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Spinal Acetylcholine Release

Mechanisms and receptor involvement

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Abstract

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Impulses coming from peripheries are modified in the spinal cord and transmitted to the brain. Several neurotransmitters have been involved in the processing of impulses in the spinal dorsal horn. Acetylcholine (ACh) is one of many neurotransmitters involved in the regulation of nociception in the spinal cord. In this study we investigated the role of nicotinic, muscarinic, serotonergic and GABA receptors in the regulation of spinal ACh release since these receptors are reported to be involved in spinal nociceptive processes.

Different receptor ligands were infused intraspinally via microdialysis and the spinal ACh release was measured by on-line HPLC. Receptor-ligand binding studies were performed with spinal cord homogenates as well as receptors expressed in cells.

In the first study, we found that nicotine and some of the nicotinic antagonists used increased ACh release suggesting that spinal ACh release is regulated by different nAChRs. Nicotine and nicotinic agonists may act on different types of receptors with different affinity to produce the observed net effect of increased ACh release. We propose the possibility of an involvement of three different nicotinic receptor subtypes in the regulation of spinal ACh release.

The effect of epibatidine, which is regarded as a nicotinic agonist, on muscarinic receptors was investigated in the second study. We propose that epibatidine, in μM concentrations, is a partial muscarinic receptor agonist that may interact with spinal muscarinic receptors to increase ACh release. The dual action on both nAChRs and mAChRs may explain the potent analgesic effect observed after intra-spinal epibatidine administration.

In the third study, we investigated the role of serotonin receptor involvement in ACh release control. The results suggest that only 5-HT_{1A} and 5-HT_{2A} receptors are involved in spinal ACh release. Considering current knowledge, the most probable location of 5-HT_{2A} receptors is on cholinergic neurones. On activation of the 5-HT_{2A} receptors the cellular excitability of cholinergic neurones is increased which results in an increasing ACh release. The 5-HT_{1A} receptors might be located on cell bodies of GABA neurones which inhibit the firing rate of the GABA neurones when activated by serotonin.

In the fourth study, we investigated the GABA receptor involvement in the regulation in spinal ACh release. We found that GABA_A receptors are tonically inhibiting spinal ACh release. The results further suggest that GABA_B receptors also are involved in the regulation of spinal ACh release. However, unlike GABA_A antagonists, GABA_B antagonists do not increase ACh release. This suggests that GABA_B receptors are not tonically regulating the spinal ACh release.

Keywords: acetylcholine release, spinal antinociception, nicotinic receptor, serotonin, GABA

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To my father....

List of Papers

This thesis is based on the following publications.

- I Kommalage M, Höglund AU. Nicotinic acetylcholinergic receptors regulate the intraspinal release of acetylcholine in male rats. *Pharmacol Toxicol.* 2003 Oct;93(4):169-73.
- II Kommalage M, Höglund AU. (+/-) Epibatidine increases acetylcholine release partly through an action on muscarinic receptors. *Basic Clin Pharmacol Toxicol.* 2004 May;94(5):238-44.
- III Kommalage M, Höglund AU. Involvement of spinal serotonin receptors in the regulation of intraspinal acetylcholine release. *Eur J Pharmacol.* 2005 Feb 21;509(2-3):127-34.
- IV Kommalage M, Höglund AU. Involvement of spinal GABA receptors in the regulation of intraspinal acetylcholine release. *Eur J Pharmacol.* In press.

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Abbreviations

5-HT	5-hydroxytryptamine/Serotonin
ACh	Acetylcholine
AMPA	α -amino-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
CGRP	Calcitonin gene related peptide
EAA	Excitatory amino acid
GABA	γ -amino butyric acid
HPLC	High Performance liquid chromatography
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NRM	Nucleus raphe magnus
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor
PAG	Periaqueductal gray matter
PG	Prostaglandin
SP	Substance P
VIP	Vasoactive intestinal peptide

Introduction

Pain

Pain is both a sensory and emotional experience generally associated with some type of tissue damage. Pain is one of the critical components of the body's defence system and is a part of a rapid warning and defence mechanism instructing the motor neurones of the central nervous system to minimise detected physical 'harm'. As pain is associated with emotional components, it is always subjective, each individual learns from the early experiences in the life. Emotional components of pain are often accompanied by desires to terminate, reduce, escape, distress or fear (Price, 2000).

