The Impact of Substance P (SP) N-Terminal Metabolite SP$_{1-7}$ in Opioid Tolerance and Withdrawal

BY

QIN ZHOU
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ABSTRACT


The heptapeptide SP1-7, a metabolite of the neuroactive peptide substance P (SP), is suggested to play a role in opioid addiction and memory function. These two dimensions are known to involve dopamine and glutamate transmissions mediated through dopamine receptors and N-methyl-D-aspartate (NMDA) receptors, respectively. Research on interactions between SP1-7 and these two neurotransmitter systems may therefore be of importance to increase our understanding of the mechanisms behind opioid tolerance and dependence as well as memory processes. New knowledge in this area may lead to the discovery of new therapeutic routes for treatment of opioid addiction and other neuropsychiatric disorders, as well.

Studies described in this thesis include investigation of adaptive changes during morphine tolerance and withdrawal in brain levels of SP1-7 and in the activity of substance P endopeptidase (SPE), an enzyme responsible for the generation of this fragment. In morphine tolerant and abstinent rats, the SP1-7 level and SPE activity were significantly increased in discrete areas of the brain, which are crucial for the development of opioid tolerance and dependence. Furthermore, significant correlations between the SPE activity and some morphine withdrawal signs were observed. This finding was indicative of an endogenous modulatory mechanism involving both the enzyme and its active peptide product.

The effects of SP1-7 on the expression of morphine withdrawal and its interaction with dopaminergic pathways were examined in this thesis by behavioural tests, microdialysis as well as Northern blot and autoradiography techniques. Pre-treatment of morphine dependent rats with SP1-7 was found to stimulate dopamine release in nucleus accumbens and to inhibit the intensity of withdrawal behaviours. It was further shown to regulate both the dopamine D2 receptor gene transcript and the density of dopamine receptor proteins in mesolimbic dopamine pathways, confirming an interaction between SP1-7 and the dopamine system.

The influence of SP1-7 on glutamate transmission was investigated in morphine naive rats. The expression of the gene transcripts of the NMDA receptor subunits NR1, NR2A and NR2B was regulated in several brain regions involved in opioid withdrawal reactions and memory functions. The result is consistent with a possible decrease glutamate transmission in these areas.

It was concluded that SP1-7 may function as an endogenous modulator of the expression of opioid withdrawal by influencing both dopaminergic and glutamatergic transmission.

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谨将此博士论文集献给
我的父亲周建新，母亲赵桂荣
丈夫彭忠，儿子彭兴
周勤
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   \item \textit{SP₁₋₇} regulates the dopamine D2 receptor gene transcript and inhibits morphine withdrawal signs in rats \textit{(Paper V)}
   \item \textit{SP₁₋₇} regulates dopamine D1 and D2 binding sites in morphine abstinent rats \textit{(Paper VI)}
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PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to the text by their Roman numerals:


IV Q Zhou and F Nyberg: Injection of substance P (SP) N-terminal fragment SP1-7 into the ventral tegmental area modulates the levels of nucleus accumbens dopamine and DOPAC in male rats during morphine withdrawal (2001), submitted.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>CAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAMGO</td>
<td>(D-(\text{Ala}^2), NMe-Phe(^4), Gly-ol)-enkephalin</td>
</tr>
<tr>
<td>DOPAC</td>
<td>dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>EAA</td>
<td>excitatory amino acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NEP</td>
<td>neutral endopeptidase</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PPCE</td>
<td>post proline cleaving enzyme</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>SP(_{1-7})</td>
<td>substance P(_{1-7})</td>
</tr>
<tr>
<td>SPE</td>
<td>substance P endopeptidase</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
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</table>
INTRODUCTION

Substance P (SP) is one of the most extensively studied peptides, which is present in the central nervous system (CNS) and peripheral systems as well. It is commonly accepted as a putative pain transmitter. In pain transmission and in other actions, the peptide interacts with several classical neurotransmitter systems including excitatory amino acids (EAA), dopamine (DA) and noradrenaline (NA) systems. Several studies have shown that the SP system exhibits a modulatory effect on opiate tolerance and dependence. This characteristic is suggested to depend on the SP N-terminal fragment SP1-7. Both the EAA and DA system are known to interact with SP. The studies included in this thesis, were initiated in order to investigate adaptive changes during opioid tolerance and dependence in both endogenous SP1-7 and in the activity of SPE, one of the enzyme responsible for SP1-7 formation. In order to test the hypothesis that DA and EAA systems may be involved in modulatory effects of SP1-7 on morphine actions, we treated morphine dependent rats with SP1-7 and examined abstinence signs, DA transmission, DA receptor gene transcript and DA receptor binding during morphine withdrawal. We also investigated the expression of NMDA receptor subunit mRNAs after SP1-7 injection.

1. Opioids

1.1. Historical background

Opioids are well known for their powerful analgesic and strong reinforcing effects. They are perhaps the oldest drugs abused by human. Nowadays, opioid abuse is a serious society problem. Research on opioid tolerance and dependence forms one of the most attractive branches in the field of drug addiction.

The time when opium originally appeared in human's life can be traced back to at least 5000 years ago (Brownstein, 1993). Opium is obtained from the juice of the unripe seed capsule of opium poppy. At first, opium was applied as an euphoriant, which was called 'gil', the word for joy. In the middle age, it was employed medically as a painkiller and in dysenteric treatment. In 1803 the first active substance isolated by
Sertürner from opium was named morphinum. Today more than 40 alkaloids have been extracted and characterised from opium. In the effort to search for safer, more potent and non-addicting opiates, the first artificial opiate, heroin was synthesised in 1898. It was followed by methadone and the first opioid antagonist nalorphine. In 1971, the existence of a receptor for opiates was suggested (Goldstein, et al., 1971). In 1973, three groups independently and almost simultaneously described stereospecific opiate binding sites in the mammalian brain (Pert and Snyder, 1973, Simon, et al., 1973, Terenius, 1973). Later studies revealed the presence of three distinct opioid receptor subunits, mu, delta and kappa. The confirmed existence of endogenous opioid receptors led to the search for the endogenous ligands of these receptors. Only two years later, Hughes and co-workers isolated two opioid peptides Met-enkephalin and Leu-enkephalin with the ability to inhibit acetylcholine release from the myenteric plexus-longitudinal muscle of the guinea pig ileum and this inhibition was blocked by naloxone (Hughes, 1975). During the following years, three major families of opioid peptides (dynorphins, enkephalins, and β-endorphins) with different genetical origin were identified. The various peptides also were found to exhibit different receptor activation profiles. Enkephalins exhibit high affinity for the delta receptor, dynorphins bind predominantly to the kappa receptor, whereas β-endorphin is believed to be an endogenous ligand for the mu and receptors. Recently, two new endogenous opioid peptides, endomorphin-1 and endomorphin-2, with high affinity and selective preference for the mu opioid receptor were discovered (Zadina, et al., 1997).

1.2. Endogenous opioids

The endogenous opioid peptides mainly comprises β-endorphins, enkephalins (Met-enkephalin and Leu-enkephalin) and dynorphins (dynorphin A and B as well as alpha-neoendorphin), which are produced from three genetically distinct large precursors termed proopiomelanocortin (POMC), proenkephalin and prodynorphin respectively. They contain an identical sequence (Tyr-Gly-Gly-Phe) at their N terminus, followed by either Met or Leu. Recently, two high mu receptor selective opioid peptides endomorphin-1 and endomorphin-2 were discovered. (Table 1) In brain, opioid peptides and their precursors are widely distributed. For instances, β-endorphin and
POMC are found in hypothalamus, limbic pathways, brain stem and spinal cord. Enkephalins and their precursor are found in amygdala, cortex, hippocampus, locus coeruleus, PAG and spinal cord. Dynorphin is present in striatum, amygdala, hippocampus, PAG, and spinal cord. However, opioid peptides are also known to exist in peripheral tissues such as adrenal, the gastrointestinal tract, medulla pancreas and even in the immune cells. In addiction to their analgesic effect, opioid peptides also play important roles in various behaviours including stress, tolerance, dependence, mental illness, mood, learning and memory, seizure and other neurological disorders (for review see (Olson, et al., 1997)).

<table>
<thead>
<tr>
<th>Table 1 Endogenous opioid peptides</th>
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<tbody>
<tr>
<td><strong>Peptides</strong></td>
</tr>
<tr>
<td>Met–enkephalin</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
</tr>
<tr>
<td>Dynorphin A</td>
</tr>
<tr>
<td>Dynorphin B</td>
</tr>
<tr>
<td>α-neoendorphin</td>
</tr>
<tr>
<td>β-endorphin</td>
</tr>
<tr>
<td>Endomorphin-1</td>
</tr>
<tr>
<td>Endomorphin-2</td>
</tr>
</tbody>
</table>

1.3. Opioid receptors

When the opioid binds to its specific receptor depending on tissue it elicits analgesia but also other effects such as respiratory depression, mood elevation and euphoria. As mentioned above, the opioid receptors are divided into three classical types: mu, kappa, delta, each of which belonging to the family of G-protein coupled receptors. The distribution of the three receptors differs. Mu opioid receptors are mainly found in areas related to dopamine circuits and analgesic pathways. This receptor is shown to mediate reward, analgesic effects, motor control and somatic effects of opiate abstinence (Arvidsson, et al., 1995b). The other two receptors are found in spinal cord
and some areas of brain (Arvidsson, et al., 1995a). The structure of each subtype has been elucidated by molecular cloning techniques (Kieffer, et al., 1992, Thomson, et al., 1993, Chen, et al., 1993a). All of the cloned opioid receptors possess the same general structure of an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal tail structure. The mu-opioid receptor (MOR-1) gene shows approximately 50-70% homology to the genes encoding the delta-opioid (DOR-1) and Kappa-opioid (KOR-1) receptors. It seems that all of the opioid receptors share common effector mechanisms. They are coupled through Gi/Go proteins to activate an inwardly rectifying potassium conductance and to inhibit voltage-operated calcium conductance (Grudt and Williams, 1993).

