclaved double distilled water. Probes were labeled with ^{35}S-dATP (Perkin Elmer), and the specific activity, approximately 1.1 x 10^9 cpm/µg and 0.64 x 10^9 cpm/µg for PACAP and PACAP receptor probes respectively, was measured in a liquid scintillator. All probes were stored at -18°C until use.

The slides containing double, adjacent pairs of left (compressed) and right (uninjured) DRG sections were air dried, and hybridized with radiolabeled probes (final activity of 10^7 cpm/ml) in a hybridization solution, without any additional pretreatment. Hybridization, with 50-100 µl hybridization solution/slide, was conducted overnight at 37°C. Control slides were incubated with the addition of corresponding, unlabeled probe in 100-fold molar excess.

Following hybridization, the slides were dipped in 1 x saline sodium citrate (SSC), the parafilm was removed

and the remaining washes were performed in 1 x SSC. The sections were dehydrated in ethanol, air dried, and immersed in Kodak NTB2 photoemulsion (diluted 1:1 in distilled water) and stored with desiccant at 4°C in light sealed containers. After 3.5 weeks the slides were developed in Kodak D19, fixed in Kodak polymax fix, counterstained with toluidine blue, and mounted with Pertex (Histolab, Göteborg, Sweden). All sections/slides were hybridized and processed under the same conditions at the same time, to ensure similar treatment and enable comparisons between the ipsi- and contralateral side DRGs.

Quantification and image analysis

To evaluate the effect of nerve compression on the expression of PACAP mRNA in DRG neurons, all slides were first analyzed qualitatively. Relative changes in hybridization signal were noted for all sections mounted on the same slide. Sections that were to be compared, were mounted on the same slide to avoid variance in overall signal between the different slides (each slide containing double, adjacent pairs of left and right DRGs). Changes in hybridization signal were examined for all sections, and the quality of each section was noted. Representative slides (where all DRG sections mounted on the same slide contain similar numbers of neurons, and the sections reflect the overall labeling for DRGs exposed to the same conditions) were selected for quantitative analysis. Each individual neuron was examined and the relative changes in hybridization signal were determined, for the ipsi- and contralateral DRGs and for the different time periods, using computer assisted image analysis. Image analysis was performed on adjacent DRG sections processed for in situ hybridization to detect PACAP mRNA (n=4 animals, 1 rat per time point analyzed, 8 DRG sections where approximately 1900 neuronal DRG L5 profiles were examined). Montages of photomicrographs (500 X) were prepared from eight pairs of adjacent sections of left or right DRGs from animals with 3, 7, 14 or 28 days of unilateral sciatic nerve compression. Individual neurons with a visible nucleus in at least one of the two adjacent sections were identified and numbered (approximately 240 neurons/montage). Light microscopy images (60X) were collected from each individual DRG L5 neuron using an Olympus DP-50 digital camera. Cross sectional areas of each neuronal profile and the percentage of the cytoplasmic area that was covered by silver grains were measured in all the ganglia, using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health, and available from the internet by anonymous FTP from zippy.nimh.nih.gov). Individual cell diameters were calculated from the cross sectional areas on the assumption that the neurons were spherical. Neurons were defined as small when the diameter was < 35 µm, and large when the diameter was > 35 µm (Giuffrida and Rustioni, 1992). The density threshold was adjusted interactively for each image, so that the area per grain was constant for all neurons analyzed on a given slide. Neurons were considered labeled for PACAP mRNA if they contained more than five times background labeling of silver grains. The background was determined from measurements of the silver grain density over five defined areas of the neuropil devoid of positively labeled cell bodies. Background was measured and determined specifically for each quantified section.

After the quantification of DRG L5 neurons we performed a somewhat less extensive examination of the DRG L4 neurons (which also support the sciatic nerve), to compare the PACAP mRNA distribution with the ganglia at the L5 level. All sections were examined and selected according to the criteria above, and this time four new animals were quantified. Montages were made (n=4 animals, 1 animal per time point analyzed, 8 DRG sections and approximately 2300 DRG L4 profiles), neurons defined and numbered, whereafter each neuron (approximately 290 neurons/montage) was examined directly in the microscope (60X). The total percentage of PACAP mRNA labeled neurons was determined on the compressed and intact sides, respectively, but no measurements of neuronal diameter was made, i.e. no distinction of the distribution in relation to neuronal size was made for the DRG L4 neurons, and changes in silver grain density/neuron was not calculated.

The differences in numbers of neurons expressing PACAP mRNA, between the ipsi- and contralateral side DRG, were analyzed using the chi-square test, and $p<0.05$ was considered significant.

[^{125}I]-PACAP 27 binding

[^{125}I]-PACAP 27 binding was used to locate binding of PACAP to its receptor. The procedure for ligand binding has been described in detail (Pettersson et al., 2002). In brief, sections were preincubated in a Tris buffer, incubated with 150 pM [^{125}I]–PACAP 27 (Perkin Elmer), at 20°C for 1 hour. Control sections were incubated with the addition 1.5 µM synthetic PACAP 1-27 (Sigma-Aldrich) to check for non-specific binding. Slides were washed, and when dry, covered with Hyperfilm MP (Amersham, Solna, Sweden), stored at 4°C in the dark for 1 week after which the film was developed in Kodak D19, fixed in Kodak polymax, and examined in the microscope.

Results

Immunohistochemistry

Scarce immunoreactivity for PACAP was observed in a few fibers in the intact sciatic nerve, whereas PACAP immunoreactive staining in the compressed nerves was observed in nerve fibers in the sciatic nerve segment beneath and bordering to the compressed area, especially accumulated at the nodal regions (Fig. 2 a,b). The increase in PACAP immunoreactivity in the compressed nerve was most prominent 3, 7 and 14 days after injury, although some increased immunoreactivity was still visible 28 days postinjury (examined qualitatively). Inflammatory cells, resembling macrophages and mast cells (based on their general histology), were found in the endoneurium, primarily around the distal border of the compressed nerve segment. Also, an enlargement of the epineurium was observed along the length of the compressed segment of the nerve around 7 days after surgery, and persisted during the time period studied (not shown). Further, an increased PACAP immunoreactivity (number of neurons and staining density, qualitative examination) was noted in the ipsilateral DRGs when compared to the contralateral side, 3, 7 and 14 days after surgery, most prominent 7 days after injury (Fig 3 a,b). A less marked increase in immunoreactivity was observed 28 days after injury. No pronounced changes in PACAP immunoreactivity was found in the spinal cord at any of the time points examined (not shown).

