The Impact of the Neuropeptide Substance P (SP) Fragment SP\textsubscript{1-7} on Chronic Neuropathic Pain

ANNA JONSSON
Abstract

There is an unmet medical need for the efficient treatment of neuropathic pain, a condition that affects approximately 10% of the population worldwide. Current therapies need to be improved due to the associated side effects and lack of response in many patients. Moreover, neuropathic pain causes great suffering to patients and puts an economical burden on society.

The work presented in this thesis addresses SP$_{1-7}$, (Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH), a major metabolite of the pronociceptive neuropeptide Substance P (SP). SP is released in the spinal cord following a noxious stimulus and binds to the NK$_1$ receptor. In contrast to SP, the degradation fragment SP$_{1-7}$ is antinociceptive through binding to specific binding sites distinct from the NK$_1$ receptor.

The aim of this thesis was to investigate the impact of SP$_{1-7}$ on neuropathic pain. To understand how SP$_{1-7}$ exerts its effect, a series of N-truncated forms of the heptapeptide were biologically evaluated. A set of small high-affinity ligands was evaluated in animal models of neuropathic pain. To confirm a clinical relevance the levels of SP$_{1-7}$ in human neuropathic pain were assessed in cerebrospinal fluid (CSF) collected from neuropathic pain patients.

The results showed that SP$_{1-7}$ could alleviate thermal as well as mechanical hypersensitivity in three different animal models of neuropathic pain. C-terminal amidation was connected with increased efficacy. N-terminal truncation of SP$_{1-7}$ indicated a necessity of five amino acids in order to retain biological effect. One small high-affinity ligand showed a significant anti-allodynic effect. CSF levels of SP$_{1-7}$ in neuropathic pain patients were lower compared to controls. Taken together, these findings demonstrate that the formation of SP$_{1-7}$ may be attenuated in neuropathic pain. C-terminal amidation and a majority of its amino acids are necessary for stability and permeability. Clearly, SP$_{1-7}$ and SP$_{1-7}$ mimetics with high affinity to the SP$_{1-7}$ binding site ameliorate neuropathic pain-like behaviors in animal models of neuropathic pain. Overall, the findings presented in this thesis contribute to new knowledge regarding the role of SP$_{1-7}$ and related analogues and fragments in neuropathic pain. In a future perspective, this could be essential for the development of efficient strategies for managing patients with neuropathic pain.

Keywords: neuropathic pain; substance P (SP); SP$_{1-7}$; bioactive fragments; spared nerve injury; spinal cord injury; streptozotocin-induced diabetes; allodynia; hyperalgesia; peptidomimetics; cerebrospinal fluid; spinal cord stimulation, radioimmunoassay

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

(+)-PTZ  (+)-pentazocine
ACE  Angiotensin converting enzyme
Arg; R  Arginine
CGRP  Calcitonin gene-related peptide
CNS  Central nervous system
CSF  Cerebrospinal fluid
DAG  diacyl glycerol
DAMGO  Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol
DP-IV  Dipeptidylpeptidase-IV
EM  Endomorphin
Gln; Q  Glutamine
Gly; G  Glycine
HIV  Human immunodeficiency virus
i.p.  Intraperitoneal
i.t.  Intrathecal
IASP  The International Association for the Study of Pain
IP₃  Inositol triphosphate
Kᵢ  Equilibrium dissociation constant for inhibitor binding
Leu; L  Leucine
Lys; K  Lysine
MAD  Median absolute deviation
Met; M  Methionine
MPE  Maximum possible effect
N/OFQ  Nociceptin/orphanin FQ
NAcc  Nucleus Accumbens
NEP  Neutral endopeptidase
NK  Neurokinin
NLX  Naloxone
NMDA  N-Methyl-D-aspartate
nNOS  Neutral nitric oxide synthase
Nor-BNI  Nor-binaltorphimine
NTI  Naltrindole
PAG  Periaqueductal Gray
Phe; F  Phenylalanine
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<th>Abbreviation</th>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
<td>Phospholipase C</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PPCE</td>
<td>Post-proline cleaving enzyme</td>
<td>Post-proline cleaving enzyme</td>
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<tr>
<td>PPT-A</td>
<td>Preprotachykinin-A</td>
<td>Preprotachykinin-A</td>
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<tr>
<td>Pro; P</td>
<td>Proline</td>
<td>Proline</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
<td>Radioimmunoassay</td>
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<tr>
<td>RVM</td>
<td>Rostroventral medulla</td>
<td>Rostroventral medulla</td>
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<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
<td>Spinal cord injury</td>
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<td>SCS</td>
<td>Spinal cord stimulation</td>
<td>Spinal cord stimulation</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sigma-1</td>
<td>Sigma non-opioid intracellular receptor 1</td>
<td>Sigma non-opioid intracellular receptor 1</td>
</tr>
<tr>
<td>SNI</td>
<td>Spared nerve injury</td>
<td>Spared nerve injury</td>
</tr>
<tr>
<td>SNRI</td>
<td>Serotonin-noradrenaline reuptake inhibitor</td>
<td>Serotonin-noradrenaline reuptake inhibitor</td>
</tr>
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<td>SP</td>
<td>Substance P</td>
<td>Substance P</td>
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<td>SPE</td>
<td>Substance P endopeptidase</td>
<td>Substance P endopeptidase</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
<td>Streptozotocin</td>
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<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
<td>Tricyclic antidepressant</td>
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<tr>
<td>VAS</td>
<td>Visula analog scale</td>
<td>Visula analog scale</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
<td>Ventral tegmental area</td>
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<tr>
<td>β-FNA</td>
<td>β-funaltrexamine</td>
<td>β-funaltrexamine</td>
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Introduction

Pain is a subjective experience. The ability to feel pain is essential and pain plays a crucial role in protecting the body from potential harm and tissue damage. However, acute pain can become chronic and chronic pain is a major health problem that affects a large number of people worldwide. The underlying mechanisms behind chronic pain are not fully clarified and effective pharmacological treatments are lacking. Thus, further knowledge regarding the mechanisms behind chronic pain is necessary in order to develop new pharmacological therapies.

Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Pain has an emotional as well as a psychological component and cannot be objectively measured. Some pain related terms of relevance for this thesis are defined in Table 1.

Table 1. Definition of pain terms as described in the latest version (2011) by the International Association for the Study of Pain (IASP).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Pain</td>
<td>An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage</td>
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<td>Allodynia</td>
<td>Pain due to a stimulus that does not normally provoke pain</td>
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<tr>
<td>Analgesia</td>
<td>Absence of pain in response to stimulation that would normally be painful</td>
</tr>
<tr>
<td>Central pain</td>
<td>Pain initiated or caused by a primary lesion or dysfunction in the central nervous system</td>
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<tr>
<td>Hyperalgesia</td>
<td>Increased pain from a stimulus that normally provokes pain</td>
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<tr>
<td>Neuropathic pain</td>
<td>Pain caused by a lesion or disease of the somatosensory nervous system</td>
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<tr>
<td>Neuropathy</td>
<td>A disturbance of function or pathological change in a nerve: in one nerve, mononeuropathy; in several nerves, mononeuropathy multiplex; if diffuse and bilateral, polyneuropathy</td>
</tr>
<tr>
<td>Central neuropathic pain</td>
<td>Pain caused by a lesion or disease of the central somatosensory nervous system</td>
</tr>
<tr>
<td>Peripheral neuropathic pain</td>
<td>Pain caused by a lesion or disease of the peripheral somatosensory nervous system</td>
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</table>
Chronic pain

Chronic pain is often defined as pain lasting more than three months and is a common health problem with a prevalence ranging from 12 to 30% in the general population (Breivik et al., 2006; Harker et al., 2012; Moulin et al., 2002; Schopflocher et al., 2011). In Sweden, 20% of the population are reported to suffer from chronic pain (SBU, 2006). Once the pain has become chronic, it is difficult to manage. The probability to recover from chronic pain is small and in a majority of the patients the pain is indeed persisting (Andersson, 2004; Breivik et al., 2006). Chronic pain is now recognized as a major health problem in Europe as it is connected to under-treatment, unsatisfactory treatments that cause severe side effects and a reduced quality of life (Breivik et al., 2006). Pain is the most common reasons for people in Sweden to seek health care and the total public costs related to pain are estimated to be 9 billion EUR in 2003 (SBU, 2006).

Chronic pain is a heterogenic condition, and can be of nociceptive and neuropathic character. Common types of chronic pain with mainly nociceptive characteristics include musculoskeletal pain, rheumatoid arthritis, and joint pain. One kind of pain that is common is low-back pain, which can consist of both nociceptive and neuropathic components (Morlion, 2011).

Neuropathic pain is defined by IASP as pain that originates from a lesion or disease of the somatosensory nervous system. It can originate from a trauma on the nervous system or as a consequence of disease. The prevalence of neuropathic pain in the general population is estimated to be 7 – 10% (van Hecke et al., 2014) and is thus a common clinical problem. It is characterized by spontaneous pain, as well as allodynia and hyperalgesia (Woolf and Mannion, 1999) and a majority of neuropathic pain patients report a moderate to severe degree of pain (Perez et al., 2013). Neuropathic pain can be divided into central and peripheral neuropathic pain according to its origin, however, the presence of mixed neuropathic pain is not unusual (Perez et al., 2013).

Peripheral neuropathic pain

Peripheral neuropathic pain is pain caused by a lesion or disease of the peripheral somatosensory nervous system. The causes underlying peripheral neuropathic pain are diverse and include post-surgical pain, ranging from painful scars to amputation stump pain, and a wide range of pathologies such as postherpetic neuralgia, human immunodeficiency virus (HIV) and diabetes (Scadding and Koltzenburg, 2006).
Central neuropathic pain

Central pain can arise from a lesion or dysfunction in the central nervous system (CNS). It can be caused by many different kinds of lesions in the brain or spinal cord, such as multiple sclerosis or spinal cord injury (SCI). Central neuropathic pain, usually manifested at or below the site of injury (Rekand et al., 2012), causes suffering because of its constant and irritating character (Boivie, 2006). Chronic pain occurs in 26–96% of the SCI patients (Dijkers et al., 2009) and can be both nociceptive and neuropathic.