1.4. Opioid tolerance and dependence

Opioid are among the most powerful analgesics but the clinical value is restricted due to their reinforcing property, which are highly addictive both in human and in laboratory animals. Repeated exposure to opiates results in the development of tolerance and dependence. Tolerance is defined by the need for an increased dose of the drug to maintain the same effect (Nestler, 1996). Physical dependence is evidenced by withdrawal syndrome induced by cessation of the drug or administration of an antagonist such as naloxone (Nestler, 1996). In the effort to better understand the mechanisms of tolerance and dependence and to prevent drug addiction, a number of possible mechanisms based on behaviour, as well as events at the cellular and molecular level have been proposed. But so far none of these can completely explain the mechanisms of morphine tolerance and dependence. As illustrated in Figure 1, numerous factors are involved in opioid tolerance and dependence, thus, it is evident that drug tolerance and dependence are complex biological processes that do not relate to a sole mechanism.

Following discovery of the endogenous opioid receptors, studies on the mechanisms of tolerance and dependence have mostly focused on changes at the receptor level. From the beginning, it was hypothesised that tolerance is caused by increased metabolic degradation and reduced affinity of opioids for their receptors as well as receptor down
regulation (James and Starr, 1977, Tao, et al., 1993, Jasinski, 1997). However, studies have failed to support these hypotheses, since data available are not consistent. In 1993, an uncoupling of the opioid receptor from G-proteins was observed during chronic opioid treatment (Tao, et al., 1993) which may partly contribute the mechanism of tolerance after chronic exposure to opioids.

The EAA systems have a function in behavioural and neuronal plasticity. Research suggested that the development of tolerance resemble that of learning and memory establishment. Both of them are the result of behaviour and neuronal adaptation and plasticity. A large body of evidence suggests a role of EAA (such as glutamate) in opioid effects, mediated via the NMDA receptor. Animal behavioral studies have shown that NMDA receptor antagonists attenuate the development of opiate tolerance and decrease withdrawal symptoms (Mao, et al., 1996). Investigations further suggest that the NMDA-mediated effects are linked via activation of nitric oxide synthase (NOS) with subsequent release of nitric oxide (NO), which in turn stimulates the

Figure 1 Factors contributing to opioid tolerance and dependence

formation of cGMP (Bredt and Snyder, 1992). Furthermore, blocking of the NMDA receptor results in decreases NOS and finally attenuates formation of NO. Long term
administration of opioid causes biochemical adaptive alteration in locus coeruleus and mesolimbic dopamine system, as documented by electrophysiological and molecular studies (Nestler, et al., 1993, Nestler, et al., 1994). In cells of these brain regions, adaptations of G-proteins cyclic AMP and in the protein phosphorylation system have been shown to play an important role in mediating opioid tolerance and dependence (Guitart and Nestler, 1993, Nestler, et al., 1993).

The mesolimbic DA system is one of the reward pathways, which plays an important role in opioid dependence and withdrawal (Koob, 1992a, Nestler, 1996). Stimulation of the DA neurones in the mesolimbic pathway leads to positive reinforcement. Activation of opioid receptors in VTA results in enhancing dopamine release in nucleus accumbens. In addition, after chronic opioid exposure, decreased dopaminergic neurotransmission is found during withdrawal.

Furthermore, adaptive changes in certain endogenous anti-opioid peptides were also suggested to underlie the mechanism of opioid tolerance and dependence (Rothman, 1992). Among these anti-opioid peptides are thyrotropin-releasing hormone (TRH), cholecystokinin (CCK-8), neuropeptide FF (NPFF), Tyr-Pro-Leu-Gly-NH₂ (Tyr-MIF), which function as part of a homeostatic system to antagonise opioid effects and are observed to be up-regulated during long term opioid administration. The anti-opioid peptides are believed to be partly responsible for the tolerance and withdrawal syndrome (Rothman, 1992) Studies have shown that these anti-opioid peptides may promote the withdrawal signs in opioid dependent animals, and that antagonism of these peptides attenuates the opioid abstinence reaction (Rothman, 1992).

Moreover, adaptive changes in the intracellular signalling pathways (Nestler, et al., 1993, Buccafusco, et al., 1995, Nestler, 1996), and in other neurotransmitter systems including serotonin, norepinephrine, acetylcholine and SP (Rothman, 1992, Chahl and Johnston, 1993, Bhargava, 1994, Chen and Liu, 1996, Nestler, 1996) may be involved in the mechanisms underlying opioid tolerance and dependence. Finally, increasing body of evidence have verified that genetic and environmental factors, such as stress,
should also be considered as establishing individual responsiveness to drugs of abuse (George and Goldberg, 1989).

2. Substance P

2.1. Substance P

About seventy years ago, SP was discovered by Von Euler and Gaddum, and since then this peptide has been extensively investigated (Von Euler and Gaddum, 1931). SP belongs to the tachykinin family of neuropeptides and is composed of eleven amino acids (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) (Table 2). SP is widely distributed in the mammalian CNS but is present in peripheral tissues, as well, (for review see (Cuello and Kanazawa, 1978, Pernow, 1983, Maggio, 1988, Watling and Krause, 1993)). It is commonly accepted that SP is a transmitter utilised by primary sensory affronts (Kellstein, et al., 1990, Radhakrishnan and Henry, 1995). Studies have confirmed that SP is released from the spinal cord following noxious stimulation (Brodin, et al., 1987, Duggan, et al., 1987, Yaksh, 1988). It is also known to elicit neurotrophic, regenerating as well as memory-promoting effects (Nikolaus, et al., 1997). Furthermore, SP has been implicated in the control of various neuronal-behavioural functions including reinforcement, opioid withdrawal and learning processes (Chahl and Johnston, 1993, Huston, et al., 1993, Hasenohrl, et al., 1998).

Table 2 Structure of tachykinins

<table>
<thead>
<tr>
<th>Tachykinins</th>
<th>Sequence</th>
<th>Receptor</th>
<th>Localization</th>
</tr>
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<tbody>
<tr>
<td>Substance P</td>
<td>Arg</td>
<td>NK1</td>
<td>PNS,CNS</td>
</tr>
<tr>
<td>Substance K</td>
<td>Pro</td>
<td>NK2</td>
<td>PNS,CNS</td>
</tr>
<tr>
<td>Neurokinin B</td>
<td>Pro-Glu</td>
<td>NK3</td>
<td>CNS</td>
</tr>
<tr>
<td>Eledoisin</td>
<td>Pro-Ser</td>
<td>NK2</td>
<td>PNS,CNS</td>
</tr>
<tr>
<td>Physalaemin</td>
<td>Pro-Ala</td>
<td>PNS,CNS</td>
<td>CNS</td>
</tr>
</tbody>
</table>

2.2. Distribution and pathways of SP
SP is distributed throughout the mammalian CNS but is also present in peripheral tissues including small diameter primary sensory fibres (Hökfelt, et al., 1975) and cells of the endocrine system, salivary gland and the gastrointestinal tract (Pernow, 1983, Maggio, 1988, Watling and Krause, 1993). Within the CNS SP has been localised in the dorsal horn of the spinal cord (Hökfelt, et al., 1977, Ribeiro da Silva and Hökfelt, 2000) as well as in various regions of the brain (Warden and Young, 1988, Medina and Reiner, 1995, Ribeiro da Silva and Hökfelt, 2000). In brain, neurones containing transcript for SP is present in the neocortex, hippocampus, olfactory bulb and associated areas, e.g. caudate-putamen, hypothalamus and central grey (Warden and Young, 1988). The highest concentration of SP is found in nigrostriatal pathway, a moderate density of substance P-immunoreactive fibres has been observed in the vicinity of mesolimbic DA perikaryas in the VTA and mesolimbic DA terminals in the nucleus accumbens (Kalivas and Miller, 1984). In SP pathways are shown to project from striatum to substantia nigra and from nucleus accumbens to VTA (Chang, 1988, Elliott, et al., 1992) (Figure 2). The anatomical overlapping of SP and DA suggests a link between these two systems. In fact, experiments have confirmed that SP-induced animal behaviors are modulated through dopamine activity (Stinus, et al., 1978), furthermore, dopamine transmission was stimulated by administration of SP (Reid, et al., 1990).