In situ hybridization

Signal for PACAP mRNA in DRG (L5) was increased on the ipsilateral side at 3, 7, 14 and 28 days after unilateral sciatic nerve compression (Fig. 3 c-f, 4; Table 1). The upregulation of PACAP mRNA expression on the ipsilateral side (number of neurons) was statistically significant at all time points studied, ranging from around two to almost four times the contralateral expression depending on the time point investigated. The greatest proportion of PACAP mRNA expressing neurons (3.9 times the contralateral expression) was detected 28 days post surgery (Table 1). On the intact side, 14-21% of the DRG neurons (small size) express PACAP mRNA, whereas 40-55% of the neurons express PACAP mRNA on the compressed side. The upregulation in PACAP mRNA expression after nerve compression is found in both small (< 35 µm) and large (> 35 µm) size DRG neurons (Fig 4, Table 1). The quantitative exminations of the PACAP mRNA expression in L4 DRG neurons confirmed our data from the L5 level, showing very similar levels of expression in response to nerve compression, except for 3 days post surgery when a very slight variance in the proportion of PACAP mRNA positive neurons was observed (Table 1). Also, on the contralateral side the percentage of PACAP mRNA expressing neurons was very consistent in the L4 and L5 DRG neurons and between the differents injury durations, except for 7 days after surgery when a small increase in the number of PACAP mRNA expressing neurons (L4 and L5) was found, that was not present at the other timepoints (Table 1). When the expression in heavily labeled populations (>32 X background) was compared between the both sides (ipsilateral vs contralateral proportions in parenthesis), a significant difference in the number of heavily expressing neurons was found 3 (12% vs 4.0%), 14 (10% vs 3.0%), and 28 (11.5% vs 1.8%) days after compression, but not 7 (12% vs 7.9%) days after compression. On the contralateral side heavy expression was observed exclusively in neurons with small diameter,

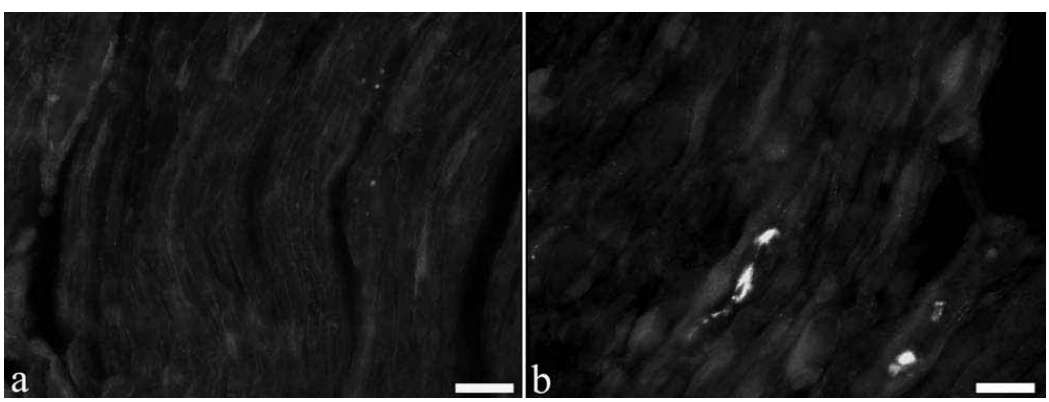

Fig. 2. Longitudinally sectioned sciatic nerves 7 days after nerve compression, contra- (a) and ipsilateral (b) sides, stained for PACAP immunoreactivity. An induction of PACAP immunoreactivity in fibers, especially accumulated at the nodal regions, was detected in the compressed nerve segment and in the surrounding areas (b). In the intact nerve, only scarce and weak immunostaining was observed (a). Scale bar (a,b) 25 µm.

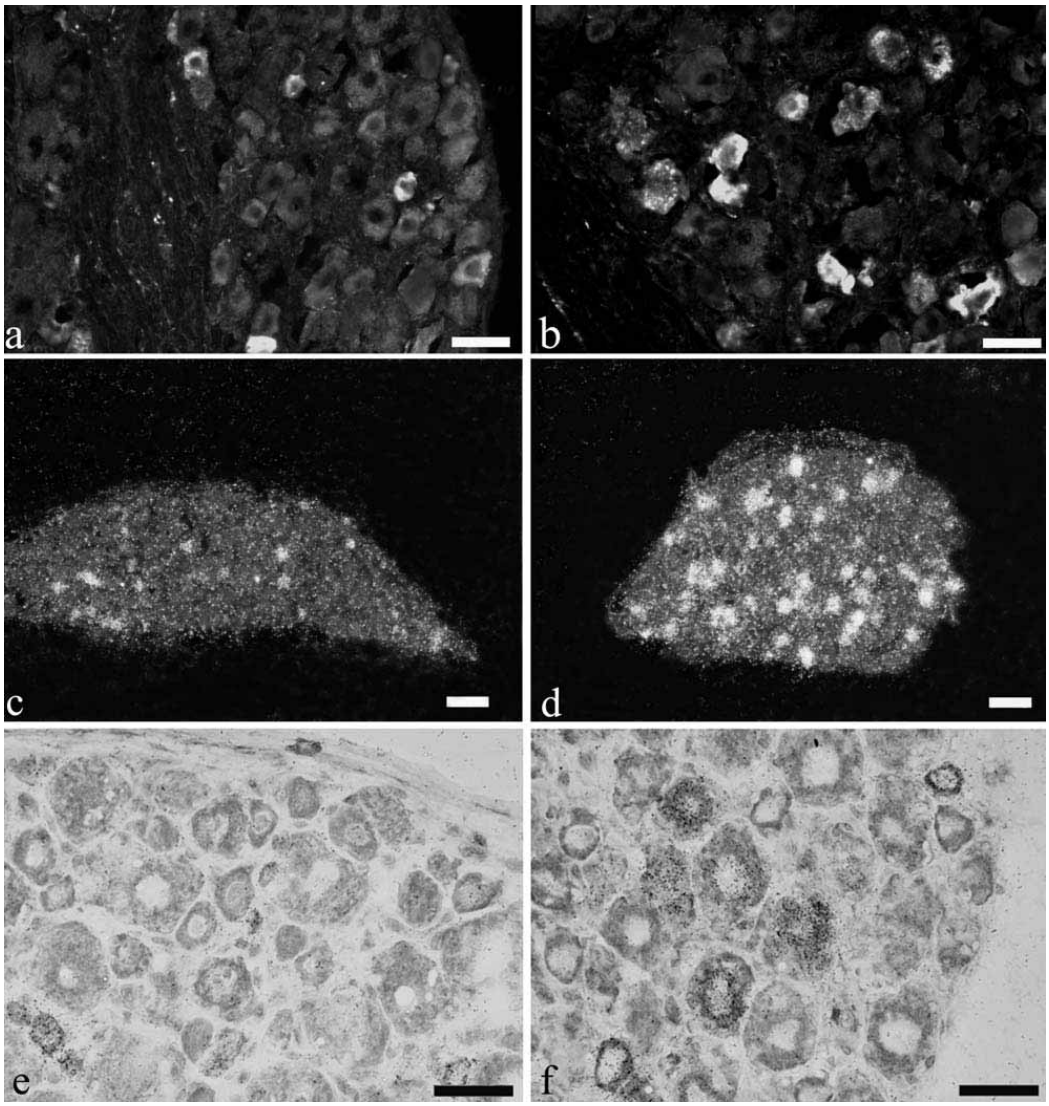

Fig. 3. Immunohistochemical staining of PACAP immunoreactivity (a,b) and in situ hybridization signal for PACAP mRNA (c,d,e,f) in rat DRG after unilateral sciatic nerve compression. An increased PACAP immunoreactivity was noted, most prominent in the ipsilateral DRG 7 days after nerve compression (b), as compared to the contralateral side (a). Dark field, low magnification pictures visualizing an upregulated PACAP mRNA expression in both small and large DRG neurons, here shown in response to 3 days of compression (d) and on the contralateral, uninjured side (c). High magnification, light field pictures visualizing the silver grain labeling of individual neruons shown in (c,d), where (e) is a close up of (c) and (f) is a close up of (d). Scale bar (a,b) 50 µm; (c,d) 100 µm; (e,f) 50 µm.

whereas after compression (3, 14 and 28 days) a significant increase in expression in both small and large diameter neurons was observed. Also, the proportion of neurons expressing PACAP mRNA at a very high level was similar in ipsilateral DRG neurons at all time points investigated.