Pain transmission

Pain signaling starts when nociceptors detect a noxious stimulus. The nociceptors, i.e. primary afferents, are free nerve endings, which are found throughout the skin and internal organs, and are divided into Aδ and C fibers. Aδ fibers are thinly myelinated fibers with high velocity and thus give rise to the “first pain”, a sharp pain that is precisely localized. The Aδ fibers respond to mechanical and thermal stimuli. C-fibers are unmyelinated fibers, and are thus more slow-conducting compared to Aδ fibers. C fibers are polymodal, meaning they respond to a variety of nociceptive stimuli, and they are responsible for transmission of the “second pain”, which can be described as burning, dull and long-lasting, and is poorly localized (Markenson, 1996). In addition, there are myelinated Aβ fibers that are large and rapidly respond to non-nociceptive mechanical stimuli (Basbaum et al., 2009).

The primary afferents are pseudo-unipolar neurons with a cell body in the dorsal root ganglion with axons that terminate in the periphery and in the dorsal horn of the spinal cord. In the spinal cord, the termination of the free nerve endings is highly organized. C-fibers terminate in lamina I and II whereas Aδ-fibers terminate in laminae I and V (Basbaum et al., 2009). The primary afferents synapse onto neurons that project from the spinal cord to the brain, where they terminate in several supraspinal sites. There are two main ascending pathways that project to the brain, the spinothalamic and the parabrachial pathway (Hunt and Mantyh, 2001). The spinothalamic tract neurons project to the thalamus, from where information is relayed to the cortex. This pathway is responsible for the discriminative and some affective components of pain. The parabrachial pathway mainly projects to the amygdala and hypothalamus, which mediate affective components of pain (Hunt and Mantyh, 2001; Woolf, 2004).

Descending pathways are responsible for pain modulating and project from the brain to the spinal cord. The descending neurons mainly originate from the cortex, amygdala and the hypothalamus and project to the peria-
queductal gray (PAG), from which neurons project to the rostroventral me-
dulla (RVM) and subsequently down to the spinal cord (Fields, 2004).

The PAG and RVM are two important relay structures with a crucial role in descending pain modulation. In these structures, two distinct types of cells, ON-cells and OFF cells, project to the dorsal horn of the spinal cord. Activation of ON-cells contributes to enhanced nociception (enhanced response to noxious stimulation) whereas OFF-cell signaling inhibits nociceptive transmission (Fields, 2004; Porreca et al., 2002).

Substance P

Several neuropeptides are shown to be involved in nociceptive transmission; one of them is the tachykinin substance P (SP), which is focused upon in this thesis. SP, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$ (Chang et al., 1971) see Figure 1, was originally discovered by von Euler and Gaddum in 1931. They found a substance that was particularly abundant in the brain and intestine of the horse that had a depressing effect on blood pressure by peripheral vasodilatation (V. Euler and Gaddum, 1931).

![Figure 1. The neuropeptide Substance P](image)

It was not until the 1980’s that other tachykinins were discovered. Four different research groups discovered, independently of each other, Neurokinin A (NKA) and Neurokinin B (NKB) (Kangawa et al., 1983; Kimura et al., 1983; Minamino et al., 1984; Nawa et al., 1983). The tachykinins all have a similar C-terminal sequence, Phe-$X$-Leu-Met-NH$_2$, where $X$ is either an aromatic (Phe, Tyr) or aliphatic (Ile, Val) amino acid (Erspamer, 1981; Maggio, 1988). Although NKA and NKB have been known for several decades, SP remains as the best characterized member of all the tachykinins.

Biosynthesis of neuropeptides is a complex process that is different from the synthesis of classical neurotransmitters. Neurotransmitter synthesis can take place throughout the neuron through enzymatic processes, whereas neuropeptide synthesis begins at the ribosomes (Hökfelt et al., 2003). SP is de-
derived from at least three different gene transcripts (α, β and γ TAC1), which originate from the tachykinin-1 TAC1 gene, previously known as preprotachykinin-A (PPT-A) (Carter and Krause, 1990; Krause et al., 1987; Nawa et al., 1983). NKA can be formed from β and γ TAC1, whereas NKB is derived from a separate gene, the TAC3 gene. The gene transcripts encode for large proteins, which are immature prepropeptides that are synthesized in the ribosomes and packed in large dense core vesicles (Hökfelt et al., 2003). Following posttranslational conversion and modifications by convertases, carboxypeptidases and an alfa-amidating enzyme, the mature peptide is released from the neuron (Harrison and Geppetti, 2001; Page, 2013). Amidation of the C-terminal contributes to the stability of SP, and is essential for biological activity (Eipper et al., 1992; Hanley et al., 1980).

**Biological effects of Substance P**

Although SP was discovered for having a role in blood pressure and vasodilation (V. Euler and Gaddum, 1931), several other effects have been attributed to the undecapeptide. The discovery of the amino acid sequence of SP made it possible to synthesize SP (Tregear et al., 1971) and subsequently develop methods for biochemical, as well as pharmacological studies, of SP in order to further study its distribution and biological effects.

SP is widely expressed both in the peripheral nervous system (PNS) as well as in the CNS. As early as 1953, Pernow published data on the distribution of SP, showing a high concentration in the CNS, but also in the dorsal roots in the PNS (Pernow, 1953). Already at the time of his pioneering work on SP in 1953, Lembeck suggested that this peptide was a neurotransmitter in primary sensory neurons (Pernow, 1983). Subsequent studies showed that SP was present in unmyelinated fibers and that it could be released from free nerve endings in the periphery (Hökfelt et al., 1975) as well as in the spinal cord after nerve stimulation ex vivo (Otsuka and Konishi, 1976) and in vivo (Yaksh et al., 1980). High levels of SP were found in the spinal cord, especially in laminae I and II of the dorsal horn (Hökfelt et al., 1975; Hökfelt et al., 1977), and SP-reactive cell bodies have been found in the dorsal horn (Hökfelt et al., 1977). This further strengthened the theory about the presence of SP in primary afferents. Pearson et al. (1982) revealed that patients with Riley-Day’s syndrome, characterized by diminished pain sensitivity, were devoid of SP-containing nerve endings in the spinal cord. This gave additional support to the hypothesis that SP is involved in nociceptive signaling. Since then, numerous studies have confirmed the role of SP in nociceptive signaling.

With the development of knock-out animals and a SP-conjugated toxin, it was possible to further study the mechanism of SP (Cao et al., 1998) and NK₁ receptors (De Felipe et al., 1998) in nociceptive signaling. In addition, it also showed the importance of SP and the NK₁ receptor in the maintenance
of neuropathic pain, because depletion of the NK$_1$ receptor lamina I neurons resulted in an attenuated response to highly noxious stimuli as well as mechanical and thermal hyperalgesia (Mantyh et al., 1997; Nichols et al., 1999).

Activation of primary afferents results in the release of SP in the periphery. The release of SP together with other neuropeptides such as calcitonin gene-related peptide (CGRP) has been suggested to promote neurogenic inflammation. When SP is released in response to a noxious stimulus, it stimulates the release of histamine, which in turn results in plasma extravasation and vasodilatation (Foreman, 1987; Pedersen-Bjergaard et al., 1991).

In addition to its pronociceptive and inflammatory effects, SP has been implicated in many diverse functions in the brain. As previously mentioned, SP and the NK$_1$ receptor are present in several areas of the brain. SP has been detected in the ventral tegmental area (VTA), hypothalamus, amygdala, nucleus accumbens (NAcc), PAG, striatum and substantia nigra (Hallberg et al., 2000). SP is also involved in addiction and the rewarding effects of opioids (Murtra et al., 2000).

NK$_1$ receptor

SP and other tachykinins exert their effects through the tachykinin receptors. Three different receptors have been characterized and cloned, namely, NK$_1$, NK$_2$ and NK$_3$ (Masu et al., 1987; Shigemoto et al., 1990; Yokota et al., 1989). Similar to most neuropeptide receptors, the NK receptors are G-protein coupled receptors. Activation of the G$_{q/11}$ coupled NK$_1$ receptor, results in activation of phospholipase C (PLC), which in turn results in formation of (IP$_3$) and diacylglycerol (DAG). The C-terminal is of importance for the tachykinins to bind to the NK receptors. Due to the similarities in the C-terminal for the tachykinins, SP and other tachykinins are able to bind and exert their effects at any NK receptor. However, SP binds preferentially to the NK$_1$ receptor, whereas NKA and NKB have preferred binding to NK$_2$ and NK$_3$, respectively (Maggi, 1995; Regoli et al., 1994).

Substance P metabolism

Neuropeptides do not have reuptake mechanisms like classic neurotransmitters, but are substrates for extracellular peptidases. Following its action on the NK$_1$ receptor; SP is subject to enzymatic degradation. Several enzymes are involved in the metabolism of SP, the main enzymes are dipeptidylpeptidase-IV (DP-IV), post-proline cleaving enzyme (PPCE), neutral endopeptidase (NEP), angiotensin converting enzyme (ACE) and substance P endopeptidase (SPE). DP-IV and PPCE act on the N-terminal part of the peptide, to yield C-terminal fragments SP$_{3-11}$ and SP$_{5-11}$ (Ahmad et al., 1992; Wang et al., 1991; Yoshimoto et al., 1983), whereas NEP and ACE cleave the peptide
in the middle or closer to the C-terminal. ACE cleaves mainly at the Phe$^7$-Phe$^8$ and Phe$^8$-Gly$^9$ bonds, and possibly also at the Gly$^9$-Leu$^{10}$ bond (Skidgel et al., 1984; Yokosawa et al., 1983). It may also subsequently act as a dipeptidyl peptidase at the C-terminal of SP fragments (Yokosawa et al., 1983). NEP can metabolize SP at four different sites; Gln$^6$-Phe$^7$, Phe$^7$-Phe$^8$ and Phe$^8$-Gly$^9$ and Gly$^9$-Leu$^{10}$ (Karlsson et al., 1997; Matsas et al., 1983), whereas SPE acts at Phe$^7$-Phe$^8$ and Phe$^8$-Gly$^9$ bonds (Nyberg et al., 1984).

Metabolism of neuropeptides generally results in inactive peptide fragments. However, it is also possible that enzymatic conversion can result in bioactive fragments with retained or modified biological activity. The latter has been shown to be the case for a number of neuropeptides in the CNS, including SP. Examples of other neuropeptides with bioactive metabolites are dynorphin, CGRP and nociceptin/orphanin FQ (N/OFQ) (Hallberg, 2014; Hallberg and Nyberg, 2003), which mimic as well as counteract the effects of the mother peptides. In the case of SP, several N-terminal and C-terminal fragments are formed, many with retained biological activity. C-terminal fragments mimic the effect of SP, whereas N-terminal fragments in some cases mimic, but more often have opposing effects when compared with SP (Hallberg and Nyberg, 2003).