**Figure 2** The pathways of SP in rat brain
There are four main SP pathways in brain, which have close relationship with dopamine systems. They are: from nucleus accumbens (NAC) to ventral tegmental area (VTA) (Medina and Reiner, 1995); from striatum to substantia nigra (SN) (Krause, et al., 1984); from striatum to pallidum (Medina and Reiner, 1995); from habenular (Hbe) to VTA (Cuello, et al., 1978).
By using immunohistochemistry and in situ hybridization techniques, it is demonstrated that SPergic neurons are co-localised with other neurotransmitters, including NO, as indicated by the presence of NOS (Kimura and Steinbusch, 1996), glutamate (Barraglia and Rustioni, 1988), opioid peptides (Hökfelt, et al., 1977, Chang, 1988, Elliott, et al., 1992, Kimura and Steinbusch, 1996), calcitonin gene-related peptide (CGRP) (Gibson, et al., 1984) and serotonin (Tomas, et al., 2000) in certain neuronal pathways. Behavioural and neurochemical studies have further shown that SP widely interacts with these systems (Chang, 1988, Smullin, et al., 1990, Elliott, et al., 1992, Tiong, et al., 1992, Boix, et al., 1994).

2.3. Substance P Receptor and functions

In 1980, high affinity binding sites for SP were found in mammalian CNS (Hanley, et al., 1980). The tachykinin receptors have then been cloned and classified as NK-1 (neurokinin-1), NK-2 (neurokinin-2) and NK-3 (neurokinin-3). All these receptors are G-protein coupled receptors which, exhibit different affinity for the various tachykinins (Ohkubo and Nakanishi, 1991). SP is the endogenous ligand to the NK-1 receptor. Whereas, neurokinin A (NKA) and neurokinin B (NKB) preferentially bind to the NK-2 and NK-3 receptors, respectively (Table 2). The distribution of mRNA for the substance P receptor (NK-1) has been investigated in the rat brain by in situ hybridization using synthetic oligonucleotide probes. NK-1 positive cells were distributed throughout the brain. A high intensity of the hybridization signal was observed in the basal ganglia and the dorsal tegmental area. A moderate and weak signals was detected in other regions, such as hippocampus, hypothalamus, midbrain, and medulla oblongata (Maeno, et al., 1993). All these areas are critical for the regulation of pain influx, affective behavior and stress. Usually, NK-1 receptors are located in the same area as SP, however, in substantia nigra almost no NK-1 receptor transcript was detectable.

Studies have revealed that NK-1 receptors are implicated in several physiological and pathological behaviors. It was also demonstrated that several C-terminal fragments of SP share the same receptor as SP to exert their effects (Burcher and Chahl, 1988,
Regoli, et al., 1988). Behavioral studies showed that stimulation of the NK-1 receptor in normal rat elicits behaviors mimicking some of those seen during morphine abstinence (Elliott, et al., 1986). In fact the NK-1 receptor is shown to be involved in opioid withdrawal reactions, thus blocking of the NK-1 receptor in morphine dependent guinea pigs decreases withdrawal behaviors (Maldonado, et al., 1993). Besides, the NK-1 receptor has been implicated in opioid reward (Murtra, et al., 2000). In NK-1 knockout mice, the rewarding properties of opiate were lost (Murtra, et al., 2000). Moreover, the withdrawal reactions in the transgenic mice were reduced (Murtra, et al., 2000). Furthermore, NK-1 receptor was recently found to be involved in depression (Kramer, et al., 1998). Thus, blocking of the NK-1 receptor with antagonist decreases the above mentioned behaviors. In addition, aggressive behavior was decreased in mice with a genetic disruption of the NK-1 receptor (De Felipe, et al., 1998).

2.4. Interaction between SP and opioids
Interactions between SP and opioids have been observed in various studies. For example, SP concentration is decreased in the rat CNS after a single morphine injection (Le Grevès, et al., 1990) and a similar change was also measured in guinea-pigs (Chahl and Chahl, 1994). However, in contrast to acute experiment, the SP level was shown to increase during chronically administration of morphine (Nylander, et al., 1991). Enhanced SP concentration was thus detected in dorsal spinal cord, striatum and medulla (Bergstrom, et al., 1984, Nylander, et al., 1991), suggesting that compensatory mechanisms are activated to maintain SP levels. In the dorsal horn of the spinal cord, morphine and the opioid peptide enkephalin inhibit release of SP from primary afferent terminals by a presynaptic action on SP-ergic nerve terminals (Jessell, et al., 1979, Yaksh, 1988). On another hand, studies have demonstrated that SP modulates the withdrawal reaction to opioids and has also been identified as a mediator of the abstinence reactions in guinea pigs (Chahl and Johnston, 1993) and rat (Sharpe and Jaffe, 1989). Kreeger and co-worker reported that SP inhibits morphine withdrawal signs in mice but this effect was later demonstrated to be caused by an N-
terminal fragment of SP which rapidly is released from the parent peptide in the extracellular fluid of the brain (Kreeger and Larson, 1993).

2.5. Substance P metabolism and SPE

The action of SP is terminated by enzymatic degradation after the release and binding to the NK-1 receptor (Lee, et al., 1981, Matsas, et al., 1983, Nyberg and Terenius, 1991). There are a number of proteases in human CSF and spinal cord which have the capacity to inactive or cleave SP to produce bioactive fragments (see Fig. 3) (Nyberg, et al., 1987, Nyberg and Terenius, 1991). In rat spinal cord and CSF, enzymes with similar action such as SPE, NEP, and ACE have been thoroughly studied (Nyberg, et al., 1984, Persson, et al., 1992a, Persson, et al., 1995). SPE was described as a metal-dependent and thiol-sensitive endoprotease, which was first identified and isolated from human cerebrospinal fluid by Nyberg and co-workers (Nyberg, et al., 1984). In rat, the SPE-like activity has been found both in CSF (Persson, et al., 1995) and in spinal cord tissues (Karlsson, et al., 1997). This enzyme has comparatively high substrate and cleavage specificity for SP, but not for neurokinin A, neurokinin B, eledoisin and opioid peptides (Nyberg, et al., 1984, Nyberg, et al., 1986). The enzyme cleaves SP at its Phe$^7$-Phe$^8$ and Phe$^8$-Gly$^9$ bonds to yield the N-terminal fragments SP$_{1-7}$ and SP$_{1-8}$, both with bioactivity either similar or opposite to SP. SPE is not inhibited

Figure 3 Summary of metabolism of Substance P
by phosphoramidon and captopril, which inhibit NEP and ACE, respectively. However it is suggested that SPE can be inhibited by CGRP, a neuropeptide that coexists with SP both in CNS and PNS (Le Grèves, et al., 1985, Nyberg, et al., 1988a). In the process of measuring the activity of SPE, a cocktail of phosphoramidon and captopril is usually included in the reaction mixture to prevent for degradation by NEP and ACE.

In rat CSF, a SPE-like enzyme was found to be affected during chronic pain conditions (Persson, et al., 1992a). In human CSF, the level of SPE activity was altered in the patients with pain from herniated lumbar discs (Lindh, et al., 1996) and in the patients with low back pain (Hyyppä, et al., 1990). In women at term pregnancy, the activity of the enzyme was also changed (Liu, et al., 1997). Furthermore SPE was modulated in CSF of morphine tolerant rat (Persson, et al., 1989).

3. Substance P$_{\text{1-7}}$

SP$_{\text{1-7}}$ is a major metabolic product of SP, which released from SP by enzymatic degradation (Lee, et al., 1981, Matsas, et al., 1983, Nyberg and Terenius, 1991). The SP$_{\text{1-7}}$ fragment has been the subject for many studies. This fragment has been suggested to retain some of the biological activity of its parent peptide (Stewart, et al., 1982, Huston, et al., 1993). Actually, some effects of SP are encoded by its N-terminal

![Figure 4](image-url) Substance P and its N- and C-terminal fragments: proposed mechanism of action
part, e.g. memory-enhancing effect (Huston, et al., 1993, Huston and Hasenohrl, 1995, Tomaz, et al., 1997). However, in many other cases, SP1-7 has opposite effects to SP (Persson, et al., 1995) or to bioactive C-terminal SP fragments (Skilling, et al., 1990). Extensive research has shown that SP1-7 is implicated in several functions of the mammalian nervous systems including antinociception (Stewart, et al., 1982), motor behavior (Reid, et al., 1990), stress behavior (Roske, et al., 1986), memory (Tomaz, et al., 1997) and opioid withdrawal reactions (Kreeger and Larson, 1993) (Fig. 4). In this thesis, the effect of SP1-7 on morphine abstinence rats has received a particular focus.

3.1. CNS distribution of SP1-7
The presence of SP1-7 has been confirmed in rat CNS (Sakurada, et al., 1985, Le Grevès, et al., 1990) as well as in human CSF (Rimon, et al., 1984). In brain, SP1-7 is found in substantia nigra, striatum, VTA and nucleus accumbens. In rat spinal cord, SP1-7 is concentrated in dorsal horn, the ratio between SP and SP1-7 is ranged from approximately 5:1 to 10:1, whereas the corresponding figures in brain tissues were approximately between 10:1 and 25:1 (Sakurada, et al., 1985, Sakurada, et al., 1991).