Acute pain is the normal physiologic response to a noxious chemical, thermal, or mechanical stimulus, and in most cases, it is time limited. Chronic pain, however, is a state in which pain persists beyond the usual course of the disease and may cause intermittent or continuous pain for months or years. Chronic pain is a complex phenomenon with various causes and issues associated with it. Chronic pain can result from inflammation and nerve damage and is mediated by structural, physiological and functional changes in the central nervous system as a result of damage.

Nociception and nociceptors

The technical term 'nociception' describes only the physiological aspect of the general term 'pain', not the psychological aspect. In Latin, nocere is an injury, so sensation or feeling of injury is nociception.

Nociceptors are the receptors, which are activated by nociceptive stimuli such as mechanical, thermal, or chemicals. Nociceptors are free nerve endings present in skin, muscles, joints and viscera (Willis and Westlund, 1997). There are three major types of nociceptors. Mechanical nociceptors which are activated only by strong mechanical stimulation, heat nociceptors which are activated by temperature greater than 45°C in human and mixed nociceptors which are activated by various types of noxious stimuli (Martin, 1982).

Sensitisation of nociceptors depends on activation of second messenger systems by the action of inflammatory mediators- prostaglandines, bradykinin, serotonin and histamine (Birrell et al., 1993; Davis et al., 1993; Dray et al., 1988; Schepelmann et al., 1992).

The quality of the pain sensation depends on the tissue innervated by nociceptors. As an example, electrical stimulation of a muscle produces aching pain (Torebjork et al., 1984), electrical stimulation of a visceral nerve at lower intensity produces fullness and nausea and at higher intensity the stimulation cause pain (Ness and Gebhart, 1990; Willis and Westlund, 1997).

Some nociceptors are 'silent' or 'sleeping' under normal circumstances and can be 'awaken' or made more sensitive under certain conditions such as inflammation or tissue injury (Schaible and Schmidt, 1983a; Schaible and Schmidt, 1983b). These silent nociceptors are about 10%-20% subset of unmyelinated C fibres in skin, viscera, and joints (Millan, 1999).

Impulses from nociceptors are transmitted to spinal cord via two types of nerve fibres - A δ smaller myelinated fibres or C unmyelinated fibres. A stimulus of receptors connected to an A δ fibre results in a 'sharp', 'first' or 'pricking' pain whereas a stimuli of receptors connected to a C fibre results in 'dull', 'slow', or 'second' pain. The majority of A δ and C neurones terminate in the superficial region of the dorsal horn innervating cells of laminae I and II.

Interestingly, pain can result from lesions in central or peripheral nervous system without involving peripheral receptors as neuropathic pain (Boivie et al., 1989). Neuropathic pain is characterized by hypersensitivity at the site of damage and in adjacent normal tissue associated with allodynia and hyperalgesia.

Spinal Cord

Spinal cord is the main nerve tract, which connect the peripheral nervous system to the brain. Via the spinal cord, sensory impulses reach the brain and impulses from the brain travel down to motor neurones. The peripheral nerves are connected to the spinal cord via the spinal nerves containing both sensory and motor fibres. In humans, there are 31 pairs of spinal nerves: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal. All afferent information to the spinal cord is conveyed via the dorsal root fibres and efferent information via ventral root fibres. Other than motor and sensory information, most of the sympathetic pathways and the lower (i.e. non-vagal) parasympathetic pathways also go through the spinal cord.

The spinal cord is comprised of an outer zone of white matter and a butterfly-shaped central component of cells and fibres called gray matter. The white matter consists of three columns - dorsal, lateral and ventral. The gray matter is divided into three zones – dorsal horn, intermediate zone, and ventral horn.