Figure 2. Processing of β-preprotachykinin to SP and subsequently SP$_{1-7}$. Modified from (Nyberg, 2005)
SP\textsubscript{1-7}

The N-terminal SP fragment SP\textsubscript{1-7} (Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH) represents one of the major bioactive fragments after SP degradation (Sakurada et al., 1999; Sakurada et al., 1985; Zhou et al., 1998) and has received particular interest. Already in 1975 Benuck and Marks (1975) revealed an endopeptidase in the rat brain that was capable of metabolizing SP to yield SP\textsubscript{1-7} and SP\textsubscript{1-8} and the corresponding C-terminal fragments SP\textsubscript{8-11} and SP\textsubscript{9-11}. Later, it was recognized that the SP\textsubscript{1-7} generating enzyme SPE, an enzyme acting specifically on SP, existed in the human brain as well as cerebrospinal fluid (CSF) with an activity similar to that discovered by Benuck and Marks (Lee et al., 1981; Nyberg et al., 1984), suggesting the presence of SP\textsubscript{1-7} in the human CNS. Over the years several new enzymes have been shown to be involved in the formation of SP\textsubscript{1-7}, including ACE, NEP and SPE, see Figure 2.

Biological effects of SP\textsubscript{1-7}

Soon after the discovery of the first SP cleaving enzyme, it was recognized that SP\textsubscript{1-7} could exert biological effects, in many cases opposite to SP (Hall and Stewart, 1983). Central effects of peripherally administered SP\textsubscript{1-7} were proposed based on the finding that SP\textsubscript{1-7} was able to induce antinociception. The N-terminal fragment SP\textsubscript{1-7} was found to exhibit antinociceptive activity, as opposed to SP and its C-terminal fragment SP\textsubscript{5-11} (Stewart et al., 1982). The fragment was found to be abundant in the dorsal spinal cord and has been suggested to be a modulator of SP. In fact, the delayed antinociceptive effect seen from SP (Stewart et al., 1976) has later on been attributed to its N-terminal heptapeptide fragment. In addition to its antinociceptive effect, SP\textsubscript{1-7} can potentiate morphine analgesia (Komatsu et al., 2009).

SP\textsubscript{1-7} has been localized in several areas of the rat brain, including the VTA, hypothalamus, hippocampus, amygdala, NAcc, PAG, striatum and substantia nigra (Hallberg et al., 2000; Sakurada et al., 1991). In addition to its antinociceptive effect, SP\textsubscript{1-7} has been shown to reduce the development of morphine tolerance and morphine withdrawal (Kreeger and Larson, 1993, 1996; Zhou et al., 2009; Zhou et al., 2011), effects that are opposite to the biological effects of SP. Both SP and SP\textsubscript{1-7} have been suggested to be involved in memory function, although the memory-promoting effect of SP is mediated by the N-terminal (Huston and Hasenöhrl, 1995; Tomaz and Nogueira, 1997). In addition to its opposing effects on opioid actions, SP\textsubscript{1-7} has been shown to alleviate the actions of SP. For example, SP-induced biting and scratching (Igwe et al., 1990a; Igwe et al., 1990b; Sakurada et al., 1999; Sakurada et al., 1988) and vasodilatation (Wiktelius et al., 2006) is inhibited by SP\textsubscript{1-7}. This has further strengthened the idea of SP\textsubscript{1-7} as a modulatory compound on opioid actions as well as on SP-induced effects. Alt-
hough SP$_{1-7}$ has been shown to alleviate morphine-induced hyperalgesia (Sakurada et al., 2007), the intrinsic effects of SP$_{1-7}$ on hyperalgesia and allodynia have not been investigated.

**SP$_{1-7}$ binding site**

Several biological effects have been attributed to SP$_{1-7}$, but still a molecular target is yet to be cloned. SP$_{1-7}$ does not bind to the NK$_1$ receptor (Geraghty and Burcher, 1993; Michael-Titus et al., 1999), which is most likely due to the missing amino acid sequence Gly-Leu-Met-NH$_2$ in the C-terminal, a sequence crucial for binding to the NK$_1$ receptor. The existence of a receptor that recognizes the N-terminal part of the receptor was initially proposed (Stewart et al., 1982), and thought to be responsible for the biological effects of the N-terminal SP fragments, including SP$_{1-7}$. The effect could be blocked by the opioid receptor antagonist naloxone (Stewart et al., 1982; Wiktelius et al., 2006) and SP$_{1-7}$ was believed to bind to or modulate the µ opioid receptor (Krumins et al., 1993; Krumins et al., 1989). However, binding sites for this heptapeptide have been found in the rat brain and spinal cord (Botros et al., 2006; Botros et al., 2008; Igwe et al., 1990a). Other SP fragments and naloxone had low or negligible affinity for the binding site, as did ligands for the NK receptors, see Table 2 (Botros et al., 2006). Interestingly, the synthetic opioid ligand Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO) and the highly potent endogenous opioid peptide endomorphin-2 (EM-2) possess high affinity for the SP$_{1-7}$ binding site in both the brain and spinal cord (Botros et al., 2006; Botros et al., 2008), see Table 2.

Table 2. Binding affinity of neurokinin and opioid ligands toward the SP$_{1-7}$ binding site in the spinal cord. Data from (Botros et al., 2006).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid sequence</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP$_{1-7}$</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH</td>
<td>0.75</td>
</tr>
<tr>
<td>SP$_{1-6}$</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-OH</td>
<td>1830</td>
</tr>
<tr>
<td>SP$_{1-8}$</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-OH</td>
<td>74</td>
</tr>
<tr>
<td>SP</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH$_2$</td>
<td>159</td>
</tr>
<tr>
<td>D- SP$_{1-7}$</td>
<td>Arg-d-Pro-Lys-Pro-Gln-Gln-d-Phe-OH</td>
<td>1.78</td>
</tr>
<tr>
<td>[Sar$^9$, Met(O$<em>2$)$</em>{11}$]SP</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Sar-Leu-Met(O$_2$)-NH$_2$</td>
<td>814</td>
</tr>
<tr>
<td>R-396</td>
<td>Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH$_2$</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Senktide</td>
<td>Suc-Asp-Phe-N-Me-Phe-Gly-Leu-Met-NH$_2$</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>DAMGO</td>
<td>Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol</td>
<td>13.1</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>Tyr-Pro-Trp-Phe-NH$_2$</td>
<td>1030</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Phe-Phe-NH$_2$</td>
<td>7.5</td>
</tr>
<tr>
<td>Naloxone</td>
<td></td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>
Opioid peptides

The use of opium stretches back thousands of years, and there are early reports on the use of opium juice from the opium poppy *papaver somniferum*, being used as an analgesic. The term opioid refers to all compounds related to opium, whereas opiates are drugs derived from opium, including natural products such as morphine and codeine, but also semisynthetic analogues thereof (Yaksh and Wallace, 2011).

Early opioid research pointed towards central effects from opioids. In 1973, specific receptors for the opioids were detected (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Three different subtypes of opioid receptors were suggested (Martin et al., 1976) and were later identified and classified as the µ, δ and κ receptors. The existence of the opioid receptors led to the search for and discovery of the endogenous opioid ligands. Leu-enkephalin and Met-enkephalin were the first endogenous opioids to be discovered (Hughes et al., 1975) and were followed by β-endorphin and the dynorphins among others (Akil et al., 1984). The effect of opioids was shown to be naloxone-reversible, which became a hallmark for the opioids. β-endorphin binds preferentially to the µ and δ receptors, whereas the enkephalins and dynorphins are δ and κ receptor preferring, respectively (Yaksh and Wallace, 2011). Later on, N/OFQ (Meunier et al., 1995; Reinscheid et al., 1995) and the corresponding NOP receptor (Mollereau et al., 1994) were discovered. The effect of N/OFQ differs from the classical opioid peptides and its role in pain processing is complex since it acts pronociceptive at a supraspinal level but is antinociceptive in the spinal cord (Zeilhofer and Calo, 2003). However, this peptide is not the focus of this thesis and will therefore not be discussed any further.

Opioid peptides are involved in pain modulation and can act at both a spinal and supraspinal level. E.g., enkephalins are expressed in interneurons in the spinal cord and can modulate nociceptive signaling on a pre- as well as postsynaptic level (Hökfelt et al., 1977; Yaksh and Wallace, 2011). Release of opioids in the spinal cord prevents the release of SP from primary afferents (Brodin et al., 1983). Enkephalins are also able to modulate descending neurons at supraspinal sites, where they act mainly on the midbrain structures PAG and possibly RVM through inhibition of the ON-cells and disinhibition of the OFF-cells (Fields, 2004).

Approximately 20 years after the discovery of the classical opioid peptides and their receptors, two endogenous opioid peptides with high affinity to the µ opioid receptor were discovered - endomorphin-1 (EM-1) and endomorphin-2 (EM-2) (Zadina et al., 1997). EM-2 has been localized in primary afferents and in laminae I and II of the spinal cord (Martin-Schild et al., 1998, 1999; Martin-Schild et al., 1997). As for EM-1, the highest density is in supraspinal sites, e.g. in the midbrain and brain stem (Martin-Schild et al., 1999). In contrast to the classical opioid peptides that are produced from
the large precursor proteins proopiomelanocortin, proenkephalin and prodynorphin, the precursor for the endomorphins remains unknown. Nevertheless, they are the most potent endogenous ligands for the µ opioid receptor to which they bind and produce biological activity (Zadina et al., 1997).

Sigma-1 receptor

In the work by Martin et al. (1976) attempting to find the different opioid receptors, the sigma receptor was discovered and was suggested to be an opioid receptor subtype, responsible for the paradoxical effects of opiates. It was soon identified that this was not the case. As opposed to opioid receptors, the sigma receptor is not a G-protein coupled receptor and neither does it bind naloxone. In fact, the sigma receptor sequence does not resemble any other protein (Su, 2015). The receptor that today is recognized as the sigma-1 receptor was discovered in 1982, showing to bind the (+)-isomer of benzomorphan such as pentazocine (Su, 1982).