3.2. Specific binding sites of SP1-7
In 1990, Igwe and colleagues performed an experiment to test the hypothesis that the SP1-7 has its own distinct receptor that could specifically account for its pharmacological and physiological effects. The results strongly supported the existence of a specific N-terminal directed SP receptor, which mediates the effect of SP1-7. This binding was found to be specific, saturable, and reversible (Igwe, et al., 1990). In the brain, SP1-7 has high affinity to this binding site (Ki = 2.6 nM; IC50 = 5.4 nM), whereas the C-terminal 3-11 and 5-11 fragments show very low affinity to the same binding site (Ki > 10,000 nM; IC50 > 10,000 nM). In addition, SP also exhibits low binding to this receptor, (Ki = 28 nM; IC50 = 49 nM). This study further suggest that the SP1-7 binding site shares some characteristic of the mu receptor (DAMGO shows some binding affinity), although it does not seem to be identical as mu-opioid receptor (Krumins, et al., 1989). Research shows that β-FNA, a specific non-equilibrium or irreversible mu-opioid antagonist, failed to alter the binding of
DAMGO to the N-terminal site, indicating that the SP\textsubscript{1-7} binding site is not identical to the opioid receptor. Further evidence is provided by the observation that Nalz (a highly selective and irreversible antagonist for mu\textsubscript{1}-opioid receptor) was not capable to affect \textsuperscript{3}H-SP (1-7) binding to brain membranes. Moreover, naloxone and sufentanil, which both have high affinity for the mu-opioid receptor, did not compete with SP\textsubscript{1-7} binding (Igwe, et al., 1990). This strongly demonstrated that SP\textsubscript{1-7} has its own distinct receptor. However, so far no successful attempt to clone the SP\textsubscript{1-7} receptor has been reported. The putative SP\textsubscript{1-7} receptor was suggested to be implicated in modulating opioid withdrawal signs in mice (Kreeger and Larson, 1993) and in modulating the NMDA receptor-induced activity in mice spinal cord (Hornfeldt, et al., 1996).

3.3. Interaction between SP\textsubscript{1-7} and opioids

Behavior test (Kreeger and Larson, 1993) has demonstrated that SP\textsubscript{1-7} mimics (Stern and Hadzovic, 1973) the ability of SP to inhibit or modulate the abstinence reaction to acute opioid withdrawal in mice (Kreeger and Larson, 1993). Thus, intrathecal injection of SP\textsubscript{1-7} prior to naloxone-precipitation of withdrawal reactions in morphine-dependent mice, was found to cause a dose-related attenuation of withdrawal jumping response (Kreeger and Larson, 1993). In 1996, the same research group adduced evidence that SP\textsubscript{1-7} has the unique characteristic of inhibiting the development of tolerance and attenuating withdrawal signs when given systemically to the morphine dependent mice (Kreeger and Larson, 1996). This effect was blocked by the specific SP\textsubscript{1-7} antagonist (D-Pro\textsuperscript{2}, D-Phe\textsuperscript{7})-SP\textsubscript{1-7}, suggesting the effect to be mediated through specific receptors for the heptapeptide fragment of SP (Igwe, et al., 1990, Larson and Sun, 1993). Kreeger et al suggested that the N-terminal fragment, which is rapidly released from the parent peptide in the extracellular fluid of the brain, should be responsible for the effect seen following SP injection (Kreeger and Larson, 1996). Although it seems that SP\textsubscript{1-7} receptors may mediate the modulatory effect of SP\textsubscript{1-7} on opioid withdrawal signs, an indirect interaction between SP\textsubscript{1-7} and the opioid receptor has also been suggested to contribute some effects of the heptapeptide, e.g. SP\textsubscript{1-7} produced antinociception in mouse, which was reversed by naloxone (Stewart, et al.,
1982). As mentioned above, an influence of SP\(_{1-7}\) on opioid receptor binding was observed in mouse brain membrane (Krumins, et al., 1989, Krumins, et al., 1993).

3.4. Interaction between SP\(_{1-7}\) and dopamine

The nigrostriatal dopamine pathway is described as a crucial circuit related to motor behaviour. In rats, activation of dopamine neurones in substantial nigra by SP\(_{1-7}\) will enhance behaviour such as locomotion (Hall and Stewart, 1992). Studies also confirmed that SP\(_{1-7}\) stimulates dopamine release in striatum (Reid, et al., 1990, Hall and Stewart, 1992). However, as to our knowledge, no experiments have been so far carried out to measure the effect of SP\(_{1-7}\) on dopamine release in morphine dependent rats.

4. NMDA receptor

The NMDA receptor is a voltage-dependent calcium channel which under normal resting conditions is blocked by magnesium (Dingledine, et al., 1999). In the cell membrane the NMDA receptor constitutes a tetrameric heteromeric assemblies of five subunits, NR1 and NR2A-D, that have different physiological and pharmacological properties and are differentially distributed throughout the CNS (Nakanishi, 1992, Dingledine, et al., 1999). All these five subunits have been cloned and their amino acid sequences have been determined. There are two broad subtypes of glutamate receptor. One of them is called ionotropic glutamate receptors, including kainate, NMDA and AMPA receptors. The other is the metabotropic glutamate receptor, which transfers information via second messengers (Sheng, 1997). NMDA receptors play a key role in brain function including synaptic plasticity and long-term potentiation, which is believed to be the basis of learning and memory (Hollmann and Heinemann, 1994, Dingledine, et al., 1999). It also underlies both the behavioral and neuroadaptive effects of morphine (Koyuncuoglu, et al., 1994).

4.1. NMDA receptor in opioid tolerance and dependence

As mentioned above, it is well known that the NMDA receptor plays an important role in development of morphine tolerance and in morphine withdrawal (Marek, et al.,
Accordingly, studies have shown that application of NMDA receptor antagonists prevents or reduces morphine tolerance as well as attenuates morphine withdrawal symptom. During exposure to morphine, the affinity and sensitivity of ligands for the NMDA receptor are changed, and this change may be due to alterations in the endogenous level of glutamate. As a result, the Ca\(^{2+}\) current influx will be influenced, which in turn affects the release of neurotransmitter e.g. acetylcholine (Cheney, et al., 1975, Zocchi and Pert, 1994), a neurotransmitters believed to be involved in morphine withdrawal syndrome. Further evidence to support the involvement of NMDA receptor in opioid dependence emerges from the observations that chronic and acute treatment of morphine regulates the NMDA receptors in mouse CNS (Bhargava, et al., 1995). Moreover, the NMDA receptor is linked to an increased NOS activity (Bredt and Snyder, 1992, Zocchi and Pert, 1994) and NOS is shown to be involved in opioid withdrawal as well.

4.2. Expression of NMDA receptor subunit mRNAs in opioid treated animals

It is well known that the pharmacological characteristics of the NMDA heteromeric receptor depend on the expression of NR2 subunits (Kutsuwada, et al., 1992, Nakanishi, 1992, Hollmann and Heinemann, 1994, Jang, et al., 1998). The NR2 subunits strongly influence the electrophysiological properties and the drug binding profile of the recombinant NMDA receptor. Therefore it seems that any change in the levels of NR2 will affect the function of NMDA receptor. The acute effect of morphine on the expression of the mRNAs for NMDA receptor subunits in the rat hippocampus, hypothalamus and spinal cord has been examined (Le Grevès, et al., 1997). This may reflect an adaptive change that leads to opioid dependence (Le Grevès, et al., 1998). In general, chronic opioids induce a down-regulation in the message of the NR2A and NR2B receptor. The down-regulation of the NMDA receptor transcripts would be consistent with an enhanced glutaminergic activity (Pulvirenti, et al., 1991, Huang, et al., 1997).

5. Dopamine and dopamine receptors

5.1. Dopamine in the reward system
The mesocorticolimbic pathway is well known by the role it played in natural and drug rewards (Koob, et al., 1998). The key role of this system in actions of opioids is also well established (Koob, 1999). This system originates in the A10 dopamine cells of the VTA and projects to the nucleus accumbens (NAc), olfactory tubercle (Koob, 1992a). Mesolimbic DA system has been functionally implicated in a variety of behaviors (e.g. arousal, motor activity, selfadministration of drugs, and some cognitive behaviors).

The majority of neurons in this pathway are between VTA and NAc, which described as central components of the circuitry underlying reward and related memory (Koob, 1992a, Kalivas and Nakamura, 1999, Mansvelder and McGehee, 2000). The activity of dopaminergic neurones in the VTA appears to be linked to reward prediction (Vezina and Stewart, 1985). Moreover, VTA is believed to be necessary for the elicitation of conditioned morphine reward (Vezina and Stewart, 1984). Previous studies have confirmed that nucleus accumbens served as a critical substrate for reinforcement of opioids (Koob, et al., 1998). NAc has been implicated not only in opioid reward, but is also believed to serve as substrate for the aversive stimulus effects of opioid withdrawal (Stinus, et al., 1990). It was therefore suggested that dopamine transmission in this is associated with abstinence behaviours (Koob, et al., 1989, Stinus, et al., 1990).

In addition to VTA and NAc, the frontal cortex also plays an important role in drug addiction. Dopaminergic innervating frontal cortex is strongly associated with regulation of working memory (Robbins, 2000). This area was described to be associated with drug-seeking and self-administration behaviours (Shaw, et al., 1984, Everitt and Robbins, 2000). D2 receptors in frontal cortex are shown to be implicated in working memory processes, behavioural flexibility and exploratory activity (Robbins, 2000) and activation of the D2 receptor with a specific agonist was found to increase cognitive performance in human (Robbins, 2000).