The qualitative examinations of the DRG sections hybridized for detection of PAC_1 mRNA did not indicate that any changes in PAC_1 mRNA expression had taken place during nerve compression, and therefore, no further quantifications of these sections were made. Further, no obvious differences in the distribution pattern of either

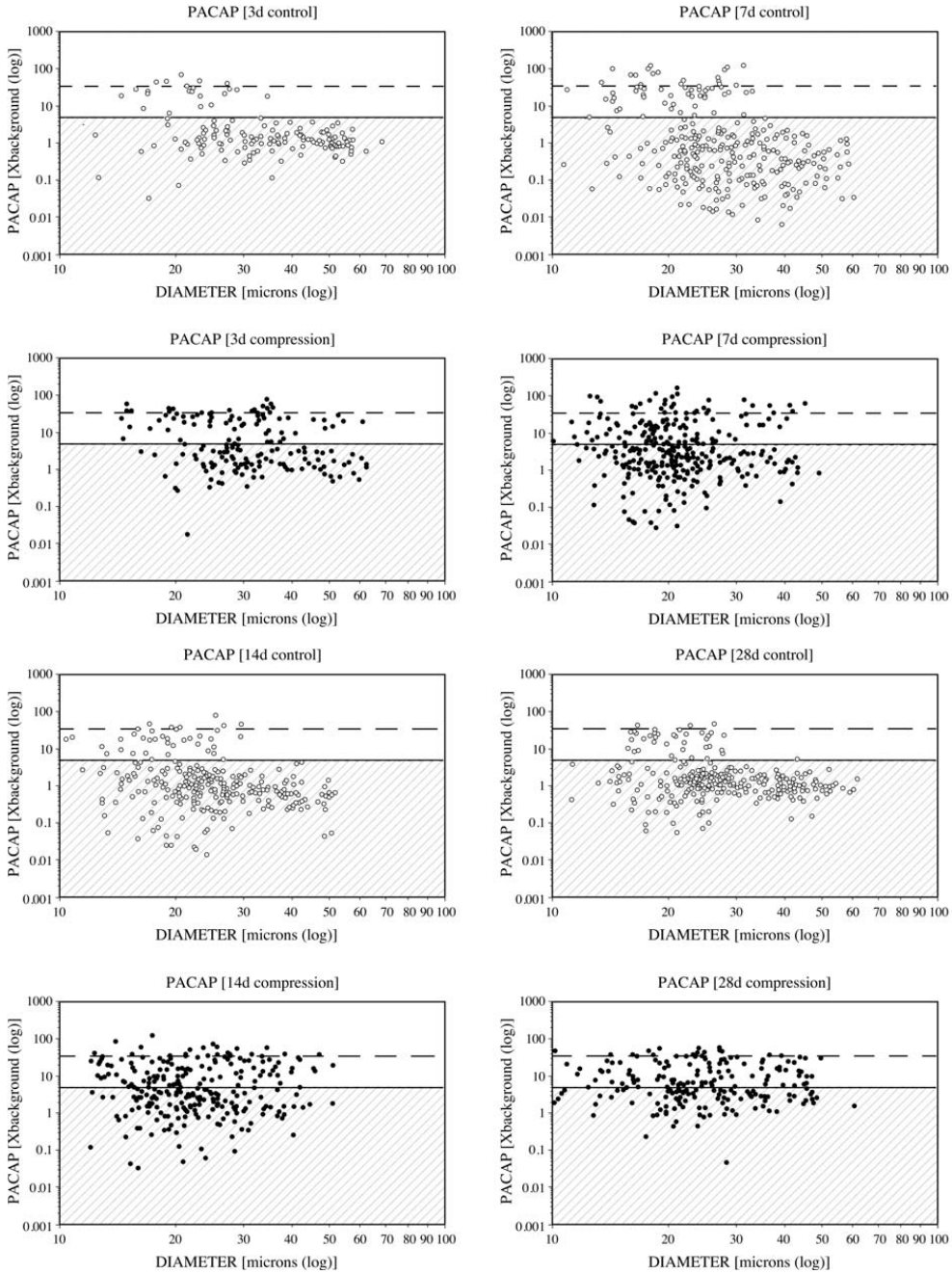

Fig.4. Scatterplot diagrams of the in situ hybridization labeling for PACAP mRNA density in relation to neuronal size in individual neuronal profiles in the ipsi- and contralateral L5 DRGs; 3 and 7 (a), and 14 and 28 (b) days after nerve compression. Each dot represents a quantified neuron, and neurons are considered labeled when the ratio of silver grain density over the neuronal cytoplasm is > 5 X the grain density over areas of the neuropil devoid of nerve cell bodies. Solid lines divide the plots into presumed labeled (> 5 X background labeling), and unlabeled (shaded) populations, whereas dashed lines separate heavily labeled (> 32 X background) from lightly and moderately labeled neuronal populations.

Table 1. PACAP mRNA expression in rat DRG neurons after 3, 7, 14, and 28 days of unilateral sciatic nerve compression; PACAP mRNA expression is correlated to the neuronal size

Sciatic nerve compression	Experimental state	Labeled cells (%)	Small cells (%) (<35μm)	Large cells (%) (>35 μm)
3 days	Control (L5)	15 (22/149)	15 (22/149)	0 (0/149)
	(L4)	16 (43/266)	–	–
	Compressed (L5)	43 (72/167)	33 (55/167)	10 (17/167)
	(L4)	48 (138/285)	–	–
7 days	Control (L5)	21 (62/291)	21 (61/291)	0 (1/291)
	(L4)	19 (59/317)	–	–
	Compressed (L5)	40 (121/305)	37 (112/305)	3 (9/305)
	(L4)	41 (122/294)	–	–
14 days	Control (L5)	14 (38/268)	14 (38/268)	0 (0/268)
	(L4)	14 (43/298)	–	–
	Compressed (L5)	46 (118/257)	39 (101/257)	7 (17/257)
	(L4)	43 (130/300)	–	–
28 days	Control (L5)	14 (38/278)	13 (37/278)	0 (1/278)
	(L4)	17 (45/269)	–	–
	Compressed (L5)	55 (114/208)	43 (89/208)	12 (25/208)
	(L4)	52 (142/273)	–	–

Summary of the data presented in Fig. 4 (DRG L5), and the data from the quantifications of the L4 DRG neurons. Total number of neurons (i.e. neuronal profiles) positive for PACAP mRNA expression (>5 X background) is noted in relation to the total number of neurons quantified in each ganglia (in parenthesis), as well as the proportion of neurons expressing PACAP mRNA (%). For the L5 ganglia, the proportions of small respectively large PACAP mRNA expressing neurons were also investigated. At all time points, a statistically significant increase in the number of neurons (both L4 and L5) expressing PACAP mRNA was found at the compressed side when compared with the control side (chi-square test, $p<0.05$).

PACAP mRNA or PAC_1 mRNA were observed when the the ipsi- and contralateral spinal cords were examined (not shown).

[^{125}I]-PACAP 27 binding

No obvious changes in [^{125}I]-PACAP 27 binding between ipsi- and contralateral sides of the spinal cord were observed at any of the time points investigated after nerve compression (not shown), i.e. the distribution pattern of [^{125}I]-PACAP 27 binding was essentially similar to that in earlier studies of intact rat spinal cords (Jongsma et al., 2000). Further, no induction of PACAP binding to its receptor was observed in the DRGs.