Although two subtypes of sigma receptors exist, sigma-1 and sigma-2 receptors (Quirion et al., 1992), the focus here will be the sigma-1 receptor. The sigma-1 receptor is an intracellular protein located at the endoplasmic reticulum where it regulates the flow of \( \text{Ca}^{2+} \) through \( \text{IP}_3 \) receptors (Hayashi and Su, 2007; Wu and Bowen, 2008). In addition, it has modulatory actions on several neurotransmitter receptors and ion channels (Cobos et al., 2008). From these actions it has received the current term sigma non-opioid intracellular receptor 1.

The sigma-1 receptor has been implicated in a variety of diseases, including depression, Alzheimers disease, Parkinsons disease and addiction but also in pain (Maurice and Su, 2009). Sigma-1 receptors have been shown to possess anti-opioid actions; sigma-1 receptor agonists attenuate the analgesic effects of opioids whereas antagonists increase the effect (Chien and Pasternak, 1993, 1994, 1995a, b). Moreover, several studies have suggested a role of the sigma receptor in neuropathic pain. Injection of sigma-1 agonists produced mechanical allodynia in mice (Yoon et al., 2009), and sigma-1 receptors were up-regulated in animal models of chronic pain (Roh et al., 2008). In addition, the receptor appeared to be crucial for the development of allodynia and hyperalgesia (de la Puente et al., 2009). It is thus evident that sigma receptor system is of interest in order to understand the underlying mechanisms of, as well as developing new treatment strategies for, neuropathic pain.
Treatment of chronic neuropathic pain

Treatment of chronic neuropathic pain is complicated. There are two main challenges associated with pain treatment. The first is to identify the underlying cause or mechanisms of the pain, and the second is to find ways to normalize the hypersensitivity that has developed, and thus to prevent the hypersensitivity becoming permanent (Woolf, 2004). Once the pain has become chronic, it is difficult to treat and the pain relief achieved in neuropathic pain patients is generally only partial.

Pharmacological treatment

Although different drugs are used based on the underlying condition, it has not been possible to show any distinct differences in treatment efficacy for most drugs on a specific condition. Tricyclic antidepressants (TCA), serotonin-noradrenaline reuptake inhibitors (SNRI), and the antiepileptic drugs pregabalin and gabapentin have been proposed as first-line treatments (Finnerup et al., 2015). Gabapentin is one of the most commonly used drugs in the treatment of neuropathic pain and has been found to have several positive effects. In addition, despite its adverse effects, it is well tolerated and may also improve sleep (Dworkin et al., 2007; Gordh et al., 2008; Rekand et al., 2012). Opioids have been seen as “the golden standard” when it comes to analgesia and are very effective in relieving acute pain. In neuropathic pain, opioids possess a similar efficiency compared to other treatment regimens (Dworkin et al., 2007). However, because they more frequently give rise to side effects such as sedation and respiratory depression, obstipation and long-term use increases the risk of tolerance and dependence, opioids are recommended as third-line treatment (Finnerup et al., 2015).

Spinal cord stimulation

An alternative to pharmacological treatment is spinal cord stimulation (SCS). SCS is not a first-line choice for treatment of neuropathic pain, but an option when pharmacological treatments have been unsuccessful. SCS is becoming more and more common, with approximately 18000 new SCS systems implanted worldwide each year (Linderoth, 2009). SCS was first introduced in the late 1960’s and is based on the gate control theory proposed by Melzack and Wall (1965). By stimulating the non-nociceptive Aβ fibers, nociceptive input from Aδ and C fibers will be reduced and thereby reduce the ascending nociceptive signals. The mechanism by which SCS is not fully understood, but includes actions on a spinal as well as on a supraspinal level (Wolter, 2014). An electrode is placed in the epidural space right above the affected region. Mild to moderate electrical pulses (50-60 Hz) are sent to the spinal cord, which give rise to paresthesia in the painful
region of the body (Guan, 2012). More recently, paresthesia free new SCS treatment modes have been developed (De Ridder et al., 2010; Van Buyten et al., 2013) The effects of SCS are intermediate, with half of the patients reporting at least 50% pain relief (Taylor et al., 2014).

Drug development

Because pharmacological treatment regimens for neuropathic pain are unsatisfactory, drugs that can alleviate chronic neuropathic pain are highly desirable. Due to the role of SP in nociception and inflammation, a lot of effort has been put into developing NK\textsubscript{1} receptor antagonists as putative analgesics. With the finding of the genes encoding SP and the NK\textsubscript{1} receptor, genetic approaches have been used in order to further understand the role of SP and its receptor. By deleting the TAC1 gene (Cao et al., 1998; Zimmer et al., 1998) or the TAC1R gene (De Felipe et al., 1998), which encode for SP and the NK\textsubscript{1} receptor, respectively, it has become possible to further investigate their roles in e.g. pain signaling. Although some results from knock-out studies indicate a role for SP and the NK\textsubscript{1} receptor in inflammation as well as for acute and chronic pain, the results were mixed, and were only valid under some testing conditions (Berge, 2014). Clinical trials have indicated a weak antinociceptive effect of a NK\textsubscript{1} receptor antagonist on dental pain, equivalent to ibuprofen (Rupniak and Kramer, 1999). The only NK\textsubscript{1} receptor antagonist available on the market is aprepitant, which became available in 2003 for the treatment of chemotherapy-induced emesis (Hargreaves et al., 2011). To date, there are no clinical trials that indicate that classic NK\textsubscript{1} receptor antagonists could be used as analgesics (Berge, 2014).

It is evident that the development of NK\textsubscript{1} receptor antagonists as analgesics did not turn out as expected. However, dual peptide ligands have been developed, with NK\textsubscript{1} and opioid pharmacophores, some of which were found to attenuate neuropathic pain-like symptoms in rats (Guillemin et al., 2015; Nair et al., 2013).

Development of SP\textsubscript{1-7} analogues

Since SP and its bioactive fragments seem to play an important role in pain signaling, alternative approaches to target the SP system in order to target pain still remains a topic of much interest. Due to the analgesic effects of SP\textsubscript{1-7}, it is tempting to draw attention to this heptapeptide fragment and the SP\textsubscript{1-7} binding site. A medicinal chemistry program has been commenced in order to develop peptidomimetics, compounds with less peptidergic character but with retained binding affinity and biological activity, to the SP\textsubscript{1-7} binding site. An Ala scan and a subsequent N-terminal truncation study of SP\textsubscript{1-7} revealed the importance of the Phe\textsuperscript{7} in the C-terminal for binding of the
heptapeptide (Fransson et al., 2008). In addition, it was identified that C-terminal amidation increases the binding affinity approximately 5-fold (Fransson et al., 2008).

As previously mentioned, the endogenous opioid tetrapeptide EM-2 possesses relatively high affinity for the SP_{1,7} binding site. A similar truncation study performed with EM-2 resulted in discovery of the dipeptide Phe-Phe-NH\textsubscript{2}, a small compound with high binding affinity (Fransson et al., 2010). Due to its intriguing pharmacological effects after intrathecal (i.t.) administration (Ohsawa et al., 2011), Phe-Phe-NH\textsubscript{2} became the starting point for the synthesis of small peptidomimetics. Small constrained Phe-Phe-NH\textsubscript{2} analogues and phenylalanine based carbamates were primarily developed (Fransson et al., 2014; Fransson et al., 2013). Receptor binding studies and subsequent permeability and stability studies revealed one carbamate and one constrained Phe-Phe-NH\textsubscript{2} analogue with favorable characteristics (Fransson et al., 2014; Fransson et al., 2013), which have been further evaluated in this thesis.
Figure 3. SP_{1-7}, EM-2 and three of the most active lead compounds H-Phe-Phe-NH₂ (1), Z-Phe-NH₂ (2) and the constrained H-Phe-Phe-NH₂ analogue (3) identified in an ongoing medicinal chemistry program aimed at developing small peptides and peptidomimetics targeting the SP_{1-7} binding site.

Methodological aspects

Animal models of chronic pain

The animal studies that have been used in the papers included in this thesis have been chosen in order to model some of the different kinds of pain that are represented in the clinic.

Streptozotocin (STZ)-induced diabetes is an animal model of insulin-dependent diabetes mellitus. Due to its selectivity for the glucose transporter 2, STZ can selectively enter the pancreatic cells and subsequently destroy the insulin-producing beta cells (Lenzen, 2008). This model is widely used for preclinical studies of diabetic neuropathic pain. The model was established in 1993 (Courteix et al., 1993) and is designed to explore neuropathic pain due to a specific disease. The diabetic animals display a hypersensitiv-
ty to thermal, mechanical and tactile stimuli (Courteix et al., 1993; Ohsawa and Kamei, 1999).

A model of spinal cord injury (SCI) has been used in order to study neuropathic pain of central origin. The present SCI model has been developed at the Karolinska Institutet in Stockholm and is induced via ischaemic injury to the spinal cord. By using a laser towards the exposed spinal cord and simultaneously injecting erythrosine B, a photochemical reaction occurs and ischemia develops at the site of irradiation. This injury mimics the injury that can arise following i.e. stroke and with accompanying symptoms of tactile, mechanical and cold allodynia in areas around and below the level of injury (Hao et al., 2004; Hao et al., 1996; Xu et al., 1992).

Spared nerve injury (SNI) is a model of peripheral neuropathic pain, originally developed in rats (Decosterd and Woolf, 2000) and later transformed to mice (Bourquin et al., 2006). Two of three branches of the sciatic nerve, the tibial and common peroneal nerves, are ligated and subsequently resected, whereas the sural nerve remain intact. This surgery results in an increased hypersensitivity to mechanical and cold stimuli in the lateral part of the ipsilateral paw.

Human Cerebrospinal Fluid

CSF is produced in the choroid plexus of the brain and is in contact with the entire CNS. It is thus likely that the content of CSF reflects the ongoing neuropeptide activity in the brain and spinal cord (Nyberg, 1993). Since it is difficult, if not impossible, to study brain tissue from patients, the CSF would be the best mirror of the activity in the brain. In order to study disease, which is likely to cause a change in the CNS, it is more probable to see a change in the CSF than in the blood circulation. In order to investigate the possible involvement of SP1-7 in human neuropathic pain, CSF was the first choice for peptide analysis.