5.2. Dopamine receptors
The DA receptors belong to the G-protein receptor families. To date, five distinct genes encoding different DA receptor proteins have been isolated and characterized (Sibley, 1999). DA receptors are divided into D1 and D2 subfamilies depending on their structure and pharmacological functions. The D1 and D2 receptors are encoded by distinct genes that are only about 29% homologous at the amino acid level (Gerfen, et al., 1995). The D1 family includes D1 and D5 subtypes, whereas the D2 family contains D2, D3 and D4 subtypes. DA receptors are widely distributed in various CNS regions, but are also found in peripheral tissues such as the pituitary, parathyroid glands, as well as the kidney (Missale, et al., 1998). In brain, the distribution of different subtypes of dopamine receptors is not even. For example, D1 and D2 receptors are subtypes predominately found in striatum with both presynaptic and postsynaptic locations (Sibley, 1999). However, in VTA, only D2 autoreceptor can be observed (Meador-Woodruff, et al., 1991). D3, D4 and D5 receptors are expressed more restrictedly in the brain. The function of D1 and D2 are quite different, e.g. D1 and D2 receptors modulate adenylate cyclase activity in opposite way (Gerfen, et al., 1995). Activation of the D1 receptor will increase the adenylate cyclase activity, whereas D2 agonists decrease the activity of adenylate cyclase. D2 receptors in NAc are associated not only with opiate rewarding effects (Maldonado, et al., 1997) but also opiate withdrawal syndrome (Harris and Aston-Jones, 1994). Place-preference test demonstrated that D2 receptor play a crucial role in the motivational component of drug addiction, thus opiate loss its effect in D2 knocked-out mice (Maldonado, et al., 1997). It was observed in behavior test that activation of D2 receptor with a specific agonist inhibited opioid withdrawal syndrome (Harris and Aston-Jones, 1994). Moreover, D1 receptors in NAc was suggested to be implicated in memory processes (Fenu, et al., 2001).

5.3. Dopamine system in opioid withdrawal

As mentioned above, the involvement of the mesolimbic dopamine system in opioid withdrawal has been suggested by Koob and co-workers (Koob, et al., 1989, Stinus, et al., 1990). Accordingly, NAc is implicated not only in positive reinforcement elicited by opioids, but it is also known as a critical target for mediating aversive stimulus
properties of opioid withdrawal (Koob, et al., 1989, Stinus, et al., 1990). It was noticed in several studies that inhibited DA transmission in NAc is usually accompany with opiate abstinence (Acquas, et al., 1991, Pothos, et al., 1991, Crippens and Robinson, 1994). E.g. microdialysis studies have indicated that the dopamine outflow in NAc is decreased following opioid withdrawal (Pothos, et al., 1991). Decreased dopamine release in NAc during opioid withdrawal was suggested to be associated with aversive state and underlie mechanism of this aversive state. (Koob, et al., 1989, Stinus, et al., 1990, Crippens and Robinson, 1994). Furthermore, there is evidence that some drugs, such as clonidine, used for treatment of opioid withdrawal, may prevent the NAc DA decrease (Pothos, et al., 1991).

Studies have further demonstrated that both DA receptor D1 and D2 in NAc are involved in opioid withdrawal syndrome (Elwan and Soliman, 1995, Georges, et al., 1999, Druhan and Walters, 2000). Harris and Aston-Jones reported that activation of the dopamine D2 receptor with specific agonist led to potently regulated somatic symptoms of opiate withdrawal. However, blocking of the receptor with an antagonist, elicited some typical withdrawal signs, including wet dog shakes and teeth chattering, in morphine dependent animals (Harris and Aston-Jones, 1994). These effects were suggested to be mediated by D2 receptors located in the shell region of the NAc (Harris and Aston-Jones, 1994).

Besides NAc, striatum is another brain substrate of effect of opioids to be implicated in withdrawal behavior. Striatum dopamine was also found to decrease in opioid withdrawal animals (Laschka, et al., 1976). Striatum is the main part of basal ganglia, an important region that plays a key role in the control of movement (Medina and Reiner, 1995). Information that underlies the generation and initiation of voluntary movement is integrated in this area (Medina and Reiner, 1995). However, striatum is also associated with many signs of opioid withdrawal, such as wet dog shakes, withdrawal jumps and stereotyped motor behaviours (Medina and Reiner, 1995). Furthermore, stimulation of D1 receptor in striatum results in enhanced grooming behaviour (Sibley, 1999).
Several studies have demonstrated that DA transmission and DA receptor D1 and D2 are involved in the mechanism of opioid withdrawal behaviours (Elwan and Soliman, 1995). Withdrawal from morphine has been observed to change the characteristics of D1 as well as the D2 receptor in the brain. (Tidey and Miczek, 1992, Shippenberg, et al., 1993), e.g. withdrawal of morphine increases the B\textsubscript{max} value of D1 in striatum (Bhargava and Gulati, 1990, Reddy, et al., 1993).
AIMS OF THE STUDY

1. To examine the effects of morphine tolerance and withdrawal on the endogenous levels of SP1-7 in male rats.

2. To assess the activity of SPE in morphine tolerant and abstinent rats.

3. To measure the effect of SP1-7 on the expression of NMDA receptor subunit mRNAs.

4. To investigate the effect of SP1-7 on the dopamine transmission during morphine abstinence.

5. To test the effect of SP1-7 on naloxone precipitated morphine withdrawal behaviours in male rats.

6. To measure the effect of SP1-7 on dopamine D2 receptor transcript in male rat during morphine withdrawal.

7. To study the effect of SP1-7 on dopamine D1 and D2 receptor binding in the male rat brain during morphine abstinence.
METHODS AND EXPERIMENTAL PROCEDURE
The details of the methods are described in the individual papers.

1. Stereotaxic surgery and animal experiment
   1.1. Models for studies of opioid tolerance and dependence (Paper I, II, IV, V and VI)
   1.2. Brain tissue dissection process (Paper I, II, III and V)
   1.3. Observation of Morphine withdrawal behaviour (Paper II and V)
   1.4. Central and local injections (Paper III, IV, V and VI)
     1.4.1. ICV injection in anaesthetise rats (Paper III)
     1.4.2. ICV injection in conscience rats (Paper III)
     1.4.3. Intra-VTA injection (Paper IV and VI)
   1.5. Microdialysis (Paper IV)

2. Biochemistry methods
   2.1. Peptide extraction (paper I)
   2.2. Enzyme extraction (Paper II)
   2.3. Radioimmunoassay (Paper I and II)
   2.4. Enzyme activity assay (Paper II)
   2.5. High performance liquid chromatography (HPLC) (Paper I, II and IV)
   2.6. Autoradiography (Paper VI)

3. Molecularbiology method
   3.1. RNA extraction and Northern blot analysis (Paper III and V)
RESULTS AND DISCUSSION

1. Adaptive changes of SP_{1-7} in morphine tolerant and dependent rats (*Paper I*)

In morphine tolerant rat, the level of SP_{1-7} was increased in VTA. However, during morphine withdrawal, the levels of SP_{1-7} were enhanced in VTA, spinal cord, substantia nigra and hypothalamus, while in striatum, the content of the heptapeptide was significantly decreased.

<table>
<thead>
<tr>
<th>brain area</th>
<th>Saline</th>
<th>Morphine</th>
<th>Saline+naloxone</th>
<th>Morphine+naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>2.97 ± 0.20</td>
<td>1.61 ± 0.05</td>
<td>1.32 ± 0.20</td>
<td>2.08 ± 0.20</td>
</tr>
<tr>
<td>Cerebral Cortex</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.07</td>
<td>0.09 ± 0.01</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.31 ± 0.03</td>
<td>0.21 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>11.7 ± 0.50</td>
<td>10.8 ± 0.40</td>
<td>9.16 ± 0.60</td>
<td>13.8 ± 0.70*</td>
</tr>
<tr>
<td>Nucleus Accum-bens</td>
<td>5.16 ± 0.30</td>
<td>10.8 ± 0.40</td>
<td>4.91 ± 0.30</td>
<td>5.21 ± 0.20</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.94 ± 0.08</td>
<td>2.33 ± 0.50</td>
<td>0.99 ± 0.08</td>
<td>0.40 ± 0.04*</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>3.69 ± 0.60</td>
<td>2.58 ± 0.30</td>
<td>1.61 ± 0.20</td>
<td>4.68 ± 0.60a</td>
</tr>
<tr>
<td>VTA</td>
<td>8.14 ± 0.05</td>
<td>13.8 ± 0.70</td>
<td>12.8 ± 1.00</td>
<td>23.1 ± 1.00*</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>3.75 ± 0.20</td>
<td>3.22 ± 0.30</td>
<td>2.25 ± 0.10</td>
<td>7.99 ± 1.00*</td>
</tr>
</tbody>
</table>

* P < 0.05  morphine+naloxone treated animals versus saline+naloxone treated animals.
* a P = 0.051 morphine+naloxone treated animals versus saline+naloxone treated animals.
* † P < 0.05  morphine treated animals versus saline treated animals.