Dorsal Horn

The dorsal horn consists of five zones (lamina I-V) where nociceptive information begins to be processed in the central nervous system. Interneuronal networks in the dorsal horn modulate and transmit the nociceptive information to neurones projected to brain or pass to other spinal neurones such as flexor motor neurones. Some stimuli sensitise and some inhibit projection neurones and the balance of these stimuli results in nociceptive transmission to the brain. The laminae I, II, V, VI, and X of the gray matter of the spinal cord and medullary caudalis nucleus of the trigeminal system are the regions predominantly involved in the reception, processing and rostral transmission of nociceptive signals (Riedel and Neeck, 2001). Neurones processing receptive fields are organised in a somatotopic manner in the dorsal horn (Schmidt et al., 1997). Cutaneous nociceptive C fibres end heavily in lamina II and less intensely in lamina I with weak input to lamina V. Cutaneous nociceptive A δ fibres terminate predominantly in lamina I with a sparse extent to lamina II. Unmyelinated, nociceptive afferents from the viscera, joints and muscles terminate primarily in lamina I and V/VI (Millan, 1999).

Spinal receptors and mediators involved in nociception.

Several neurotransmitters have been found in the terminals of nociceptive primary afferents that terminate in the dorsal horn including glutamate and other excitatory amino acid (EAA), (De Biasi and Rustioni, 1988), substance-P, somatostatin, VIP, and calcitonin gene related peptide (CGRP) and nitric oxide (NO) (Willis and Westlund, 1997). These neurotransmitters and neuropeptides together with other enzymes display a complex pattern of co-localisation, co-modulation and co-release in the primary afferent terminals. Neurochemical composition of primary afferents varies both qualitatively and quantitatively depending on the type of tissue they come from (skin, muscle and viscera etc), state of the tissue (normal state, inflammation state, etc), and the class of primary afferent fibres (C fibres, A δ , etc) (Millan, 1999). Glutamate, aspartate, tachykinins and CGRP are mainly co-localized in a subset of capsaicin-sensitive small calibre fibres projecting to the dorsal horn and these transmitters produce excitatory post-synaptic potentials in the dorsal horn (Willis and Westlund, 1997). CGRP exist in two isomer forms and act on two receptor subtypes CGRP₁ and CGRP₂. Glutamate and aspartate act on metabotropic receptors, NMDA and AMPA receptors located in various neuronal classes in the dorsal horn, located presynaptically as well as postsynaptically to primary afferent terminals. NMDA and AMPA receptors are involved in various functions other than processing of nociception such as development of opioid tolerance and NO synthesis (Fürst, 1999; Willis

and Westlund, 1997). NMDA receptors are found as autoreceptors in terminals of primary afferent fibers, where they can facilitate the transmission of inputs to the spinal cord by increasing the release of neurotransmitters from the primary afferent terminals (Liu et al., 1994).

Substance-P is more prominent in C fibres coming from muscles and other deeper tissues than from skin while cutaneous A δ fibres contain little or no substance-P (Lawson et al., 1997). Substance-P and neurokinin-A act mainly on neurokinin1 and neurokinin2 receptors in the dorsal horn (Bentley and Gent, 1995).

There are several neuropeptides that act as neurotransmitters in the dorsal horn. Tachykinins and CGRP are the two main neuropeptides. Other than these corticotropin, cytokinin, galanin, and VIP also act as neurotransmitters in dorsal horn.

Glutamate, aspartate, neurotensin, VIP, and substance-P are the main excitatory neurotransmitters while GABA, glycine, (Lin et al., 1994; Willcockson et al., 1984a), and enkephalins (Duggan et al., 1977; Willcockson et al., 1984b) are the inhibitory neurotransmitters in the dorsal horn. GABA act on GABA_A and GABA_B receptors which form axo-axonic synapses with primary sensory afferents in the dorsal horn to produce presynaptic inhibition. Glycine is another inhibitory amino acid, found in higher concentrations in gray matter and is believed to play a role in nociception (Fürst, 1999) activating mainly glycine receptors.

Three types of opioid receptors μ , κ , and δ are found in pain transmission pathways and are involved in spinal and supraspinal analgesia. In lamina I and II, μ receptors are more abundant. These are the common type of receptors targeted in acute and chronic pain management. Endogenous opioid peptides, β -endorphin and enkephalins are found in various places in CNS including the dorsal horn (Fürst, 1999).