Radioimmunoassay

In the search for peptides in biological material, sensitivity and specificity are two important factors. Radioimmunoassay (RIA) is a method that has been used for this purpose for many years. In fact, this method dates back to the 1950’s, when the ability of measuring the levels of insulin in plasma was described (Yalow and Berson, 1959). The method is based on the interaction between an antigen and its antibody. A labeled antigen competes with the unlabeled antigen (the peptide of interest) to bind to the limited number of binding sites on an antibody. Free antigen is separated from the bound antigen with either a secondary antibody or with charcoal solution. The amount of labeled antigen bound to the antibody is measured, and the amount of unlabeled antigen can be calculated with a standard curve of known concen-
trations. The more unlabeled antigen in the sample, the less of the labeled antigen able to bind to the antibody.

An important strength with this method is the sensitivity of the assay; it is, depending on the antibody, possible to measure down to the low femtomolar range. This is especially important when measuring CSF samples, in which the levels of peptides in general are low. A drawback of the method is that cross-reactivity with similar peptides may occur. This can however be reduced with pre-separation procedures as well as antibodies that is very specific for its antigen. The SP₁₋₇ antibody used in this assay shows less than 5% cross-reactivity towards SP and N- and C-terminal fragments (Eriksson et al., 1996). In addition, by doing an ion-exchange chromatography prior to the assay, peptides with different ion strength will be eluted in different fractions, which increases the specificity.
Aims

The overall aim of this thesis was to investigate the impact of the neuropeptide SP fragment SP$_{1-7}$ in chronic neuropathic pain.

The specific aims of this thesis were:

- To study the effects of SP$_{1-7}$ in animal models of chronic neuropathic pain.
- To further characterize the binding properties of SP$_{1-7}$ to its binding site.
- To study the pharmacological effects of SP$_{1-7}$ related compounds in the search for new drugs to treat neuropathic pain.
- To study whether the CSF levels of SP$_{1-7}$ are altered in neuropathic pain patients.
Methods

Animals
ICR mice (paper I and II), NMRI mice (paper IV) and Sprague-Dawley rats (paper III) were used. Animals were housed 4-5 per cage in an animal room with a 12 hour light-dark cycle. Food and water was available ad libitum. The temperature was maintained at a constant level throughout each experiment. The experimenter was blinded to the drug treatments. All animal experiments were approved by a regional ethical committee and were carried out according to the Ethical Guidelines of IASP.

Streptozotocin-induced diabetes
Male ICR mice (Tokyo Animal Laboratory Inc, Tokyo, Japan), weighing approximately 20 g, were rendered diabetic by an intravenous injection of STZ (200 mg/kg) prepared in a 0.1 N citrate buffer at pH 4.5. Age matched animals were injected with vehicle alone. Animals with a serum glucose level exceeding 400 mg/dL (22.2 mmol/L) were considered diabetic and were included in the study. Experiments were conducted two weeks after STZ injection.

Spared Nerve Injury (SNI)
Male NMRI mice (Taconic, Denmark), weighing approximately 15 g were anesthetized with isoflurane (Abbott Scandinavia, Sweden) (4% v/v for induction and 3.5% v/v for maintenance during surgery). The surgical procedure was performed according to the protocol by Bourquin et al. (2006). Briefly, the animal was positioned on its right side with the left hindlimb immobilized. An incision was made at the mid-thigh level and the Biceps femoris muscle was separated at the mid-thigh level to expose the sciatic nerve. The common peroneal and the tibial nerves were ligated together with 6.0 silk thread (Vömel, Germany) and subsequently transected distal to the ligation. Great care was taken not to touch or stretch the sural nerve. After nerve transection, the muscles were placed back in the original position and the skin was closed with Reflex 7 mm wound clips. The animals were allowed to recover from surgery for at least one week before any post-surgical behavioral testing was performed.
Spinal Cord Injury (SCI)

Female Sprague-Dawley rats (Möllegård, Denmark), weighing 250 g at the start of the experiment, were used. An ischemic SCI was induced photochemically as described previously (Hao et al., 2004; Hao et al., 1996; Xu et al., 1992). Briefly, animals were anaesthetized with 0.5 mg/kg intraperitoneal (i.p.) medetomidine (Domitor, Lääkefarmos, Finland) and 60 mg/kg i.p. ketamine (Ketalar, Parke-Davis, Sweden), and one jugular vein was cannulated. A midline incision was made on the skin overlying the vertebral segments T12 – L1. The animals were positioned with the vertebral segment T12 or T13 (spinal segments L3–5) beneath the beam of a tunable argon laser (Innova model 70, Coherent Laser Product Division, Palo Alto, CA; operating at 514 nm with an average power output of 160 mW) and irradiated for 10 min. Immediately prior to and 5 min after the start of the irradiation, erythrosine B (Red N°3, Aldrich-Chemie, Steinheim, Germany) dissolved in 0.9 % (w/v) saline was injected intravenously at a dose of 32.5 mg/kg. The rat temperature was maintained at 37-38°C during the irradiation. The animals were allowed to recover from surgery for at least one week before any postsurgical behavioral testing was performed.

Administration of SP, SP1-7 and SP1-7 related compounds

In paper I and II, SP1-7 and SP1-7-NH2 were administered i.t. as described by Hylden and Wilcox (1980). The doses ranged between 0.5 and 4 pmol/mouse. The mice were manually restrained and the needle was inserted between the L5 and L6 vertebrae. This site is near the end of the spinal cord and minimizes the risk of spinal damage.

In paper III and IV, SP1-7 and SP1-7 related compounds were administered i.p. In paper II, compounds were administered in cumulative doses, 1.85, 18.5 and 185 nmol/kg body weight, with doses adapted from (Stewart et al., 1982; Zhou et al., 2011). The results in paper III laid the foundations for the study design in paper IV, where one dose (185 nmol/kg) was administered i.p.

SP was purchased from Bachem (Bubendorf, Switzerland) and SP1-7 was purchased from Polypeptide Group (Strasbourg, France). SP1-7-NH2 (Paper II-IV), truncated SP1-7 analogues (Paper III) were prepared as described previously (Fransson et al., 2008) and constrained SP1-7 analogues (Paper IV) were prepared by standard Fmoc chemistry as previously reported (Fransson et al., 2014).
Behavioral testing
Thermal hyperalgesia in diabetic mice
The anti-hyperalgesic response was evaluated using a tail-flick apparatus (KN-205E Thermal analgesimeter, Natume, Tokyo, Japan) as described by D'amour and Smith (1941). Briefly, the mouse was gently placed in a restrainer. The tail was positioned underneath a heat source that was focused at the tip of the tail. Latencies were determined as the mean of two trials. To study hyperalgesia, the voltage of a 50 W projection lamp was set to 50 V (Ohsawa and Kamei, 1999) giving a response from non-diabetic animals in 10-14 s. The cut-off time was set at 30 s to prevent injury of the tail. Tail-flick latencies were measured before and 5, 30, 60 and 90 min after i.t. injection of SP1-7 or SP1-7-NH2.

Mechanical allodynia in mice with SNI
Mechanical threshold was evaluated using the Von Frey test. Calibrated von Frey filaments (Bioseb, France) were applied according to the Chaplan “up-down paradigm” (Chaplan et al., 1994). Animals were placed in transparent chambers on an elevated mesh stand (IITC, USA). Prior to the testing, animals were allowed to acclimatize to the environment for approximately 30-60 min. The filament was applied for approximately three seconds to the medial plantar side of the paw. A positive response was registered if the mouse quickly withdrew the paw. If a positive response was registered, a thinner filament was applied. In the case of a negative response, a thicker hair was applied. The testing was performed until six responses around the threshold had been monitored. Two baseline values were obtained for each mouse before surgery.

On the day of compound testing, a baseline value was obtained before drug injection and the mechanical threshold was evaluated at 15, 30, 45 and 60 min after injection.

Mechanical allodynia in rats with SCI
Sensitivity to mechanical stimulation was tested by application of calibrated von Frey filaments (Stoelting, USA) onto the skin. The animal was gently restrained and the filaments were applied with increasing pressure on the back and flanks until they bent. Each filament was applied 5-10 times at a frequency of 1/s and the intensity of stimulation that induced vocalization at a > 75% response rate was considered the pain threshold.
Acute nociception in rats with SCI

The reaction of the animals to an acute nociceptive stimulus was tested with the tail-flick test. The animal was gently restrained and placed on a glass surface with a heated light source underneath that was focused at the tip of the tail. The intensity of the light was set so that the animal responded in around 6 s. The time from the onset of the heat source to the withdrawal of the tail was registered. The cut-off time was set at 10 s to prevent injury to the tail.

Motor tests in rats with SCI

A combined motor test of walking in an open field and a righting reflex to detect the potential motor and sedative effects of SP and its fragments in spinally injured rats was used, as described in Gao et al. (2013). The following scores were used in the tests. Walking: 0=Normal walking; 5=Walks with only mild deficit; 15=Hindlimb can support weight; 25=Frequent movement of hindlimb, no weight bearing; 40=Minor movement in hindlimb, no weight bearing; 45=No movement of hindlimb, no weight bearing. Righting: 0=Normal righting counter to direction of the roll; 5=Weakened attempt to righting; 10=Delayed attempt to righting; 15=No attempt to righting.

Receptor screen

Radioligand binding assays and enzyme assays were performed by Ricerca Biosciences, LCC (Ohio, USA; www.ricerca.com). The SP₁₋₇ analogue Phe-Phe-NH₂ was screened towards two panels consisting of a total of 111 targets; one core panel that included 96 common drug targets, and one pain panel that included 15 enzymes and receptors of interest with regard to pain. The concentration of the test compound was 10 μM. Assays were performed according to the standard assay protocols at Ricerca Biosciences.

Patients and CSF sampling

CSF samples from 11 patients diagnosed with chronic neuropathic pain due to peripheral nerve or root injury and reporting satisfactory pain relieving effect from their SCS system were analyzed for levels of SP₁₋₇ and compared with CSF from eleven voluntary patients (56±2 years, eight females and three males). Pain patients were recruited from a database of SCS users at the Multidisciplinary Pain Center at Uppsala University Hospital. For this study, each participant contributed one CSF sample. Prior to the sampling
procedure, the participants refrained from using their spinal cord stimulator for 48 hours. The patient’s pain sensation was rated on a visual analogue scale (VAS) 0-10 (0 = no pain; 10 = worst possible pain) before the sample was collected. Control CSF was obtained from patients undergoing minor urological surgery under spinal anaesthesia (47±4 years, five females and six males). They had no known neurological disorder, nor any pronounced pain suffering, and samples were collected according to the same procedure as for the neuropathic pain patients. The samples were frozen at -70°C until analysis was performed.