Discussion

The present study shows that experimental nerve compression induces an upregulation of PACAP expression in the number and density (expression level/neuron) of both small, and large sensory neuronal cell bodies in the DRG, as well as an induction of immunoreactivity for PACAP in the compressed nerve segment. However, no change in immunoreactivity was observed in the spinal cord. Alterations in PACAP expression have previously been shown in response to both complete sciatic nerve transection (Zhang et al., 1995; Zhang et al., 1996b; Jongsma Wallin et al., 2001) and peripheral inflammation (Zhang et al., 1998; Jongsma Wallin et al., 2003). Nerve transection induces an increase in PACAP expression (number of neurons and expression level/neuron) in medium-large sized DRG neurons (Jongsma Wallin et al., 2001). In contrast, the upregulation (number of neurons and expression level/neuron) associated with inflammation takes place primarily in small-medium sized neurons (Zhang et al., 1998; Jongsma Wallin et al., 2003). The present and previous studies indicate that there is an injury related expression of PACAP in cell bodies of differential sizes,

which is important information when discussing neuropeptide expression after various trauma.

Nerve compression is a frequently encountered condition, affecting around 4% of the Swedish adult, human population, (Atroshi et al., 1999) and this syndrome is sometimes associated with pain. The consequences of compression, on the nerve, are related to the magnitude and duration of the applied pressure (Rempel et al., 1999). Initially, compression induces an impaired intraneural microvascular circulation and formation of edema. An impediment or a complete block of the energy dependent axonal transport of e.g. structural proteins synthesised in the cell soma (anterograde transport) or trophic substances from the periphery (retrograde transport) occur in the compressed nerve (Dahlin and McLean, 1986; Dahlin et al., 1986b). Later, structural changes such as local myelin damage and axonal disintegration are induced (Rydevik et al., 1981; Lundborg and Dahlin, 1992). Biomechanical principles explain why larger fibers are more adversely affected by compression (large myelinated fibers) and ischaemia (thinner myelinated fibers) than are smaller fibers (e.g. non-myelinated C-fibers), as are fibers in superficial fascicles compared to fibers in the more deeply located fascicles. Furthermore, fascicles embedded in small amounts of connective tissue in the epineurium are more affected by compression than fascicles surrounded by a larger amount of insulating connective tissue (MacGregor et al., 1975; Battista and Alban, 1983; Dahlin et al., 1989; Lundborg and Dahlin, 1992).

The inhibition of axonal transport, caused by ischemia and/or mechanical injury during experimental nerve compression, may elicit pronounced cell body reactions (Dahlin and McLean, 1986; Dahlin et al., 1986a; Dahlin et al., 1987). Changes in neuropeptide expression in the nerve cell bodies have been observed. An increased immunoreactivity for both C-terminal flanking peptide of neuropeptide Y (CPON; primarily large size neurons) and galanin (primarily small size neurons) was found in DRGs following chronic nerve compression (Bergmark et al., 2001; Dahlin et al., 2003). In the present study we show that there is an increase in the number of both small and large size DRG neurons expressing PACAP mRNA after nerve compression. The number of small neurons that express PACAP mRNA in the ipsilateral ganglia is approximately 1.8 – 3.3 times the expression in the contralateral ganglia. The total number of large size neurons induced to express PACAP mRNA is not very high, however, the increase in expression in these neurons is quite impressive when compared to their normal PACAP mRNA expression (~0.1%). After nerve compression approximately 3–12% of the larger DRG neurons express PACAP mRNA on the ipsilateral side. The greatest number of neurons expressing PACAP mRNA was detected 28 days after surgery, indicating that there is still a pronounced upregulation at that timepoint, and suggesting that longer durations of nerve compression might lead to even greater changes in expression. In contrast, the increase in the number of sensory neurons immunoreactive for CPON during a 28 day period of chronic nerve compression, was transient (Bergmark et al., 2001). The intensity and number of PACAP immunoreactive neurons are also increased in DRGs in response to compression injury, indicating that the PACAP mRNA is translated into functional peptide in these neurons. No change in receptor mRNA expression or ligand binding in the ipsi- and contralateral DRGs was observed, indicating that there was no induction of receptor synthesis after compression. Furthermore, PAC_1 mRNA expression, as well as ligand binding to the spinal cord was virtually the same after compression (ipsi- and contralateral sides) as in intact spinal cords, suggesting that the increased amounts of PACAP expressed in the DRGs after compression executes is effect on PAC_1 receptors already present in the intact spinal cord.

Compression injuries may in some patients, although not all, be associated with pain, especially during severe nerve compression. PACAP has been suggested to be involved in the modulation of pain (Zhang et al., 1993a; Yamamoto and Tatsuno, 1995; Narita et al., 1996; Xu and Wiesenfeld-Hallin, 1996; Zhang et al., 1996a; Jongsma et al., 2001), and it is not unlikely that the increased PACAP expression in response to compression injury might affect the regulation of pain particularly since the level of PAC_1 expression was unchanged in the spinal cord dorsal horn during the compression period. This is in contrast to the reduction in ligand binding observed in the dorsal horn after nerve transection (Jongsma et al., 2000). The PACAP mRNA expression increased in relation to the duration of the compression, except for 7 days after surgery, when there was a small transient dip in upregulation. The deviation from the pattern in upregulation (7 days of compression), as well as the slight inconsistency in PACAP mRNA expressing neurons between DRG L5 and L4 (3 days of compression), might suggest that there is a period of transition somewhere between 3 and 7 days after application of the compression tube, where animals are more, or less affected by the surgery and tube application per se. These procedures may affect the expression and lead to a superimposed increase in PACAP mRNA expression initially, as well as a transient contralateral effect (see day 7), which in turn partly can mask an increased expression on the ipsilateral side when expression on both sides is compared. The increased contralateral expression at 7 days conceals the increased expression density/neuron when heavily labeled neuronal populations are compared at this timepoint. It seems as the deviation in the number of PACAP mRNA expressing neurons primarily is an effect of the increased expression in large neurons, who present a pronounced upregulation initially (3 days of compressi-

on), and then falls to a more modest PACAP mRNA expression (7 days of compression) which again slowly increases with time. As discussed above, large axons are most severely influenced by compression as a result of their greater diameter (Lundborg and Dahlin, 1992) and it might be this effect that is manifested initially after the surgery. The PACAP mRNA expression in small diameter neurons is steadily increased over time. Nerve compression is a complex condition where the nerve is exposed to both ischaemia and a certain degree of direct mechanical injury to the nerve fibers, depending on the pressure applied and its duration. In addition, an inflammatory component may be induced which might promote an increased PACAP mRNA expression in smaller neurons. This mixed condition may help to explain the dual effects we saw after the experimental nerve compression, i.e. an increased PACAP mRNA expression in both small and larger DRG neurons. The severe compression lesion inflicted in the present study induced a degeneration in the distal segment of the nerve, which elicits an inflammatory reaction with invasion of macrophages, giving evidence that there is an inflammatory component present with the possibility to contribute to the upregulation.