Other main receptors involved in nociceptive transmission in spinal cord are adrenoceptors, serotonin receptors and acetylcholine (ACh) receptors. Descending pain inhibitory pathways contain α_2 -adrenoceptors, especially of the α_{2A} subtype and subtypes of 5HT₁, 5HT₂, and 5HT₃ receptors in the dorsal horn (Millan, 1997). Norepinephrine and serotonin are involved in regulation of the nociceptive threshold obtained by opiate analgesia. Iwamoto *et al* suggested an involvement of spinal muscarinic ACh receptors (mAChR) in the descending pain inhibitory pathway (Iwamoto, 1991; Iwamoto and Marion, 1994). There are evidences for an interaction between muscarinic and adrenergic receptor mechanisms in the regulation of pain threshold (Eisenach, 1999; Pan et al., 1999).

Adenosine triphosphate (ATP), NO and prostaglandin (PG) also act as potential transmitters of nociceptive information in the dorsal horn. These substances are released from primary afferent terminals, intrinsic neurons and non-neuronal sources. NO is produced in a wide variety of cell types and exert actions both at its site of formation as well as at neighboring sites fol-

lowing extracellular diffusion and membrane penetration. Recent investigations suggest that NO plays a role in nociceptive processing in the dorsal horn with an involvement of NMDA, α_2 adrenergic or ACh receptors (Eisenach, 1999; Meller and Gebhart, 1993; Pan et al., 1998).

Acetylcholine and Cholinergic receptors

ACh, the first neurotransmitter to be identified, was identified in 1914 by Henry Hallett Dale, and later confirmed as a neurotransmitter by a German physiologist, Otto Loewi (they received the Nobel Prize in Physiology or Medicine, 1936). Originally the transmitter was called "vagusstoff" because it was found to be the substance released by stimulation of the vagus nerve.

ACh is synthesised by the enzyme choline acetyltransferase which uses acetyl coenzyme A and choline as substrates for the formation of ACh. Upon release, ACh is metabolised into choline and acetate by acetylcholinesterase, and other nonspecific esterases. ACh is one of the main neurotransmitters in the neural synapses in the brain, in the spinal cord and in peripheral nervous tissues as well as at the motor end plate of vertebrate muscles. ACh release can be excitatory or inhibitory depending on the type of tissue and the nature of the receptor with which it interacts. Cholinergic receptors, on which ACh mainly act, can be divided into two types, muscarinic and nicotinic, based on the pharmacological action of various agonists and antagonists. Muscarinic receptors originally were distinguished from nicotinic receptors by the selectivity of the agonists muscarine and nicotine respectively.

Nicotinic receptors

Nicotinic ACh receptors (nAChR) are transmembrane proteins belonging to a super family of ligand-gated ion channels that include GABA (GABA_A, GABA_C types), glycine and serotonin (5-HT₃ type) receptors. nAChRs consist of five polypeptide subunits arranged symmetrically perpendicular to the membrane with a central pore. Several subunits- α_{1-9} , β_{1-4} , δ , γ and ϵ have been found to cooperate to form nAChRs in different places in vertebrates. Each subunit has spanning regions, M₁-M₄ and M₂ forms of the wall of the channel. While open, nAChRs conduct cations, which can cause a local depolarisation of the membrane. Comparative analysis of the available nAChR subunits gene sequences suggests that the first duplication between nAChR subunits is probably older than one and a half billion years. (Corringer et al., 2000).

nAChRs are widely distributed in the central nervous system and have been implicated in multiple physiological and pathological conditions. nAChRs can be divided into two main groups depending on the location:

muscle receptors, which are found at the skeletal neuromuscular junction where they mediate neuromuscular transmission, and neuronal receptors, which are found throughout the peripheral and central nervous system where they are involved in fast synaptic transmission. In mammalian adult skeletal muscle, neuromuscular nAChR consist of two α_1 , and one of each β_1 , δ , and γ subunits where as in spinal cord and brain they consist of α and β combinations or α subunit homomeric arrangements where the $\alpha_4\beta_2$ and α_7 is the commonest combinations. Autoradiographical analysis in brain suggests that presumably $\alpha_4\beta_2$ and α_7 have largely unique but sometimes overlapping distributions (Ronald.J.Lukas, 1998). nAChRs have been shown to be involved in the release of neurotransmitters such as ACh, dopamine, norepinephrine, serotonin, glutamine, and GABA in brain (Alkondon et al., 1999; Alkondon et al., 1996; Gray et al., 1996; Guo et al., 1998; Li et al., 1998) and serotonin, substance P and excitatory amino acids in spinal cord (Cordero-Erausquin and Changeux, 2001; Khan et al., 1996; Lloyd and Williams, 2000).

nAChRs have been demonstrated in primary afferents and dorsal root ganglia (Roberts et al., 1995), and both ascending nociceptive pathways and descending pain inhibitory pathways in - dorsal horn, nucleus raphe magnus, periaqueductal gray, pedunculopontine tegmental nucleus, thalamus and cortex.