Radioimmunoassay

Separation procedures

The frozen CSF samples (1 mL) were thawed on ice and purified by ion exchange chromatography as previously described (Nyberg and Hallberg, 2011). Briefly, each CSF sample was diluted with 3 mL buffer I (0.1M formic acid and 0.018M pyridine; pH 3.0) before being applied to separate columns packed with SP-Sephadex C-25 gel (total gel volume = 1 mL) pre-washed with 10 mL buffer I. After addition of the sample, and 2 mL of buffer I, the columns were washed with 10 mL buffer II (0.1M formic acid and 0.1M pyridine). SP1-7 was subsequently eluted with 4 mL buffer IV (0.8M formic acid and 0.8M pyridine). The collected fractions were evaporated in a vacuum centrifuge (Savant, Hicksville, NY) and stored at -80°C until further analysis.

Radioimmunoassay technique

The RIA was based on the charcoal adsorption technique as described in (Eriksson et al., 1996; Nyberg and Hallberg, 2011). Briefly, standards and samples were dissolved in MeOH: 0.1M HCl (1:1). Aliquots (25 µL) were incubated in incubation tubes together with 100 µL antibody and 100 µL iodinated peptide (approximately 5000 cpm/tube). The iodinated SP1-7 was prepared from Tyr0-SP1-7, see (Hallberg et al., 2000). The antibody and the iodinated peptides were diluted in gelatin buffer. The antibody for SP1-7 (89:2D) was raised in rabbits against the thyroglobulin conjugate and was used at a final dilution of 1:200 000. After 24 hours of incubation at 4°C, 200 µl dextran-coated charcoal (750 mg charcoal and 75 mg dextran in 200 mL 50 mM phosphate buffer) was added to each tube in order to separate the bound from the free peptides. After 10 min, the samples were centrifuged (Beckman GS-15R) for 2 min at 12 000 x g, 4°C. The supernatant (300 µL) was collected and subsequently analyzed in a gamma counter (1470 Wizard, Wallac, Turku, Finland). The detection limit was approximately 1 fmol/tube.
Statistics

Statistics were performed using Graphpad Prism (Graphpad Software Inc., USA; Paper I, II, IV and V) and Statview software (SAS Institute Inc., USA; Paper III). The results are presented as the mean with standard error of the mean (SEM) or the median with median absolute deviation (MAD). In this thesis, results are also presented as % MPE (maximum possible effect). % MPE was calculated according to the following formula:

\[
\text{% MPE} = 100 \times \frac{(\text{test latency} - \text{baseline latency})}{(30 - \text{pre-drug latency})}
\]

Where 30 is the cut-off time in seconds.

Data was analyzed by one-way or two-way ANOVA followed by Bonferroni’s or Dunn’s post hoc test. Paired data was analyzed with the paired t-test or the Wilcoxon signed rank test, whereas unpaired data was analyzed with the unpaired t-test or the Mann-Whitney U test. Correlation was calculated using Pearson correlation. For all the statistics, \( p < 0.05 \) was considered significant.
Results and discussion

Due to the nature of chronic neuropathic pain and the lack of efficient pharmacological treatments, there is an unmet need for new treatment strategies based on knowledge on pain signaling and the endogenous analgesic system. In this thesis, the biological effect of SP$_{1-7}$ was investigated in order to provide further insights on the SP system in relation to chronic pain, and to biologically evaluate new SP$_{1-7}$ related compounds in the search for novel analgesics. In addition, to further understand the role of SP fragments in neuropathic pain patients, human CSF was analyzed for the peptide fragment.

Effect of SP$_{1-7}$ and SP$_{1-7}$-NH$_2$ on thermal hypersensitivity

Thermal hypersensitivity was studied in STZ-induced diabetic mice and was evaluated using the tail-flick test. The effect of SP$_{1-7}$ and its amidated analogue SP$_{1-7}$-NH$_2$ was investigated in Paper I and II, respectively. Diabetic mice displayed significant hypersensitivity to thermal stimulation, as assessed by the tail-flick test.

After i.t. administration of SP$_{1-7}$, a dose-dependent increase in tail-flick latency was seen for both diabetic and control mice, see Figure 4A. The effect was short-lived and peaked 5 min after administration. Although tail-flick latencies were affected in both diabetic and non-diabetic mice, the effect was more prominent in diabetic animals. A low dose of 0.5 pmol SP$_{1-7}$ resulted in a significant increase in tail-flick latency in diabetic mice, whereas non-diabetic mice were devoid of an effect at that dose. This result may indicate that a low dose of SP$_{1-7}$ can ameliorate thermal hypersensitivity induced by diabetes because control animals are unaffected.
Figure 4. Effect of A) SP\textsubscript{1-7} and B) SP\textsubscript{1-7-NH\textsubscript{2}} on tail-flick latency in diabetic and non-diabetic mice 5 min after i.t. administration. Data is expressed as the % MPE ± SEM. ** p <0.01, *** p<0.001 versus respective control group (One-way ANOVA followed by Dunnett’s multiple comparison test); n = 8-10 mice per group.

I.t. injection of SP\textsubscript{1-7-NH\textsubscript{2}} seemed to yield a more prominent analgesic effect than those of the native heptapeptide (see Figure 4B). A significant increase in tail-flick latency was found in diabetic animals as well as control animals. A reason for the potent effects of SP\textsubscript{1-7-NH\textsubscript{2}} could be due to the higher binding affinity for SP\textsubscript{1-7-NH\textsubscript{2}} towards the SP\textsubscript{1-7} binding site (Fransson et al., 2008). Another plausible reason for these biological effects may lie in the stability, because C-terminal amidation of peptides occurs in order to increase stability, i.e. protect from enzymatic degradation (Eipper et al., 1992).

The effect of opioid and sigma receptor ligands on SP\textsubscript{1-7}-induced behavior

In order to investigate the role of the opioid system on the prolonged tail-flick latency caused by SP\textsubscript{1-7} (Paper I) and SP\textsubscript{1-7-NH\textsubscript{2}} (Paper II), animals were pre-treated with opioid receptor antagonists. Data on SP\textsubscript{1-7} is presented in Figure 5. The non-specific opioid antagonist naloxone completely blocked the effect of SP\textsubscript{1-7}. This is in line with previous studies, in which SP\textsubscript{1-7}-induced antinociceptive and anti-inflammatory effects are reversed by naloxone (Stewart et al., 1982; Wiktelius et al., 2006). However, selective µ, δ, and κ opioid receptor block with β-funaltrexamine (β-FNA), naltrexol (NTI) or nor-binaltorphimine (nor-BNI), respectively, could not antagonize the effect of SP\textsubscript{1-7}. These results suggest that the SP\textsubscript{1-7} binding site is distinct from the classical opioid receptors, which are in agreement with receptor binding studies, in which SP\textsubscript{1-7} did not compete with specific opioid receptor
ligands (Botros et al., 2006; Igwe et al., 1990a). Nevertheless, the fact that naloxone could block the effect of SP\textsubscript{1.7} still remains.

Because naloxone has been reported to bind to a sub-type of sigma receptors (Tsao and Su, 1997), and sigma-1 receptors have been suggested to possess anti-opioid actions (Chien and Pasternak, 1993), the next step was to investigate the role of the sigma receptor system in the biological effects of SP\textsubscript{1.7} in diabetic animals. Animals were pre-treated with either the sigma-1 receptor agonist \textit{(+)pentazocine \textit{[(+)PTZ]} or the sigma-1 receptor antagonist BD1047 (see Figure 6). \textit{(+)PTZ completely blocked the effect of SP\textsubscript{1.7} in diabetic mice, whereas BD1047 did not affect the behavior induced by SP\textsubscript{1.7. The effective blocking of the heptapeptide-induced effect by \textit{(+)PTZ is indicative of the involvement of the sigma-1 receptor in the mediation of the SP\textsubscript{1.7} effect. However, as no distinct interaction between the sigma-1 receptor agonist and the binding sites of SP\textsubscript{1.7} could be confirmed it was suggested that the interaction with the sigma-1 receptor occurs downstream from the SP\textsubscript{1.7} binding sites. Another plausible explanation could be that SP\textsubscript{1.7} acts on a naloxone-sensitive sigma receptor-binding site, which would account for the biological effects seen from both naloxone as well as \textit{(+)PTZ in the present work. However, this remains to be confirmed.
Effect of SP\textsubscript{1.7} and its amidated and truncated forms

In Paper III, SP\textsubscript{1.7} was evaluated for its effect on mechanical allodynia, acute nociception and motor impairment in rats with SCI. SP\textsubscript{1.7} was administered i.p. in cumulative doses of 1.85, 18.5 and 185 nmol/kg. SP\textsubscript{1.7} could alleviate signs of mechanical allodynia in SCI rats see Figure 7. Although 18.5 nmol/kg tended to increase the mechanical threshold, a statistically significant difference was not detected until 15 and 30 min after the administration of 185 nmol/kg. SP\textsubscript{1.7}-NH\textsubscript{2} was shown to be even more potent, with a noticeable anti-allodynic effect at 18.5 nmol/kg. An increased response after SP\textsubscript{1.7}-NH\textsubscript{2} was expected according to the results in papers I and II, but also from a previous study on opioid withdrawal (Zhou et al., 2009). The increased efficacy of SP\textsubscript{1.7}-NH\textsubscript{2} correlates well to its binding affinity, showing a five-fold higher potency compared with SP\textsubscript{1.7} (Fransson et al., 2008).

Similar to the results in papers I and II, the effect on mechanical hypersensitivity appears to increase in a dose-dependent manner. This is in contrast to previous studies, which depict a bell-shaped dose-response curve for SP\textsubscript{1.7} actions (Herrera-Marschitz et al., 1990; Stewart et al., 1982), with a peak in antinociceptive effect at 18.5 nmol/kg in mice (Stewart et al., 1982). Our results in paper III are more in line with the data presented in papers I and II, which also show a dose-dependent increase in analgesia for SP\textsubscript{1.7}, as well as SP\textsubscript{1.7}-NH\textsubscript{2}. Dose-dependent effects were also seen for the heptapeptides on anti-inflammatory actions and opioid withdrawal in rats (Wiktelius et al., 2006; Zhou et al., 2009). However, detailed comparisons between the different studies are difficult due to the use of different rat and mouse strains, routes of administration and behavioral tests performed.
Figure 7. Effects of (A) substance P (SP) fragment SP₁₋₇, (B) SP₁₋₇-NH₂, (C) SP₂₋₇-NH₂, (D) SP₃₋₇-NH₂, (E) SP, and (F) saline, on vocalization thresholds (mechanical hypersensitivity measured in the von Frey test) in spinal cord-injured (SCI) rats. Data were plotted as the mean ± S.E.M; n = 8 in all except B, where n = 6. * P < 0.05 versus baseline (time 0; Wilcoxon Signed Rank test). # P < 0.05 versus the vocalization threshold for saline-treated SCI rats at the corresponding time point (Mann-Whitney U-test).