The alteration in the SP_{1-7} content indicates that there is an interaction between the opioid and the SP systems. As mentioned before, morphine treatment of rats is previously shown to affect the SP content in several CNS tissues (Bergstrom, et al., 1984, Mccarson and Goldstein, 1989, Tiong, et al., 1992, Chahl and Chahl, 1994). Following naloxone injection in morphine-maintaining animals the SP concentration was significantly enhanced in the midbrain and hypothalamus and this elevation occurred at a time correlated with the peak of the behavioral morphine withdrawal syndrome (Tiong, et al., 1992). The influence of chronic morphine administration on SP_{1-7} level is for the first time reported in our present study. The increased content of
the peptide in spinal cord may be involved in the morphine tolerance, since this tissue has previously been proposed to play a role in morphine tolerance (Gutstein and Trujillo, 1993) and SP₁₋₇ was shown to prevent the development of morphine tolerance (Kreeger and Larson, 1996). The enhanced content of SP₁₋₇ in the VTA may reflect an elevated activity in SP-containing neurons innervating this area. SP-containing neurones has also been found in NAc and in the ventromedial striatum (Napier, et al., 1995). The function of SP in these pathways has been suggested to be related to motor behavior. Furthermore, SP₁₋₇ was found to act as a very potent antagonist of these SP-induced responses (Herrera-Marschitz, et al., 1990). Moreover, infusion of the SP receptor antagonist GR73632 into the VTA significantly and dose-dependently increased the basal locomotion activity in the rat (Elliott, et al., 1992) and local injection of the undecapeptide itself into this brain area was earlier found to enhance dopamine turnover in the prefrontal cortex (Cador, et al., 1989). It thus seems that both SP and its N-terminal fragment SP₁₋₇ may interact with mesolimbic dopamine neurones related to motor activity. In addition, effects of SP on acetylcholine, as well as SP₁₋₇ on dopamine, with respect to both reward and aversion have been described (Boix, et al., 1994).

Interactions between the N-terminal fragments of SP and opioids have previously been shown both in behavioral tests and ligand binding assays. For instance SP₁₋₇ is shown to produce a naloxone reversible antinociception when given ICV or i.p. in the rat (Stewart, et al., 1982). The peptide is also shown to exhibit a long-acting antinociceptive effect in mice as described by Kreeger and Larson (Kreeger, et al., 1994). Furthermore, SP₁₋₇ was found to inhibit the binding affinity of certain opioid antagonists for mu-opioid receptors (Krumins, et al., 1993).

Studies also reveal that SP₁₋₇ may affect the naloxone-induced withdrawal action in mice (Krumins, et al., 1993). Following injection of SP₁₋₇ prior to morphine, the incidence of withdrawal jumping increased in the mice, whereas when the heptapeptide was injected prior to naloxone it caused a decrease in this kind of withdrawal behavior. This reflects the ability of SP₁₋₇ to alter the binding affinity of
opioid. According to the studies cited above, the presently observed increase in the SP_{1-7} concentration in morphine tolerant rats may contribute to an decreased sensitivity of the receptor for the opioid drug. Interestingly, the most pronounced elevation of the SP_{1-7} activity occurs in the VTA. This area of the brain has been described as an important part of the brain reward system (Koob, 1992a). The enhancement of the SP_{1-7} content in the VTA during withdrawal may also affect the morphine withdrawal syndrome.

One possible explanation for the enhanced concentration of the N-terminal SP fragment, SP_{1-7} is that during morphine dependence and withdrawal the release of the parent compound SP is increased as mentioned above. It could also be compatible with an increased activity of enzymes responsible for the generation of SP_{1-7}, or by both. As mentioned earlier, several enzymes capable of releasing SP_{1-7} from SP have been identified in the CNS (Matsas, et al., 1983, Lantz and Terenius, 1985, Lantz, et al., 1992). A specific endopeptidase, named SPE, in human (Nyberg, et al., 1984) and rat CSF (Persson, et al., 1992b) has been purified and characterized. It was found to be a metal-dependent thiol-sensitive protease hydrolysing its substrate in the vicinity of the phenylalanine residues, to generate SP_{1-7} and SP_{1-8} as major products. The activity of SPE was found to be increased in CSF collected from morphine tolerant male rats (Persson, et al., 1989) but how it is affected during withdrawal warranted further studies.

2. Adaptive change in the activity of SPE in morphine tolerant and dependent rats (Paper II)

Thus, during opioid tolerance and withdrawal, a significant up-regulation occurred in the activity of an enzyme cleaving substance P to release a heptapeptide fragment, which is evidenced by elevated content of SP_{1-7}. We have previously reported (Paper I) that the content of SP_{1-7} is significantly elevated in the rat VTA, substantial nigra, spinal cord and hypothalamus during morphine withdrawal. In our study presented in paper II, the SPE like activity is also significantly increased in these areas which is obviously compatible with the altered level of the SP heptapeptide fragment. This
finding suggests a role of SPE in regulating the SP$_{1-7}$ content in rat CNS during morphine tolerance and withdrawal.

Table 4 SPE-like activity (pmol / min / mg protein) in rat brain tissues during morphine tolerance and withdrawal. Values are means ± SEM.

<table>
<thead>
<tr>
<th>brain region</th>
<th>saline</th>
<th>morphine</th>
<th>saline+naloxone</th>
<th>morphine+naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAG</td>
<td>35.2 ± 12.6</td>
<td>19.9 ± 1.6</td>
<td>12.5 ± 2.1</td>
<td>36.3 ± 6.9**</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>29.1 ± 4.7</td>
<td>33.9 ± 7.1</td>
<td>30.3 ± 7.1</td>
<td>36.4 ± 8.0</td>
</tr>
<tr>
<td>Nucleus accum-bens</td>
<td>18.1 ± 3.0</td>
<td>18.2 ± 3.9</td>
<td>12.4 ± 3.7</td>
<td>20.5 ± 6.6</td>
</tr>
<tr>
<td>Striatum</td>
<td>39.6 ± 5.7</td>
<td>20.3 ± 1.7††</td>
<td>39.6 ± 5.5</td>
<td>47.0 ± 4.2</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>9.1 ± 1.1</td>
<td>13.3 ± 1.6</td>
<td>9.8 ± 1.3</td>
<td>22.5 ± 2.2***</td>
</tr>
<tr>
<td>VTA</td>
<td>18.6 ± 5.1</td>
<td>25.1 ± 4.4</td>
<td>26.4 ± 5.9</td>
<td>93.0 ± 29.0*</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>83.9 ± 35.2</td>
<td>76.5 ± 15.0</td>
<td>96.5 ± 19.4</td>
<td>323.0 ± 75.1**</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ morphine+naloxone treated animals vs. saline+naloxone animals.
†† $P < 0.01$, morphine treated animals vs. saline treated animals.

There appeared to be correlation between measured level of SPE-like activity and some of the recorded withdrawal signs, e.g. in VTA a negative correlation between the enzyme activity and wet-dog shake (Fig. 5) and face-washing was observed, whereas a positive correlation was found between SPE-like activity and chewing behavior. Besides, a negative correlation between the enzyme activity and scratching and ptosis was noticed in spinal cord. During opioid withdrawal the SPE-like activity displayed a 4-fold increase in VTA (Table 4) and the same dignity of elevation is present in spinal cord corresponding to a comparable elevation in the SP$_{1-7}$ level (Zhou, et al., 1998). In the substantia nigra, the enzyme activity was doubled during naloxone induced opioid withdrawal, an observation also in line with the previously shown elevation in the SP$_{1-7}$ content in this brain area, whereas we found a positive correlation between the enzyme activity and grooming. The elevated SPE like activity noted in PAG during withdrawal correlated with the frequency of stretch. This finding does support that the enzyme may be involved in a mechanism balancing the withdrawal reactions. Thus, at the same time as SPE diminishes the action of SP by proteolytic degradation it releases a fragment from SP counteracting its effect on the withdrawal reaction. This suggests
that the SPE-like activity have a regulative and modulatory function on the expression of opioid withdrawal.

The mechanism behind the increase in SPE-like activity during opioid withdrawal is not yet clear. An increased activity of cAMP system during withdrawal may be involved in enhancing the enzyme activity. According to the previous study, long time exposure to opioid will cause neuronal adaptation, which involve some changes at molecular level to oppose the effect of the opioid. Furthermore, during opioid withdrawal, due to the homeostatic function of the system, some components in the circuits will change contrary to the over-firing neurones to balance the biological system. The increased SPE-like activity may meet such requirement, hydrolysing SP to reduce its effect on the withdrawal reaction and at the same time release the N-terminal fragment SP_{1-7}, which counteract the effect of SP.

![Figure 5 Correlation of SPE-like activity and withdrawal sing in VTA](image)

3. ICV administration of SP_{1-7} alters the expression of NMDA receptor subunit mRNAs (Paper III)

Studies have indicated that activation of the NMDA receptor underlies both the behavioral and neuroadaptive effects of opioids. There is a growing body of evidence documenting the NMDA receptor as a modulator of opioid tolerance and withdrawal
(Pulvirenti, et al., 1991, Gutstein and Trujillo, 1993, Koyuncuoglu, et al., 1994, Yukhananov and Larson, 1994a). Thus, injection of non-competitive NMDA antagonists such as MK-801 and dextromethorphan or a competitive antagonist CGS 19755 in morphine tolerant rats results in a reduction of the withdrawal symptoms (Gutstein and Trujillo, 1993, Buccafusco, et al., 1995). Morphine and psychostimulants affecting the NMDA receptor subunits transcripts have been

Table 5: Effect of SP1-7 on NMDA receptor subunits transcripts. Values are means ± SEM. Values are expressed as % of control.