Previously, we have shown that PACAP mRNA expression is at least partly regulated by the neurotrophins, nerve growth factor (NGF), neurotrophin 3 (NT-3), and brain derived neurotrophic factor (BDNF) (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003; Pettersson et al., 2003). NT-3 negatively regulates PACAP in intact DRG neurons, and also mitigates the increased PACAP mRNA expression in large neurons after axotomy, wheras NGF appears to promote an upregulation of PACAP mRNA expression in intact and inflamed small DRG neurons. In addition, BDNF contributes to the upregulation in PACAP mRNA expression in response to axotomy (Pettersson et al., 2003). Nerve compression results in an impaired retrograde axonal transport (see above) and is therefore likely to induce changes in the expression of neuropeptides in DRG neurons, possibly via impairment of transport of e.g. trophic factors. In this study we have observed an upregulation in PACAP mRNA expression in both small and large size DRG neurons, which may be brought on by an interruption in axonal transport of NT–3 from cells in the periphery possibly leading to an upregulation of PACAP mRNA expression in large neurons, and a locally increased production of NGF and/or BDNF in response to the inflammation that may result in an increased expression of PACAP mRNA in small DRG neurons. An increased NGF and BDNF synthesis in the sciatic nerve and DRG respectively, has been observed after induction of peripheral tissue inflammation (Donnerer et al., 1992; Cho et al., 1997).

We observed a scarce PACAP immunoreactivity in fibers in the intact sciatic nerve. However, an increased immunostaining in the compressed segment and in the parts of the nerve bordering to that area indicates that there is an inhibition of axonally transported PACAP and/or an increased transport of PACAP from the DRG to the periphery. An increased PACAP immunoreactivity in the sciatic nerve has also been observed in response to sciatic nerve transection (Zhang et al., 1995; Zhang et al., 1996b). However, the role of PACAP in the compressed nerve segment, and in the periphery has yet to be clarified. It has been hypothesized that PACAP can act as a survival factor for axotomized neurons, and that this action in some cases might differ from that of the classical growth factors (Waschek, 2002). Furthermore, PACAP has been shown to exert an anti-inflammatory effect via inhibition of production of pro-inflammatory agents e.g. cytokines and nitric oxide (Martinez et al., 1998; Delgado et al., 1999a; Delgado et al., 1999c), and stimulation of production of anti-inflammatory cytokines (Delgado et al., 1999b) by activated macropaghes.

Taken together, our findings implicate that the neuropeptide PACAP is involved in the neuronal response to a peripheral nerve compression injury, and that the PACAP expression in sensory neurons of different size is dependent on the type of injury. Pain can be a prominent feature among patients with nerve compression lesions, and the findings of an increased PACAP expression in sensory neurons in response to nerve compression contribute to the understanding of the pathophysiology of these common lesions.

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Paper V

Endogenous BDNF regulates the expression of PACAP in rat DRG neurons after sciatic nerve injury

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Abstract

Previous studies have shown that nerve injury results in changes in the peptidergic phenotype of DRG neurons. The injury induced changes involve e.g. increased expression of neuropeptides presumed to be involved in regeneration and neuronal survival. We and others have previously shown that there is an upregulation in pituitary adenylate cyclase activating polypeptide (PACAP) expression in adult rat DRG neurons in response to both proximal and distal nerve injury and peripheral inflammation. We have also shown that this expression is modulated by NGF and NT–3. In this study we have investigated the effects of another neurotrophin, brain derived neurotorphic factor (BDNF), on PACAP mRNA expression in rat DRG after 3 days of sciatic spinal nerve (L4-L6) transection. Animals were given BDNF antibodies in conjunction with the injury, either intrathecally or intraperitoneally. Injured animals with or without additional administration of control antibody (sheep IgG) served as controls. DRGs were sectioned and hybridized with radiolabeled oligonucleotide probes, and the expression of PACAP mRNA was investigated and quantified with computer assisted image analysis. Our results demonstrate a significant downregulation in PACAP mRNA expression after intrathecal administration of BDNF antibodies, whereas the effects of intraperitoneal administration of BDNF antibodies were less clear cut. BDNF is upregulated in sensory neurons in response to nerve injury, which implies a possible role for BDNF in the modulation of the peptidergic phenotype in DRG neurons after injury. Our findings indicate that the endogenous injury-associated upregulation of BDNF is involved in the positive regulation of PACAP in response to nerve injury.

1. Introduction

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) is expressed in nerve cell bodies and fibers in both the central and the peripheral nervous systems, as well as in endocrine cells and tissues, and several further functions of the neuropeptide have been established; the peptide can act as a hormone, neurohormone, neurotransmitter or trophic factor, for an extensive review, see (Vaudry et al., 2000). In the spinal cord, PACAP immunoreactivity has been found in nerve fibers in the superficial laminae of the dorsal horn, dorso-laterally to the central canal (Moller et al., 1993; Zhang et al., 1995; Dun et al., 1996; Zhang et al., 1996; Jongsma et al., 2000; Vaudry et al., 2000), and also in fibers and neurons in the intermediolateral column (IML) (Dun et al., 1996; Hannibal, 2002). Expression of PACAP mRNA has been observed in cell somas primarily in the superficial layers of the dorsal horn, but also in some neurons around the central canal and in motorneurons in the ventral horn (Pettersson et al., 2004), as well as in neurons in the IML (Hannibal, 2002). In the intact state, roughly a fifth of the rat DRG neurons express mRNA or show immunoreactivity for PACAP, preferentially nerve cell bodies of smaller diameter (Moller et al., 1993; Mulder et al., 1994; Zhang et al., 1995; Dun et al., 1996; Zhang et al., 1996; Jongsma et al., 2000). However, the expression is very plastic and changes in response to nerve lesions or inflammation. After peripheral sciatic nerve transection, PACAP

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expression is upregulated in primarily medium-large diameter neurons (Zhang et al., 1995; Zhang et al., 1996; Jongsma et al., 2000), whereas an upregulation in both small and larger neurons is observed after both sciatic spinal nerve transection (Jongsma Wallin et al., 2001), and compression injury (Pettersson et al., unpublished findings).

Neurotrophins have been shown to affect the regulation of various neuropeptides (Verge et al., 1995; Woolf, 1996), and previous studies have established an effect of both NGF and NT-3 on the regulation of PACAP expression in DRG neurons (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003). NT-3 downregulates PACAP mRNA expression in intact DRG neurons and mitigates the increased expression in large neurons after proximal nerve transection, wheras NGF promotes an upregulation of PACAP mRNA expression in intact as well as in transected or inflamed small DRG neurons (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003). In the present study we wanted to examine the effects of another neurotrophin, brain derived neurotrophic factor (BDNF), on PACAP mRNA expression in rat DRG after 3 days of sciatic spinal nerve transection. BDNF is a member of the neurotrophin gene family of neurotrophic factors consisting of proteins with similar sequences (NGF, BDNF, NT-4/5, and NT-3). BDNF acts through the trkB receptor, is normally expressed in ~30-50% of the DRG neurons, mostly small diameter (Ernfors et al., 1990; Wetmore and Olson, 1995; Apfel et al., 1996; Michael et al., 1999), and is upregulated in response to nerve transection, inflammation, or NGF treatment (Karchewski et al., 1995; Apfel et al., 1996; Cho et al., 1997a; Cho et al., 1997b; Thompson et al., 1999; Karchewski et al., 2002). The pattern of BDNF mRNA expression in response to injury and inflammation is similar to that of PACAP.

2. Materials and methods

2.1. Experimental animals, models and tissue processing

All animal procedures were conducted in accordance with the NIH policy on the use of animals in research and the University of Saskatchewan animal care committee guidelines (protocol #19920164).