Because the amidated heptapeptide appeared to be more efficacious than native SP₁₋₇, and because C-terminal amidation is present in several endogenous neuropeptides (Eipper et al., 1992), it is tempting to speculate that amidation of the heptapeptide occurs in vivo. However, an amidated form of SP₁₋₇ has not yet been detected in vivo.

As previously mentioned, the amino acid Phe⁷ is essential for SP₁₋₇ in order to bind to its binding site and to yield biological effects and N-terminal truncations down to a tripeptide have yielded compounds with retained binding affinity to the SP₁₋₇ binding sites (Fransson et al., 2008). In this study, we investigated the role of these amino acids on the biological activity in SCI rats. As shown in Figure 7, removal of Arg¹ and Pro² could be performed without losing the anti-allodynic effect, whereas further truncation resulted
in inactive compounds. One plausible explanation for the lack of activity of the shorter peptides, despite high affinity for the binding site, could be that they are more prone to enzymatic degradation. Another explanation could be altered permeability for the shorter peptides. A decrease in permeability would result in decreased levels of peptide at the site of action, which in turn could result in a decrease, if any, in biological activity.

No compound showed any sign of sedation in the motor tests performed. This finding is of great interest; because several compounds that are effective in this animal model also affect motor performance (Xu et al., 1992). None of the compounds included in this study had any acute antinociceptive effect. The results from this study are summarized in Table 3.

Table 3. Amino acid sequences and $K_i$ values for $SP_{1-7}$ radioligand binding to rat spinal cord membrane. $K_i$ data from (Fransson et al., 2008), SP data from (Botros et al., 2006), and effects of the respective peptides on mechanical hypersensitivity (von Frey test), acute nociception (tail-flick test) and motor impairment. Statistically significant effect at (+) 185 nmol/kg; (++) 18.5 nmol/kg body weight.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>$K_i \pm$ SEM (nM)</th>
<th>Mechanical allodynia</th>
<th>Acute nociception</th>
<th>Motor impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>RPKPQQFFGLM-NH$_2$</td>
<td>159 ± 12</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{1-7}$</td>
<td>RPKPQQF</td>
<td>1.6 ± 0.06</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{1-7}$-NH$_2$</td>
<td>RPKPQQF- NH$_2$</td>
<td>0.3 ± 0.02</td>
<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{2-7}$-NH$_2$</td>
<td>PKPQQF- NH$_2$</td>
<td>2.8 ± 0.25</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{3-7}$-NH$_2$</td>
<td>KPKQQF- NH$_2$</td>
<td>4.4 ± 0.1</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{4-7}$-NH$_2$</td>
<td>PQQF- NH$_2$</td>
<td>4.5 ± 0.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$SP_{5-7}$-NH$_2$</td>
<td>QQF- NH$_2$</td>
<td>1.9 ± 0.05</td>
<td>--</td>
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<td>--</td>
</tr>
</tbody>
</table>

Effect of $SP_{1-7}$ and small constrained $SP_{1-7}$ analogues

In paper IV, $SP_{1-7}$ was evaluated for its effect on mechanical hypersensitivity in SNI mice. The dose 185 nmol/kg was chosen according to the results in paper III, in which a dose of 185 nmol/kg was required to detect an antiallodynic effect with $SP_{1-7}$. Although the effect was quite short-lived, the mechanical threshold showed a tendency to increase at 15 min after administration and a significant effect was detected 30 min after administration of $SP_{1-7}$. There was no significant difference compared with baseline after 45 min, see Figure 8A. A similar effect was seen for $SP_{1-7}$-NH$_2$, Figure 8B. Thus, exogenously administered $SP_{1-7}$ and its amidated analogue appear to attenuate mechanical hypersensitivity in a similar fashion to what was reported in SCI rats (Paper III).

Three small constrained $SP_{1-7}$ analogues, lead compounds in the medicinal chemistry program, were evaluated for their effect on mechanical hypersensitivity. The same dose, 185 nmol/kg, was used for these compounds. The amidated dipeptide Phe-Phe-NH$_2$ did not induce any antiallodynic effect.
Although there was a tendency towards an increase in mechanical threshold, see Figure 8C, no statistically significant difference was seen. The lack of effect for Phe-Phe-NH$_2$ was explained by a high *in vivo* clearance and the compound is most likely rapidly degraded in plasma (Fransson et al., 2014). While this compound is suggested to have poor drug-like properties (Fransson et al., 2014), its anti-allodynic effect was still of interest to this study. In fact, it has been shown to alleviate the signs of both thermal and mechanical hypersensitivity in diabetic mice after i.t. administration (Ohsawa et al., 2011).
Figure 8. Effect of A) SP$_{1-7}$, B) SP$_{1-7}$-NH$_2$, C) Phe-Phe-NH$_2$, D) Z-Phe-NH$_2$ E) the constrained Phe-Phe-NH$_2$ analogue and F) control on mechanical hypersensitivity in SNI mice. # p < 0.05, ## p < 0.01 compared to after surgery (baseline) (Wilcoxon signed rank test). * p < 0.05, ** p < 0.01 compared to baseline (Friedman test followed by Dunn’s multiple comparison test).

Z-Phe-NH$_2$ in Figure 8D was the only analogue to induce a slight increase on the mechanical threshold; however, this increase was not statistically significant. There was great variation in response within the group, which most likely contributed to the fact that no statistical difference could be seen.
Similar to Phe-Phe-NH$_2$, Z-Phe-NH$_2$ had high plasma instability in rats with a clearance higher than the rat liver after i.v. administration. The compound was not measurable in rat plasma after oral administration because of extensive metabolic degradation (Fransson et al., 2014). Therefore, it is reasonable to believe that the plasma stability is poor in the mouse, which would explain the lack of efficacy. Interestingly, when Z-Phe-NH$_2$ is infused in rats, it is capable of entering the CNS. Furthermore, the stability in human plasma is extremely high (Fransson et al., 2014), which makes this compound interesting to study further despite its instability in plasma from rats.

The constrained Phe-Phe-NH$_2$ had an anti-allodynic effect similar to the heptapeptides, with a significant effect 30 min after administration, see Figure 8E. Although the significant effect was short lasting, the mechanical threshold did not revert to baseline levels after 60 min. A significant effect was expected with Z-Phe-NH$_2$ because rigidification in the C-terminal yielded a compound with retained binding affinity for the SP$_{1-7}$ binding site, together with improved metabolic stability and cell permeability compared with Phe-Phe-NH$_2$ (Fransson et al., 2014; Fransson et al., 2013). With the improved stability, it would have been expected that the effect would be more long lasting. However, it should be noted that this compound is subject to blood-brain barrier efflux (Fransson et al., 2014), which could account for a decreased anti-allodynic effect.

Conclusive remarks on the behavioral tests

SP$_{1-7}$ and SP$_{1-7}$-NH$_2$ yield significant analgesic effects after spinal administration in low picomolar doses. Our results talk in favor of the finding that specific binding sites are present in the mouse and rat spinal cord (Botros et al., 2006; Igwe et al., 1990a), to which SP$_{1-7}$ and its synthetic analogues bind and exert anti-hyperalgesic-like effects. This is strengthened by previous studies, in which biological effects of spinally administered SP$_{1-7}$ in picomolar doses have been detected (Komatsu et al., 2009; Sakurada et al., 2004; Sakurada et al., 2007). It was previously demonstrated that the SP fragment was antinociceptive when administered peripherally and that the mechanism of antinociception is central (Stewart et al., 1982). Studies on the effects of peripheral administered SP$_{1-7}$ on e.g. opioid tolerance and memory (Huston and Hasenöhrl, 1995; Kreeger and Larson, 1993, 1996; Tomaz and Nogueira, 1997; Zhou et al., 2009; Zhou et al., 2011) are also in favor of a central mechanism of action for this peptide, even though local peripheral effects have been detected in the rat paw (Wiktelius et al., 2006). Interestingly, peripheral SP and NK$_1$ receptors have been shown to have a role in neuropathic pain (Teodoro et al., 2012), suggesting that peripheral actions of the SP system may yield central effects. However, further studies need to be performed in order to verify this hypothesis.
Screen for the SP\textsubscript{1-7} binding site

In paper IV, an attempt to further characterize and identify the SP\textsubscript{1-7} binding site was performed. The high-affinity dipeptide Phe-Phe-NH\textsubscript{2} was screened towards a number of enzymes and receptors that are involved in nociception, but also common drug targets. Results from the behavioral studies presented in this thesis indicate that SP\textsubscript{1-7} and SP\textsubscript{1-7} related compounds act on or downstream to sites sensitive for naloxone and (+)-PTZ. SP\textsubscript{1-7} has previously been suggested to interact with several receptor and enzyme systems, e.g. µ opioid receptors (Krumins et al., 1993; Krumins et al., 1989), NMDA receptors (Zhou et al., 2000), NK\textsubscript{1} (Vigna, 2001; Yukhananov and Larson, 1994) dopamine receptors (Zhou et al., 2003; Zhou et al., 2004) sigma receptors (Hornfeldt et al., 1996) and neuronal nitric oxide synthase (nNOS) (Kovacs et al., 2001). However, the screening results obtained revealed a low or negligible binding of Phe-Phe-NH\textsubscript{2} to the included targets. This is indicative of a poor ability for the targets to recognize the SP\textsubscript{1-7} ligands. However, there is still a possibility that the SP\textsubscript{1-7} binding site is located on a known receptor, but separate from the classical binding site.

Table 4. Binding affinity of the SP\textsubscript{1-7} analogue Phe-Phe-NH\textsubscript{2} (10 \textmu M) to a selection of enzymes and receptors related to pain. For a complete list, see Paper IV.