<table>
<thead>
<tr>
<th>tissues</th>
<th>2h after ICV injection</th>
<th>4h after ICV injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.6 nmol SP1-7/ rat</td>
<td>28 nmol SP1-7/ rat</td>
</tr>
<tr>
<td>NR1 mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>85.7±12.6%</td>
<td>176.1±28.2%</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>102±12.5</td>
<td>97.1±14.5</td>
</tr>
<tr>
<td>Nucleus accum.</td>
<td>144±37.3</td>
<td>138±9.5</td>
</tr>
<tr>
<td>PAG</td>
<td>113±36.0</td>
<td>112±35.2</td>
</tr>
<tr>
<td>VTA</td>
<td>121±6.6</td>
<td>120±7.9</td>
</tr>
<tr>
<td>Striatum</td>
<td>85±19.3</td>
<td>84.1±15.9</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>44.8±4.6*</td>
<td>44.6±2.3*</td>
</tr>
<tr>
<td>NR2A mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>123±15.7</td>
<td>203±35.4*</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>92.3±19.8</td>
<td>111±25.5</td>
</tr>
<tr>
<td>Nucleus accum.</td>
<td>111±34.8</td>
<td>108±13.4</td>
</tr>
<tr>
<td>PAG</td>
<td>169±39.6</td>
<td>225±32.7*</td>
</tr>
<tr>
<td>VTA</td>
<td>145±13.8</td>
<td>172±21.0*</td>
</tr>
<tr>
<td>Striatum</td>
<td>130±3.7</td>
<td>66.9±9.3</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>50.9±4.7</td>
<td>49.3±3.2</td>
</tr>
<tr>
<td>NR2B mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>112±21.7a</td>
<td>197±29.8*</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>102±12.5</td>
<td>97.1±14.5</td>
</tr>
<tr>
<td>Nucleus accum.</td>
<td>104±25.7</td>
<td>113±11.3</td>
</tr>
<tr>
<td>PAG</td>
<td>152±11.2</td>
<td>106±23.3</td>
</tr>
<tr>
<td>VTA</td>
<td>89.3±9.5</td>
<td>87.3±8.1</td>
</tr>
<tr>
<td>Striatum</td>
<td>103±25.4</td>
<td>129±38.5</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>39.6±2.5*</td>
<td>40.9±2.5*</td>
</tr>
</tbody>
</table>

* P < 0.05  SP1-7 treated animals versus controls.
α P < 0.05  low dose group versus high dose group of same survival time.
nd not detectable.
confirmed by several studies (Le Grevès, et al., 1998, Ghasemzadeh, et al., 1999). Adaptive changes at the NMDA receptor level would contribute to drug tolerance and drug addiction. In normal rats, ICV injection of SP$_{1-7}$ affected the expression of NMDA receptor subunits NR1, NR2A and NR2B mRNAs in CNS, including VTA and NAc (Table 5), which as mentioned above are important areas which are implicated in opioid reward and withdrawal. (de la Baume, et al., 1979, Ahtee, et al., 1987, Koob, 1992). It is well known that glutamate modulates dopamine release in the striatum, substantia nigra, and VTA (Mount, et al., 1989, Imperato, et al., 1990, Krebs, et al., 1991). Accordingly the SP$_{1-7}$ induced attenuation of opioid withdrawal signs may be due to an alteration in the glutamate mediating activity on dopaminergic neurones. In the present study, an altered level of the NR1 was also found in the spinal cord and PAG, areas, which is involved in morphine tolerance and some withdrawal signs, respectively. Therefore, a change in the NMDA receptor in this region should be involved in the mechanisms by which SP$_{1-7}$ decrease morphine tolerance and attenuates the withdrawal reaction. Besides, the three receptor subunits mRNAs were found to be significantly increased in hippocampus which is a brain area involved in learning and memory (Pitkanen, et al., 1997, Pikkarainen, et al., 1999). The NR2B subunit was suggested be as a molecular switch in the memory process both in human and mice (Tang, et al., 1999). SP or its N-terminal fragment SP$_{1-7}$ were previously reported to enhance memory capabilities in rat (Huston, et al., 1993, Tomaz and Nogueira, 1997). Therefore, the increased expression of the in NR2B transcript in hippocampus following ICV injection of SP$_{1-7}$ may underlie a previously observed effect of the heptapeptide on memory and learning (Tomaz and Nogueira, 1997).

Since the NMDA receptor complex composition of NR1 and NR2A-D have different physiological and pharmacological, properties (Nakanishi, 1992, Dingledine, et al., 1999) changes in the subunit level may cause the functional alterations in the NMDA receptor. In the present study, ICV injection of SP$_{1-7}$ altering the expression of the NR1, NR2A, and NR2B transcripts probably affects the function of the NMDA receptor. It seems that the peptide may regulate the glutamate system by acting on NMDA subunit mRNA expression. This may be one of mechanisms by which SP$_{1-7}$
attenuates the intensity of opioid withdrawal symptoms, as previously observed in mice (Kreeger and Larson, 1993).

4. SP₁₋₇ regulates withdrawal signs and dopamine systems in morphine
Abstinence (Paper IV, V and IV)

Paper IV, V and VI describe a series of studies, which explored possible interactions of SP₁₋₇ with dopamine pathways. Mesocorticolimbic and nigrostriatal dopamine systems have been confirmed as substrates of drug addiction and opioid withdrawal reactions. A link between the substance P system and mesolimbic dopamine circuits has been indicated by our previous studies. It was therefore hypothesised that the mesolimbic dopamine system may serve as a mediator for the modulatory effects of SP₁₋₇ on withdrawal.

4.1. SP₁₋₇ increases dopamine release during morphine withdrawal (Paper IV)

In this study, we administered SP₁₋₇ into VTA of morphine dependent rats in order to investigate the effect of the peptide on dopamine release in NAc by using microdialysis method. We found that the levels of both dopamine and its metabolite DOPAC were significantly increased in the rats receiving intra-VTA SP₁₋₇ injection compared to the saline injected group. The time course of changes in dopamine and DOPAC are show in Figure 6 and Figure 7 respectively.

The result shows an obvious increase in extracellular dopamine as well as in DOPAC in NAc after SP₁₋₇ injection. The recorded time courses indicate a matched increase in dopamine release and dopamine metabolism (reflected by DOPAC) at 60, 120 and 150 min after administration of the peptide. A decrease in the level of dopamine (at 30, 90, 150 min) and in DOPAC (at 120, 150 min) was observed over time in the saline treated group compared with that of base line. A clear evidence for the ability of the heptapeptide to stimulate the activity in dopamine neurons in VTA and to increase dopamine metabolism in the NAc was obtained. The result demonstrates that during morphine withdrawal, SP₁₋₇ modulates dopaminergic actions by opposing the decrease

Figure 6 Extracellular dopamine in the nucleus accumbens of rats during morphine withdrawal. Rats were pre-treated with SP1-7 (28 nmol/ side, intra-VTA) or with saline. * P < 0.05, SP1-7 treated animals vs. saline treated control animals before naloxone challenge. †† P < 0.01, dopamine levels in control group after naloxone injection vs. that of baseline.

Figure 7 Extracellular DOPAC in the nucleus accumbens of rats during morphine withdrawal. Rats were pre-treated with SP1-7 (28 nmol/ side, intra-VTA) or with saline. ** P < 0.05, SP1-7 treated animals vs. saline treated control animals before naloxone challenge. †† P < 0.01, DOPAC levels in control group after naloxone injection vs. that of baseline.
This result keeps in line with previous studies indicating that SP1-7 excitatively stimulates dopamine neurons to cause an increased transmitter outflow from their terminals (Hall and Stewart, 1992). It further suggests that the previously reported (Kreeger and Larson, 1996) attenuating effect of SP1-7 on the reaction to opioid withdrawal may be due to SP1-7-induced enhancement of dopamine in NAc. NAc is implicated not only in positive reinforcement of opioids but it is also serves as a critical target for mediating aversive stimulus properties of opioid withdrawal (Koob, et al., 1989, Harris and Aston-Jones, 1994). Microdialysis studies have indicated that the decreased dopamine outflow in NAc is associated with opioid abstinence (Pothos, et al., 1991). Dopamine level in this brain region seems to be significantly involved in the expression of opioid withdrawal (Pothos, et al., 1991). Some drugs, such as clonidine, used for treatment of opioid withdrawal, may prevent the NAc dopamine decrease (Pothos, et al., 1991).

The mechanism by which SP1-7 affects the release of dopamine is not yet clear. SP1-7 perhaps acts on specific binding sites in VTA. Specific binding of SP1-7 to CNS tissues was previously suggested by Igwe and co-worker, (Igwe, et al., 1990). The peptide may also react with mu-opioid or NK-1 receptor in the VTA to modulate dopamine activity. Interactions of SP1-7 with mu-opioid receptor and NK-1 receptor have previously reported (Krumins, et al., 1989, Yukhananov and Larson, 1994b). Both these receptors are recognised for their important roles in connection with opioid withdrawal (Koob, et al., 1989, Murtra, et al., 2000).

The present finding confirms the ability of SP1-7 modulates mesolimbic dopamine in morphine dependent rat, providing evidence for an excitatory effect of the heptapeptide on VTA dopamine neurones during morphine withdrawal. The result is indicative of a dopamine dependent mechanism by which SP1-7 modulates opioid withdrawal reactions.

4.2. SP1-7 regulates the dopamine D2 receptor gene transcription and inhibits morphine withdrawal signs in rats (Paper V)
The effects of ICV injection of SP<sub>1-7</sub> on the withdrawal behaviours and dopamine D2 receptor transcript were studied in morphine abstinent rats. Behaviour test showed that SP<sub>1-7</sub> significantly decreased at least some withdrawal signs (Figure 8). The expression of withdrawal signs such as teeth chattering (decreased by 62%, P < 0.05), ptosis (100%, P < 0.001) and digging (65%, P < 0.05) was decreased compared to that observed in saline injected animals (Figure 8).