Male Wistar rats (~300 g) were anaesthetized with sodium pentobarbital (Somnitol®, 65 mg/kg, MTC Pharm, Canada), and all animals were exposed to sciatic spinal nerve transection; the sciatic nerve was transected unilaterally, at its origin from the L4 and L5 spinal nerves, and a 5 mm segment was resected to prevent regeneration. In one experimental group, animals were given either sheep anti-BDNF IgG (n = 3; anti-BDNF, Chemicon International, Temecula, CA, USA) or control IgG (n = 3; control IgG, Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally (i.p.). The systemic injections (1 mg/ml) of 0.75 ml anti-BDNF (in sterile distilled water) or sheep control IgG (in a 150 mM sodium saline solution) were given 1 hour before, and 24 and 48 hours after the injury. In another experimental group, continuous intrathecal infusions of anti-BDNF (1.5 μg/μl in sterile distilled water; n = 4) or control IgG (1.5 μg/μl in a 150 mM sodium saline solution; n = 4) were administered at 1.5 μg/μl/hour for 3 days, via mini-osmotic pumps (Alzet® model 2001, Durect Corporation, Cupertino, CA, USA). Immediately after nerve transection the pump was inserted into the dorsal lumbar subcutaneous space and attached to a silicone tubing (0.3 mm outer diameter) that was passed through the dura and arachnoid at the lumbo-sacral junction and ran cranially along the spinal cord for approximately 1.5 cm, delivering the antibody solution at the level where the L4-L6 spinal nerves connect to the spinal cord. The pump contents and the silicone tubing were handled with care as to assure the sterility. Animals treated with control IgG were used as controls and compared with anti-BDNF treated animals. All animals were given buprenorphine (Temgesic; 0.05 mg/kg) to relieve any pre- and postoperative discomfort. Three days after surgery, animals were deeply anaesthetized and perfused with cold phosphate buffered saline (PBS, 0.1M), to clear away the blood, and 4% PF for tissue fixation. Dissected tissues (lumbar spinal cord, and L5 DRGs) were postfixed in 4% PF (1-1.5 hours), repeatedly rinsed in 20% sucrose, and cryoprotected in 20% sucrose (overnight). Tissues were placed in cryomolds, embedded and stored at -80°C.

Sections were analyzed and photographed with an Olympus BX-60 microscope connected to an Olympus DP-50 digital camera. Brightness and contrast were adjusted with the software Adobe Photoshop® 5.0.

2.2. In situ hybridization

Radiolabeled (^{35}S) oligodeoxyribonucleotide (oligoDNA) probes for detection of PACAP mRNA, and the mRNA for the high affinity receptor for BDNF, tyrosine kinase B (trkB), were used for in situ hybridization. OligoDNA probes complementary to and selective for PACAP mRNA -nucleotides 700-747 (Hurley et al., 1995), and trkB mRNA -nucleotides 1361-1408 (Meakin et al., 1992), were synthesized and purified (University of Calgary DNA services, Alberta, Canada). All cDNA regions used were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm.nih.gov, October 2003); no greater than 60% homology was found to sequences other than the cognate transcript. Labeling of probes (at a concentration of 3.1 ng probe/μl), with ^{35}S–dATP (39%, Perkin Elmer) and terminal transferase enzyme (11.8%; Amersham, Canada) was performed in a terminal transferase buffer, containing sodium cacaodylate 500 mM, $CoCl_2$ (pH 7.2), 10 mM, mercaptoethanol 1 mM, for 1.5-

2 hours at 37°C. The reaction was stalled by adding 500 µl, 0.1 M Tris Cl (pH 8.0), and probes were purified through NENSORB-20 columns (New England Nuclear, USA), and dithiotthreitol added to a final concentration of 10 mM. The activity was measured to approximately 0.27-0.28 x 10^9 cpm/ml, and probes were stored at –18°C.

To prevent degradation of RNA by external RNAses, all steps prior to hybridization were performed under RNase free conditions, and all dilutions were performed in autoclaved double distilled water. The perfused tissues were sectioned (6 µm) and slides were air-dried and post-fixed in 4% PF (20 minutes), washed in PBS (3 x 5 minutes), treated with proteinase K at 37°C (20 µg/ml; 7-8 minutes), rinsed in PBS (5 minutes), fixed in 4% PF (5 minutes), rinsed in PBS (2 x 5 minutes), rinsed in diethyl pyrocarbonate (0.1%) -H_2O (5 minutes), and dehydrated in increasing ethanol concentrations (70%, 90%, 100%; approximately 1 minute in each). Sections were hybridized with radiolabeled probes at a concentration of 10^7 cpm/ml in a hybridization solution consisting of 50% formamide, 4 x saline sodium citrate (SSC), 1 x Denhardt´s solution (0.02% bovine serum albumin (BSA), 0.02% Ficoll and 0.02% polyvinylpyrrolidone), 10% dextran sulphate, 0.5 mg/ml salmon sperm DNA, 1% sarcosyl and 0.2 M DTT. Hybridization, with approximately 100 µl hybridization solution/slide, was conducted over night at 43°C in air sealed moist chambers to prevent evaporation. Control slides were incubated with the addition of the corresponding unlabeled probe in 100-fold molar excess. Following hybridization, the slides were washed in 1 x SSC (4 x 15 minutes, 55°C, and an additional 30 minutes, room temperature), dipped twice in distilled water, dehydrated in ascending ethanols and air dried. Slides were immersed in Kodak NTB2 photoemulsion (diluted 1:1 in distilled water), to generate autoradiograms. After 4.5 weeks storage, with desiccant in light sealed containers at 4°C, the slides were developed in Kodak D19 (3-5 minutes), rinsed in tap water, fixed in Kodak rapid fix (5 minutes) and rinsed in tap water (20 minutes). For darkfield viewing and photography, slides were left unstained, whereas slides for brightfield examination were counterstained with 0.5 % toluidine blue (in an acetate buffer; pH 4-4.5), and mounted in Permount (Fisher, Canada).

2.3. Quantification and image analysis

To evaluate the effect of endogenous BDNF on the expression of PACAP mRNA in DRG neurons after axotomy all slides were first analyzed qualitatively. All sections that were to be compared were processed at the same time during identical conditions, to avoid variance in the overall signal between the slides. Relative changes in hybridization signal were noted for all sections mounted on the same slide. Changes in hybridization signal were examined for all sections, further, the quality of each section was noted. Representative slides (where all DRG sections contained similar numbers of neurons, and the sections reflected the overall labeling for DRGs exposed to the same conditions) were selected for quantitative analysis. In the first analysis, animals were examined after intrathecal infusions of antibodies (n = 4, 2 rats per treatment analyzed), and after i.p. injections (n = 2, 1 rat per treatment analyzed). Montages of photomicrographs (500 X) were made and neuronal profiles, individual neurons with a visible nucleus in at least one of the two adjacent sections, were identified and numbered. Thereafter, each neuron (approximately 410 neurons/montage, intrathecal infusions, and 495 neurons/ montage, i.p. injections) was examined directly in the microscope (60X). The total percentage of PACAP mRNA labeled neurons was determined on the ipsilatral side after the different treatments, but no measurements of neuronal diameter was made, i.e. no distinction of the distribution in relation to neuronal size, or changes in silver grain density/neuron was calculated in these early examinations. These first and somewhat less extensive analyses were performed to establish the effects of BDNF on PACAP mRNA expression in spinal cord. An effect was observed, and therefore, further extensive analyses were made.