<table>
<thead>
<tr>
<th>Target</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate, NMDA, agonism</td>
<td>16</td>
</tr>
<tr>
<td>Glutamate, NMDA, Glycine</td>
<td>12</td>
</tr>
<tr>
<td>Glutamate, NMDA, Phencyclidine</td>
<td>1</td>
</tr>
<tr>
<td>nNOS</td>
<td>6</td>
</tr>
<tr>
<td>Opiate, δ</td>
<td>17</td>
</tr>
<tr>
<td>Opiate, κ</td>
<td>-3</td>
</tr>
<tr>
<td>Opiate, µ</td>
<td>-11</td>
</tr>
<tr>
<td>Sigma-1, σ₁</td>
<td>-5</td>
</tr>
<tr>
<td>Tachykinin NK₁</td>
<td>4</td>
</tr>
</tbody>
</table>

SP\textsubscript{1-7} in human CSF

In paper V, human CSF was analyzed for SP\textsubscript{1-7}-like immunoreactivity using RIA. The mean level in CSF from 11 patients with severe neuropathic pain was significantly lower than that recorded in samples from controls, see Figure 9. The level of SP\textsubscript{1-7}-like immunoreactivity in the neuropathic pain group was 11.7 ± 1.7 fmol/mL (range 3.4 – 22.4 fmol/mL) compared with the controls that had 18.1 ± 2.0 fmol/mL (range 8.7 – 34.5 fmol/mL). This is, to our knowledge, the first time SP\textsubscript{1-7} has been measured in CSF from patients with neuropathic pain. The finding that SP levels are reduced in chronic pain pa-
tients has been presented previously in CSF (Almay et al., 1988; Nutt et al., 1980), as well as in saliva (Parris et al., 1990). In addition, another study has shown that SP levels are reduced supraspinally, as measured in ventricular fluid (Jost et al., 1991), which suggests decreased synthesis of SP, as well as SP release, not only at the spinal level. The levels of SP in lumbar CSF mainly reflect release from primary afferents (Nutt et al., 1980). Thus, our results revealing lower SP₁₋₇ levels in neuropathic pain patients are probably due to low levels of the precursor SP in primary afferents.

Figure 9. Levels of SP₁₋₇-like immunoreactivity in CSF from neuropathic pain patients and controls. n=11 in each group. * p<0.05 (unpaired t-test).

Because SP is known as a pain transmitter, it is plausible that the levels of SP, and subsequently SP₁₋₇ levels, would increase in chronic pain patients. Instead, several studies have failed to show an increase of SP in CSF in patients suffering from pain with a neuropathic character (Almay et al., 1988; Bäckryd et al., 2014; Lindh et al., 1997; Nutt et al., 1980). In the case of fibromyalgia and osteoarthritis, the SP levels are increased in CSF (Russell et al., 1994; Vaeroy et al., 1988). Thus, it seems that SP levels differ depending on the etiology of the pain.

In previous studies, correlations between pain score and SP-immunoreactivity have been observed in patients with osteoarthritis (Lindh et al., 1997) and with postoperative pain, peaking in their pain intensity (Sjöström et al., 1988). The patients included in this study had refrained to use SCS for 48 hours prior to CSF sampling. They did not use any other analgesics and suffered from severe pain. The possibility of finding an inverse correlation between the VAS score and the SP₁₋₇-like immunoreactivity was investigated. However, no correlation was found. This could be due to several reasons. Firstly, the number of samples was small, and secondly, the possibility of finding a correlation between SP₁₋₇ levels and pain score is hypothesized based on knowledge about SP in neuropathic pain. As mentioned previously, SP can be metabolized to SP₁₋₇ by SPE, ACE and NEP (Hallberg and Nyberg, 2003). SPE-like activity is present in human CSF.
(Nyberg et al., 1984), as well as in spinal cord tissue (Karlsson et al., 1997), and in various other areas of the brain (Zhou et al., 2001). A decrease in the activity of SPE-like enzymes would result in decreased conversion of SP to bioactive fragments, including SP1-7, and subsequently an increase of SP. It seems however less likely that the decrease can be explained by altered enzymatic activity only, but it could possibly contribute.

Although the underlying cause of a decrease in SP1-7 levels in CSF from neuropathic pain patients is not clear, it is an interesting finding, which we believe is mainly a consequence of a reduced amount of its precursor SP. This, together with previous studies on SP in different chronic pain conditions (Almay et al., 1988; Bäckryd et al., 2014; Lindh et al., 1997; Russell et al., 1994; Sjöström et al., 1988), suggest a possible role for the SP system, the native undecapeptide, as well as the N-terminal fragment SP1-7 in chronic neuropathic pain.
Conclusions

The studies presented in this thesis have identified that the endogenous SP N-terminal fragment SP$_{1-7}$ has the ability to attenuate signs of thermal and mechanical hypersensitivity in three different animal models of neuropathic pain. In addition, the SP system, in particular SP$_{1-7}$, appears to be affected in humans with neuropathic pain. Specifically, the conclusions were:

- The effect of i.t administered SP$_{1-7}$, could reduce thermal hypersensitivity in diabetic mice and the effect increased in a dose-dependent manner. The effect was more pronounced in diabetic mice compared with controls, which suggests that the hypersensitivity is due to a dysregulation in the SP system. An anti-allodynic effect of SP$_{1-7}$ was observed even after i.p. administration in SCI and SNI rodents.

- C-terminal amidation of SP$_{1-7}$ led to an increase of analgesic effects, a result that can be attributed to the increased binding affinity to the binding site, but most likely also to increased stability. Similar to SP$_{1-7}$, there was a dose-dependent effect on thermal hypersensitivity, and the biological effect was observed in animals with SNI and SCI.

- Blocking the effect of SP$_{1-7}$ with the opioid receptor antagonist naloxone, as well as the sigma-1 receptor agonist (+)-PTZ, could be indicative of an involvement of the opioid system in the mechanism by which SP$_{1-7}$ exerts its effects. However, selective opioid receptor antagonists failed to mimic the effect of naloxone, and a large receptor screen revealed that neither sigma-1 nor the opioid receptors are the target for SP$_{1-7}$. The characteristics of SP$_{1-7}$ binding sites remain to be clarified, although it seems plausible that a down-stream effect of SP$_{1-7}$ involves the opioid or the sigma receptor system.

- N-terminal truncation of SP$_{1-7}$-NH$_2$ indicated the necessity of at least five amino acids (Lys-Pro-Gln-Gln-Phe-NH$_2$) in order to retain biological activity. Further truncation led to inactive compounds, despite the fact that they still bind to the SP$_{1-7}$ binding site with high affinity. This indicates that these five amino acids are necessary for the peptide to reach its binding sites. The binding sites are suggested to be located in the CNS, but most likely also in the periphery.
• In the evaluation of small SP\textsubscript{1-7} analogues, one of the compounds yielded a significant anti-allodynic effect after i.p. administration. This was in agreement with previous \textit{in vitro} studies on stability and permeability. These results can be useful in further modification studies of the lead compound Phe-Phe-NH\textsubscript{2} in the search for novel compounds targeting the SP\textsubscript{1-7} binding site.

• Measurement of the SP\textsubscript{1-7}-like immunoreactivity in CSF from neuropathic pain patients revealed a decrease when compared with that of control patients. This suggests that an imbalance in the SP system may contribute to neuropathic pain, and this finding might be important in order to further understand the role of the SP system/bioactive SP fragments in chronic neuropathic pain.

Finally, although preclinical data are not easily transformed to humans, the finding that exogenously administered SP\textsubscript{1-7} alleviates thermal and mechanical hypersensitivity, together with the fact that SP\textsubscript{1-7} levels may be reduced in neuropathic pain patients, points toward a role of SP\textsubscript{1-7} in chronic neuropathic pain. Overall, these results indicate that the SP\textsubscript{1-7} binding site is a promising target for the development of peptidomimetics and drug-like molecules as potential drugs with the aim to alleviate neuropathic pain.
Svensk sammanfattning


Substans P (SP) är en neuropeptid som finns i nervceller och som frisätts i ryggmärgen vid ett smärtsamt stimuli. Genom att binda till sin receptor, NK₁ receptorn, på en närliggande nervcell bidrar SP till att smärtsignalen skickas vidare upp till hjärnan. Efter att SP har aktiverat sin receptor bryts den ner av enzymbindningar och mindre peptidfragment. Ett av dessa fragment kallas SP₁₋₇, vilken har studerats i detta avhandlingsarbete. SP₁₋₇ består av sju aminosyror (Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH), binder till ett bindningsställe som skiljer sig från NK₁ receptorn och har biologiska effekter som i många avseenden är motsatta till SP. Bland annat har det påvisats att SP₁₋₇ har förmågan att dämpa akut smärta.

Syftet med denna avhandling var att studera SP₁₋₇:s förmåga att kunna motverka smärta av neuropatisk karaktär. SP₁₋₇ och syntetiska SP₁₋₇-analoger har studerats i olika modeller för mätning av smärta med avseende på deras förmåga att hämma just neuropatisk smärta. För att öka förståelsen om hur SP₁₋₇ utövar sin effekt i kroppen, utvärderades effekten av peptider där man tagit bort en aminosyra i taget, s.k. trunkerade varianter av peptiden. Dessutom studerades små syntetiska SP₁₋₇-analoger som binder specifikt till SP₁₋₇:s bindningsställe och som tidigare utvärderats med avseende på deras stabilitet.
i blodet och permeabilitet över biologiska membran. För att få en inblick i SP₁₋₇:s roll vid kronisk neuropatisk smärta, mättes nivåerna av SP₁₋₇ i ryggmärgsvätska från smärtpatienter.


Smärtpatienterna visade sig ha sänkta nivåer av SP₁₋₇ i ryggmärgsvätska jämfört med kontroller, något som skulle kunna bero på en obalan i SP-systemet hos dessa personer. Dessa resultat bidrar till att öka förståelsen om att bioaktiva metaboliter av SP kan spela en roll i utveckling och ”maintenance” av vissa typer av neuropatisk smärta.

Sammantaget visar resultaten i denna avhandling att nivåerna av det bioaktiva peptidfragmentet SP₁₋₇ är sänkta hos en grupp patienter med svår kronisk neuropatisk smärta. Peptidomimetika som binder till samma bindingsställe som SP₁₋₇ kan dämpa tecken på neuropatisk smärta av perifer och centralt ursprung, vilket är ett steg i rätt riktning i sökandet efter nya läkemedel mot kronisk neuropatisk smärta. Mer specifikt kan dessa resultat bidra till ökad kunskap för framtida utveckling av nya läkemedel mot smärta som verkar på samma ställe som SP₁₋₇.
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