At 4h after ICV injection of SP<sub>1-7</sub>, the level of the dopamine D2 receptor mRNA was significantly decreased by 61% (P < 0.05) in NAc compared to controls. In frontal cortex, a similarly reduction in the D2 receptor gene expression was detected, i.e. a decrease by 46% (P < 0.05). However, no significant change in the transcript was measured in the striatum (Table 6).

### Table 6

<table>
<thead>
<tr>
<th>Area</th>
<th>Saline treated</th>
<th>SP&lt;sub&gt;1-7&lt;/sub&gt; treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>9.6±1.9</td>
<td>3.7±0.4</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>56.6±9.5</td>
<td>30.1±6.5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Striatum</td>
<td>19.0±2.4</td>
<td>27.0±9.2</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

In frontal cortex, we also found a positive correlation between the level of the mRNA and wet dog shakes ($r^2 = 0.657$, P < 0.05), however, only in rats treated with SP<sub>1-7</sub>. In this area, agonist stimulation of the D2 receptor was found to increase cognitive performance in human (Robbins, 2000). The functions of dopamine neuron in this area are thought to be associate with working memory processes, behavioural flexibility and exploratory activity (Robbins, 2000). However, there is little information about effects mediated through the dopamine D2 receptor on opioid withdrawal signs in this area. Considering the previously reported promoting effect of SP<sub>1-7</sub> on memory (Tomaz and Nogueira, 1997), It is easy to suggest that SP<sub>1-7</sub> may cause a change in
dopamine transmission in frontal cortex that contribute to the cognitive performance of the animals during morphine withdrawal.

Of particular interest in this study is the observation that there seems to be a correlation between the level of dopamine D2 receptor mRNA and teeth chattering ($r^2 = 0.802, P < 0.05$) in NAc, indicating a regulatory role of D2 dopamine receptor with regard to withdrawal signs recorded in SP$_{1-7}$ treated animals. This finding is in line with previous studies reported by Harris and Aston-Jones. They found that the D2 receptor in NAc has an important role in regulating opioid withdrawal behaviours (Harris and Aston-Jones, 1994). Thus pre-treatment with a dopamine D2 agonist results in attenuated withdrawal signs, including teeth chattering, wet dog shake, writing, vocalise, ptosis, eye twitch and diarrhoea. The evidence that NAc serves as a substrates for the aversive stimulus effects of opioid withdrawal was also described by Koob and co-workers (Koob, et al., 1989, Stinus, et al., 1990). A positive correlation between the level of mRNA and teeth chattering in NAc implies a negative relationship between dopamine activity and withdrawal sign in this area.
The lack of a remarkable effect of SP$_{1-7}$ on level of D2 receptor mRNA in striatum in morphine dependent rat, does not imply an exclusion of a functional role of the peptide in mediating effects via the dopamine pathways in this area. Instead, we should considering the restriction of the Northern blot method in measuring changes in brain subregions. The nigrostriatal dopamine pathway is described as a crucial substrate related to motor behaviour. Previous reports have described the effect of SP$_{1-7}$ on striatum dopamine release and behavior performance (Reid, et al., 1990, Pothos, et al., 1991). Furthermore, we previously observed elevated levels of endogenous SP$_{1-7}$ and enhanced activity of SPE in the rat substantia nigra of abstinent rats, suggesting an involvement of this pathway in the action of the morphine withdrawal (Zhou, et al., 2001).

Thus, this study (Paper V) suggests a modulatory effect of SP$_{1-7}$ on morphine withdrawal signs together with an action and on the activity in dopamine circuits. The correlation between the dopamine D2 receptor transcripts and score of some withdrawal signs suggests an involvement of the D2 receptor in the mechanism for the modulatory function of SP$_{1-7}$ on morphine withdrawal.

4.3. SP$_{1-7}$ regulated dopamine D1 and D2 binding sites in morphine abstinence rats (Paper VI)

Treatment of morphine dependent rats with intra-VTA injection of SP$_{1-7}$ was found to affect the dopamine receptor density in various brain regions during morphine withdrawal. The density of D1-like receptors was significantly decreased in striatum, NAc shell, NAc core, substantia nigra and medial globus pallidus (MGP) (Fig. 9). Meanwhile, the density of D2-like receptor was decreased in VTA but increased in the substantia nigra and frontal cortex (Fig. 10).

The alterations in dopamine receptor binding may due to the enhanced activity in dopamine neurones in VTA and substantia nigra after intra-VTA injection of SP$_{1-7}$. It is well established that dopamine transmission and dopamine receptors (D1 and D2) are implicated in many of the opioid-induced behaviors (Sibley, 1999). Decreased
dopamine release in NAc is associated with opioid abstinence (Pothos, et al., 1991). A recent study suggested that the dopamine D2 receptor in NAc plays an unnegligible role in the somatic signs of opiate withdrawal (Harris and Aston-Jones, 1994), whereas, activation of the D1 receptor in NAc will decreases the conditional aversive reaction (Fenu, et al., 2001). Modulation of central dopaminergic activity by SP\(_{1-7}\) during morphine withdrawal was confirmed by microdialysis study (*Paper IV*), and is suggested to be responsible for an altered expression of the dopamine D2 receptor.

**Figure 9** The density of D1 like receptor (fmol/mg) in tissues of SP\(_{1-7}\) treated and control rats. *** P < 0.001, ** P < 0.01,* P < 0.05

**Figure 10** The density of D2 like receptor (fmol/mg) in tissues of SP\(_{1-7}\) treated and control rats. ** P < 0.01,* P < 0.05.
gene transcript (Paper V). The alteration in dopamine D1 and D2 receptor binding could result from various feedback mechanisms. NAc is known to send negative feedback projections to VTA (Kalivas, et al., 1993) by activating a long and a short negative feedback loops (Rahman and McBride, 2001). Activity in the short feedback loop is mediated by D2-like autoreceptors at the somatodendritic level (Kalivas and Duffy, 1991). The majority of neurons projecting from nucleus accumbens to VTA express mRNA for D1 receptor (Lu, et al., 1998). The expression of mRNA for both D1 and D2 receptors has been found in major GABAergic output from the NAc to the pallidum (Kalivas, et al., 1993, Lu, et al., 1998). Stimulation of these feedback loops could be associated with altered dopamine receptor density (Kindlundh, et al., 2001).

In conclusion, we found that the density of both dopamine D1 and D2-like receptors are altered by intra-VTA injection of SP1-7. Changes in density were found in VTA, MPG, NAc, striatum, substantia nigra and frontal cortex, which all are important areas associated with various aspects of morphine actions. Thus, the results reveal an ability of SP1-7 to modulating the activity in dopamine systems during the morphine withdrawal. It therefore gives strong support for our previous hypothesis that SP1-7 alters morphine withdrawal syndromes through dopamine pathways that are linked to drug addiction and aversive reactions.
CONCLUSIONS

1. This study shows that the SP metabolite SP_{1-7} is significantly elevated during morphine tolerance and withdrawal in several areas of rat brain including VTA. It was suggested that the enhanced level of the peptide may reflect an endogenous mechanism to counteract adaptive changes induced by the opioid and its antagonist.

2. The activity of SPE, an enzyme controlling the CNS levels of SP_{1-7}, was significantly altered in several brain areas during opioid tolerance and withdrawal. This increase is compatible with enhanced content of SP_{1-7} as seen in an identical protocol study and correlated to some withdrawal signs, suggesting a regulatory role of this enzyme on SP_{1-7} level and withdrawal syndrome in chronic morphine dependent rats.

3. ICV injection of SP_{1-7} caused a significant alteration in the expression of the gene transcripts for the NR1, NR2A and NR2B subunits of the NMDA receptor in various brain regions related to the functional anatomy of opioid reward and memory functions, indicating the involvement of glutamate transmission in the mechanism behind the actions of SP_{1-7}.

4. Pre-treatment of morphine dependent rats with SP_{1-7}, decreased some typical morphine withdrawal signs. The level of Dopamine D2 receptor mRNA was found to correlate with some withdrawal behaviours, suggesting that the inhibiting effect of SP_{1-7} is at least partly mediated by the D2 receptor.

5. In morphine abstinent rats SP_{1-7} elicited an increased dopamine release, consistent with a stimulating activity on mesolimbic dopamine neurones, in contrast to what is normally found during morphine abstinence. This peptide-induced effect could be responsible for the observed changes in dopamine receptors.
6. The effect of SP$_{1-7}$ on the dopamine D2 receptor transcript suggests an ability of the heptapeptide to modulate dopamine transmission in the brain of opioid withdrawal animal. The ability of SP$_{1-7}$ to suppress the opioid withdrawal reactions may be associated with this modulatory effect on dopamine neurons. The mesocorticolimbic dopamine system may thus serve as a target for SP$_{1-7}$ to modulate opioid withdrawal reactions.

7. SP$_{1-7}$ alters the density both of D1 and D2 receptor proteins in morphine abstinent rats. This reflects a strong regulatory effect of the peptide on dopamine system. Changes in the receptors and dopamine transmission could be involved in the mechanisms behind the modulatory effect of the SP fragment on opioid withdrawal.
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