Antinociception has been observed after administration of the nAChR agonist nicotine (Iwamoto, 1991; Sahley and Berntson, 1979), epibatidine (Curzon et al., 1998), A-85380, (Curzon et al., 1998) and ABT-594 (Bitner et al., 1998) to some areas in the brain. At least some of these agonists activate multiple, descending, pain modulatory pathways that terminate in the spinal dorsal horn involving multiple neurotransmitter systems. Whether spinal cord nAChRs act as a primary site for nicotinic agonist induced antinociception is controversial. There are findings in favour as well as against an antinociceptive effect of intrathecally administered nicotine and epibatidine. Nicotine and epibatidine, the main agonists on nAChRs, have overlapping as well as distinct pharmacological activities.

A characteristic property of nAChRs is the desensitisation, which is well described in brain related to smokers and treating patients with Alzheimer's disease using acetylcholinesterase inhibitory drugs. It has been shown that nicotine, choline and ACh itself, desensitise nAChR (Alkondon et al., 1997). Long-term exposure to nicotine has been shown to result in an increase in the density of nicotine-binding sites in brain tissue. Post-mortem autoradiographs of human brain have revealed that the density of [3H]-epibatidine and [3H]-cytisine binding is increased in brains from smokers compared to matched controls (Perry et al., 1999). This effect of nicotine is unusual in that it is contrary to the general accepted phenomena, over exposure to agonist produce receptor down regulation (Paterson and Nordberg, 2000).

Muscarinic receptors

Muscarinic receptors (mAChR) belong to the seven transmembrane-spanning receptor family with G-protein coupled. The receptors consist of an extracellular amino terminus, seven putative transmembrane domains (I-VIII), and an intracellular carboxy terminus. Now there are five receptors defined based on pharmacological criteria (Waelbroeck et al., 1990) as well as molecular cloning (Bonner et al., 1987; Bonner et al., 1988). It is recommended to use “M1, M2, M3, M4, and M5,” when referring to both proteins and pharmacological activities (Matsui et al., 2004). mAChRs can be divided into two broad functional categories: the M1, M3, and M5 receptors that preferentially couple to the Gq family of G-proteins which activates phospholipase C whereas the M2 and M4 receptors preferentially couple to the Gi family of G-proteins which inhibit adenylate cyclase activity. Muscarinic receptors are present in neurones in the central and peripheral nervous system, cardiac and smooth muscles, and a variety of exocrine glands.

Among their functions and locations M1 receptors are predominant in all forebrain areas including the cerebral cortex, hippocampus and corpus striatum, M2 receptors control cardiac function and M3 receptors control salivation and intestinal motility. The M2 receptor is also the major subtype in sympathetic ganglia, ileum, uterus and atrium. The M4 receptor is mainly localised in striatum (Ince et al., 1997). The M5 receptor is distributed at very low levels throughout several brain regions including midbrain regions (Ince et al., 1997). Spinal mAChRs has been shown to be involved in antinociception initiated by muscarinic agonists (Iwamoto and Marion, 1993b; Machelska et al., 1999; Naguib and Yaksh, 1997) and the antinociceptive effect of neostigmine has been described by the activation of mAChRs (Naguib and Yaksh, 1994). An involvement of spinal mAChRs in blood pressure control has been shown (Carp et al., 1994).

Serotonin receptors

5-hydroxytryptamine (5-HT; serotonin) receptors are assigned to at least seven families, 5-HT₁–7, comprising a total of 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes. The 5-HT receptor family is mostly a seven transmembrane-spanning receptor family with G-protein coupled except for the 5-HT₃ receptor, which is a ligand-gated ion channel. 5-HT₁ receptors are negatively coupled to adenylyl cyclase and exert an inhibitory influence upon neuronal excitability where as 5-HT₂ receptors are positively coupled to phospholipase and exert excitatory influence upon neuronal activity (Barnes and Sharp, 1999).