In the further studies, additional animals (intrathecal infusions of antibodies) were analysed; slides were examined qualitatively and representative slides were selected according to the criteria above. Montages were made, neurons were identified and numbered, and each individual neuron was examined. The relative changes in hybridization signal were determined with the use of computer assisted image analysis on adjacent sections processed for in situ hybridization to detect PACAP mRNA (n = 4 animals, 2 rats per treatment analyzed, 4 DRG sections where approximately 900 neuronal DRG L5 profiles were examined), and trkB mRNA (sections adjacent to the ones analyzed for PACAP (n = 2 animals, 2 DRG sections, approximately 460 neuronal DRG L5 profiles). Montages were prepared from pairs of adjacent sections of ipsilateral DRGs hybridized for detection of PACAP and trkB mRNA, respectively. This was made both for ganglia from animals infused with anti-BDNF and ganglia infused with control IgG. Neuronal profiles were identified and numbered (approximately 240 neurons/montage), and light microscopy images (60X) were collected from each individual neuron using an Olympus DP-50 digital camera. Cross sectional areas of every single neuronal profile and the percentage of the cytoplasmic area covered by silver grains were measured for all the neurons in all the ganglia, using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health, and available from the internet by anonymous FTP from zippy.nimh.nih.gov). Individual cell diameters were calculated from the cross sectional areas

on the assumption that the neurons were spherical. Neurons were defined as small (diameter < 35 µm), and large (diameter > 35 µm) (Giuffrida and Rustioni, 1992). The density threshold was adjusted interactively for each image, so that the area per grain was constant for all neurons analyzed on a given slide. Neurons were considered labeled for PACAP and trkB mRNA if they contained more than six times background labeling of silver grains. The background was determined from measurements of the silver grain density over five defined areas of the neuropil devoid of positively labeled cell bodies. Background was measured and determined specifically for each quantified section.

Differences in the number of PACAP or trkB mRNA expressing neurons between the control IgG treated and anti-BDNF treated DRGs, were analyzed using the chi-square test, and $p<0.05$ was considered significant.

3. Results

3.1. Spinal sciatic nerve transection in conjunction with intrathecal infusions of sheep anti-BDNF IgG or sheep control IgG

Examination of the slides showed that expression of PACAP mRNA was increased 3 days after sciatic spinal nerve transection as well as after infusion of control IgG in conjunction with the nerve injury (Fig. 1a). A reduced upregulation of expression after injury was observed when animals were infused with anti-BDNF (Fig. 1b).

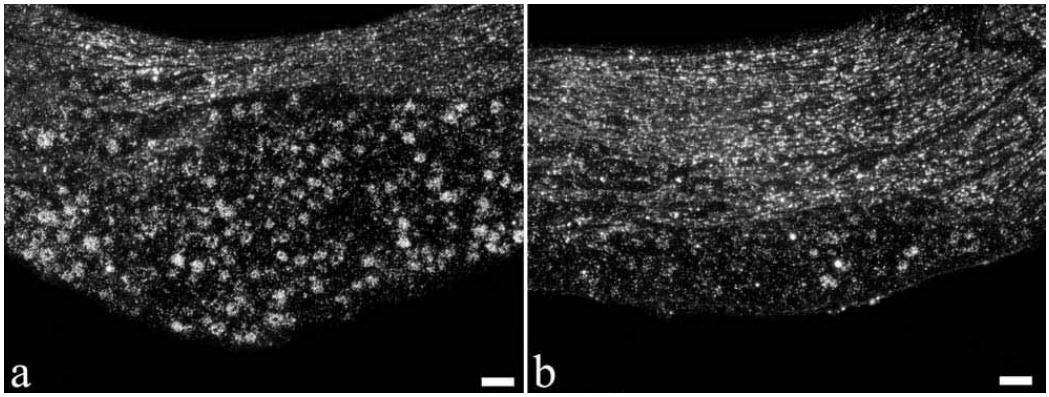

Figure 1. Darkfield images visualizing the induction of PACAP mRNA expression in DRG neurons after spinal sciatic nerve transection and 3 days of intrathecal control IgG (a) or anti-BDNF (b) infusions. The increase in PACAP mRNA expression observed after nerve transection is mitigated by anti-BDNF infusion. Scale bar 100 µm.

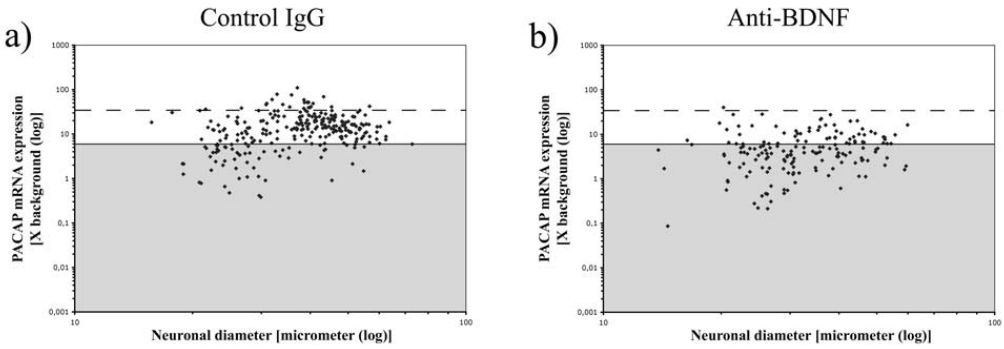

Figure 2. Scatterplot diagrams of the in situ hybridization labeling for PACAP mRNA density in relation to neuronal size in individual neuronal profiles in DRG neurons after nerve injury and intrathecal control IgG (a), or anti-BDNF (b) infusion, respectively. Each dot represents a quantified neuron, and neurons are considered labeled when the ratio of silver grain density over the neuronal cytoplasm is >6 X the grain density over areas of the neuropil devoid of positively labeled cell bodies. Solid lines divide the plots into presumed labeled (>6 X background labeling) and unlabeled (shaded) populations, whereas dashed lines separate lightly and moderately labeled (<32 X background) populations from heavily labeled neuronal populations (>32 X background). A decreased number of neurons expressing PACAP mRNA, as well as a relative decreased expression level/neuron was observed with anti-BDNF treatment as compared to control IgG treatment.

Sciatic nerve transection (3 days)	Experimental state	Labeled cells (%)	Small cells (%) (<35μm)	Large cells (%) (>35 μm)
PACAP	Control IgG (ith)	60 (815/1369)	27 (132/482)	36 (172/482)
	Anti-BDNF (ith)	35 (409/1168)	15 (61/416)	8.7 (36/416)
Trk B	Control IgG (ith)	11 (24/214)	3.7 (8/214)	7.5 (16/214)
	Anti-BDNF (ith)	10 (26/249)	3.6 (9/249)	6.8 (17/249)
PACAP	Control IgG (i.p.)	65 (301/460)	-	-
	Anti-BDNF (i.p.)	67 (356/530)	-	-

Numbers and percentages of DRG neurons expressing detectable PACAP or trkB mRNA after axotomy and intrathecal or i.p. antibody treatments. PACAP or trkB mRNA expressing neurons/total number of neurons counted are indicated in parenthesis for each condition stated. Control IgG and anti-BDNF refers to DRGs treated with sheep control IgG or sheep anti-BDNF IgG, respectively, and ith and i.p. refers to intrathecal or intraperitoneal routes of antibody administration. The increase in PACAP mRNA expression after axotomy (small and large diameter DRG neurons) is mitigated by intrathecal anti-BDNF infusion, and a significant decrease is observed in both small and large diameter DRG neurons. A statistically significant difference, in the number of neurons was found between the control IgG treated DRGs and the DRGs treated with anti-BDNF (chi-square test). No obvious change in trkB mRNA expression after intrathecal anti-BDNF infusion, or in PACAP mRNA expression after i.p. administration of anti-BDNF was observed, when these animals were compared to control IgG treated animals.