5-HT receptors are one of the most complex receptors on the basis of their structural, functional and to some extent pharmacological characteristics.

There are emerging evidences that many 5-HT receptor subtypes have naturally occurring polymorphic variants making 5-HT receptors a major source of biological variation within the nervous system. 5-HT receptors are widely distributed in the central nervous system, peripheral nervous system, non-neuronal tissues and have been implicated in multiple physiological and pathological conditions.

In the dorsal spinal cord, 5-HT elicits a spectrum of pro- and antinociceptive actions with an involvement of different receptor subtypes (Millan, 2002). 5-HT receptors play a major role in descending pain inhibitory pathways with direct inhibitory and excitatory effects on projection neurones and inhibitory interneurons. Cell bodies of almost the entire spinal serotonergic nervous system are located in supraspinal sources (Kirifides et al., 2001; Li et al., 1997).

GABA receptors

GABA is the main inhibitory neurotransmitter in the mammalian central nervous system originally demonstrated in 1950. GABA activates three major types of GABA receptors, GABA_A, GABA_B and GABA_C. GABA_A and GABA_C are ligand-gated ion channels while GABA_B is a seven transmembrane receptor.

GABA_A receptors mediate fast synaptic inhibitory neurotransmission in the nervous system. GABA_A receptors consist of five subunits arranged to form an ion channel. By changing the combination of these subunits, GABA_A receptors with different pharmacological and physiological properties can be formed and are found in different places in the central nervous system (Johnston, 1996). Structurally diverse substances interact with GABA_A receptor via different sites in the receptor making the GABA_A receptor one of the most common drug target in the central nervous system. Drugs such as benzodiazepines and barbiturates exert their actions through an interaction with the GABA_A receptor.

GABA_B receptors are located pre- and postsynaptically in the brain and spinal cord. Presynaptic receptors function as autoreceptors, regulating GABA release or function as heteroreceptors, regulating the release of other neurotransmitters. Postsynaptic receptors results in slow, prolonged inhibitory potentials.

In the dorsal horn of the spinal cord, GABA_A and GABA_B receptors are found in high concentrations in laminae I – III. Both GABA_A and GABA_B receptors have a presynaptic location on primary afferent fibres, where GABA_B receptors act as autoreceptors at the GABA-containing interneurons which synapses on primary afferent fibres.

Ascending nociceptive tracts

The nociceptive impulses from the dorsal horn transmit via projection neurons to various regions in the brain such as the thalamus, periaqueductal gray (PAG), reticular formation, parabrachial region, and limbic structures. The axons are arranged in tracts in the white matter of the spinal cord.

The Spinothalamic tract is located in the anterolateral system in spinal cord and ends mainly in the contralateral thalamus. The lateral spinothalamic tract contains sensory neurones for pain, temperature and light touch; and the anterior spinothalamic tract contains sensory neurones for touch and pressure. Two types of projections are found in the spinothalamic tract, which end in different nuclei in the thalamus. The lateral projection originates mainly in laminae II and V in the dorsal horn and ends in the ventral posterior lateral, ventral posterior inferior and central lateral nucleus in the thalamus. The medial projection originates mainly in deep dorsal and ventral horns and end mainly in the central lateral nucleus in the thalamus.

The Spinomesencephalic tract originates in lamina I and IV – VI and terminates at different areas in midbrain such as the PAG, nucleus cuneiformis, intercolliculus nucleus, deep layer of the superior colliculus, anterior and posterior pretectal nuclei etc. Different components of this tract has different functions as projections to PAG can activate descending nociceptive inhibitory systems and the projection to the superior colliculus can play a role in orientation.

The Spinoreticular tract originates in deep layers of the dorsal horn and in some areas in the ventral horn and terminates in the reticular formation of medulla and pons. This tract transmits inputs to the brainstem autonomic centres.

Other than these, tracts have been described projecting to the limbic system, hypothalamus, and to amygdala from the dorsal horn (Willis and Westlund, 1997).