Quantitative examinations showed that after infusion of control IgG during nerve transection the expression of PACAP mRNA was increased in DRG L5 neurons and approximately 63% of the neurons were estimated to express PACAP mRNA (Fig. 2a and Table 1), compare with the expression on the uninjured side where approximately one fifth of the DRG neurons express PACAP mRNA (Zhang et al., 1996; Jongsma Wallin et al., 2001). In contrast, treatment with antibodies against BDNF resulted in a mitigation of the injury induced upregulation in PACAP mRNA expression. Approximately 23% of the neurons expressed PACAP mRNA after inactivation of endogenous BDNF, and the reduction in expression was observed in both small and large diameter DRG neurons (Fig. 2b and Table 1). Further, a decreased level of PACAP mRNA expression/neuron was found. Less neurons were expressing PACAP mRNA at high levels (>32x background) after anti-BDNF infusion (Fig. 2), i.e. a significant difference in both the number of neurons, as well as the level of PACAP mRNA expression/neuron, was established when anti-BDNF treated DRGs and DRGs treated with control IgG where compared (chi-square test).

The reduction in PACAP mRNA expression was observed primarily in neurons expressing trkB mRNA, although a reduction was found also in non-trkB expressing neurons. No statistically significant change in the number

Experimental state	Labeled cells (%) colocalisation PACAP & trkB	Labeled cells (%) PACAP positive (non trkB)
Control IgG (ith)	7.5 (16/214)	38 (82/214)
Anti-BDNF (ith)	0.4 (1/249)	14 (35/249)

Correlation of PACAP and trkB mRNA expression in DRG neurons after sciatic spinal nerve transection and additional antibody infusions. The reduction in PACAP mRNA upregulation effected by anti-BDNF infusion takes place both in trkB mRNA expressing neurons, and neurons not obviously expressing trkB mRNA.

of trkB mRNA expressing neurons or percentages of small vs large neurons expressing trkB mRNA was observed when anti-BDNF and control IgG treated animals were compared (Table 2).

3.1. Spinal sciatic nerve transection in conjunction with intraperitoneal injections of sheep anti-BDNF IgG or sheep control IgG

The expression of PACAP mRNA was increased also in animals subjected to spinal sciatic nerve transection and i.p. injections of sheep control IgG, where 65% of the DRG neurons express PACAP mRNA, as compared to the much lower PACAP mRNA expression in intact animals (see above). However, after i.p. injections of antibody against BDNF, around 67% of the neurons still expressed PACAP mRNA. Since the i.p. injections of anti-BDNF after nerve transection did not have any obvious effect on PACAP mRNA expression, further examinations of these tissues were not performed.

4. Discussion

Brain derived neurotrophic factor (BDNF) is normally expressed in small, trkA positive sensory neurons in the DRG (Ernfors et al., 1990; Wetmore and Olson, 1995; Apfel et al., 1996). After axotomy, BDNF expression is down-regulated in a subpopulation of small neurons and up-regulated, primarily in medium-large diameter (trkB and trkC positive) DRG neurons (Michael et al., 1999; Zhou et al., 1999). This injury induced upregulation in BDNF expression suggests a role for BDNF in modulation of the injury response. Since we previously have observed that the regulation of PACAP is very plastic and changes in response to axotomy and inflammation, and that it at least in part is regulated by other neurotrophins such as NGF and NT-3, we wanted to examine whether upregulation in BDNF in conjunction with nerve transection also affects the expression of PACAP. Furthermore, BDNF and PACAP expression is elevated in similar populations of DRG neurons in response to injury and inflammation, indicating a possible relation (Thompson et al., 1999; Jongsma Wallin, 2002).

On the intact side, PACAP is expressed in roughly one fifth of the DRG neurons, predominantly of small diameter (Zhang et al., 1996; Jongsma Wallin et al., 2001), whereas an increase in expression is observed after nerve transection. We see an increased PACAP mRNA expression in both small and large DRG neurons after nerve transection with and without control IgG treatment. The increase is mitigated by anti-BDNF infusion, resulting in decreased expression levels where approximately half of the neurons, that where induced to express PACAP mRNA after injury and control IgG infusion, express PACAP mRNA after anti-BDNF treatment. The reduction takes place in both small and large diameter DRG neurons. Furthermore, the reduction in expression is observed both in trkB and non-trkB expressing neurons. This suggests that the effect of endogenous BDNF on the upregulation in PACAP mRNA expression after axotomy is not exclusively a direct effect on neurons expressing detectable levels of the BDNF receptor, trkB. It might also be an effect of BDNF signalling via trkB expressing neurons or non-neuronal cells that in turn regulate the PACAP mRNA expression in non-trkB neurons through other mechanisms, e.g. upregulation of cytokines and growth factors. Alternatively, BDNF may effect more global responses via the common neurotrophin receptor, p75, which is expressed by ~80% of the DRG neurons, and in other cell types (Karchewski et al., 1999), and has been shown to effect signalling in response to BDNF.

The upregulation in BDNF expression in response to axotomy (Tonra et al., 1998; Michael et al., 1999; Thompson et al., 1999; Zhou et al., 1999), might be regulated partly by a reduction in retrograde supply of NT-3 to the DRG from the periphery. This is supported by findings that infusion of NT-3 have a negative effect on BDNF expression in intact and injured DRG neurons (Karchewski et al., 2002). An effect of NGF has also been observed on the regulation of BDNF expression in conjunction with nerve injury (Verge et al., 1996; Shadiack et al., 2001; Karchewski et al., 2002) and inflammation (Cho et al., 1997b; Thompson et al., 1999), and it might be that the effects of NGF and NT-3 on the regulation of PACAP expression are mediated at least in part via BDNF.

The reason that we did not see any effects on PACAP mRNA expression after i.p. injections of anti-BDNF, may be either that the systemic dose was not sufficient to neutralize the endogenous BDNF, or as opposed to after the intrathecal infusions where we were able to effectively target the central sources that influence the sensory neuron function after injury, the systemic injections will not influence central sources as effectively. This suggests that the primary endogenous source of BDNF in regulation of PACAP mRNA expression after nerve injury would be central, either from the spinal cord, or more likely from the DRG neurons where an upregulation in BDNF mRNA expression takes place in response to injury (Tonra et al., 1998; Michael et al., 1999; Zhou et al., 1999).

We have demonstrated an increase in PACAP mRNA expression in rat DRG neurons in response to sciatic spinal nerve transection. Furthermore, BDNF is upregulated in sensory neurons in response to nerve injury, which implies a possible role for BDNF in the modulation of the peptidergic phenotype in DRG neurons after axotomy. Our findings indicate that the endogenous injury-associated upregulation of BDNF is involved in the positive regulation of PACAP in response to nerve injury, and that this regulation may take place in both trkB and non-trkB expressing neurons

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