Descending pain modulatory tracts

Reynolds *et al.* described the midbrain PAG as an important pain modulatory centre in 1969 and originated the idea of descending pain modulator pathways (Reynolds, 1969). Several areas are involved in this system in the brain such as PAG, nucleus raphe magnus (NRM), locus coeruleus, and some nuclei in the bulbar reticular formation. Stimulation of these areas with electrical stimuli or receptor agonists like nicotine, ABT-594, baclofen, or

carbachol has been shown to produce antinociception (Bitner et al., 1998; Brodie and Proudfit, 1986; Hammond et al., 1998; Ishizawa et al., 2000; Iwamoto, 1991). Most of the connections from PAG to the dorsal horn are not direct but passes via the NRM, locus coeruleus, A5 cell group, and nuclei in the pontine and medullary reticular formation (Cameron et al., 1995). Most of the fibres terminate in the inner layer of lamina II and a significant amount in lamina I, the outer layer of lamina II, and lamina III as shown in c-fos studies (Sandkuhler, 1996). These descending modulatory pathways act on terminals of primary afferents, projection neurones, excitatory and inhibitory interneurons in the dorsal horn (Millan, 2002) and result in inhibition or facilitation on stimuli transmitted from primary afferents to projection neurones.

Descending pain inhibitory pathways consist mainly of serotonergic and noradrenergic neurones and to lesser extent dopaminergic neurones. Cell bodies of these neurones are located in different regions in the brain stem. Stimulation of these serotonergic and adrenergic neurones in the brain-stem results in antinociception.

There are evidences on a tonically active nature of this system in various experimental models (Cervero et al., 1991; Dickhaus et al., 1985; Hall et al., 1982). Increased release and synthesis of GABA, glycine and nitric oxide have been observed in the dorsal horn after stimulation of this pathway (Peng et al., 1996; Xu et al., 1996).

Spinal acetylcholine release and pain modulation

Spinal administration of cholinergic receptor agonists (Gillberg et al., 1989; Iwamoto and Marion, 1993b; Qian et al., 1993; Yaksh et al., 1985) as well as choline esterase inhibitors (Bouaziz et al., 1995; Hood et al., 1995; Naguib and Yaksh, 1994) has been shown to produce antinociception in animals as well as in humans. The mechanisms underlying spinal cholinergic antinociception as well as spinal ACh release are not well understood. There are evidences for an involvement of mAChRs (Iwamoto and Marion, 1993b, Naguib and Yaksh, 1997) as well as nAChRs (Khan et al., 1998) in spinal antinociception. Other than the two cholinergic receptors, mAChR and nAChR, behavioural studies show an involvement of serotonin (Crisp et al., 1991; Eide and Hole, 1991; Xu et al., 1994) and GABA receptors for spinal pain transmission. There are evidences for an interaction of serotonin and GABA receptors with the cholinergic nervous system in relation to spinal nociceptive mechanisms (Iwamoto and Marion, 1993a ; Li et al., 2002 ; Obata et al., 2002 ; Obata et al., 2003 ; Rashid and Ueda, 2002).

In the experiments discussed in this thesis, we studied the role of mAChR, nAChR, serotonin and GABA receptors in the regulation of spinal

ACh release using mainly a spinal microdialysis model to measure ACh in the spinal cord.

Aim of the study

The general aim of this thesis was to study the mechanisms that regulate spinal ACh release.

Specific aims

1. To study the spinal nicotinic receptor involvement in intraspinal ACh release.
2. To further study the effect of the potent nicotinic agonist, epibatidine, on spinal ACh receptors.
3. To investigate the possible involvement of muscarinic receptors on the potent analgesic effect of epibatidine.
4. To study the serotonin receptor modulation of spinal ACh release and to examine the serotonin receptor subtype involved.
5. To investigate the GABA receptor involvement in the regulation of spinal ACh release.

Materials and Method

Animals

Male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) weighing 330–400g were provided with free access to food (R36, Ewos, Vadstena, Sweden) and tap water at all times. The animals were kept in the animal facility at BMC, Uppsala University, Sweden, on a 12 hr light/dark cycle (lights on 6 am to 6 pm) at 20 ± 1 °C for one week before use. All experiments were conducted after approval by the animal ethics committee in Uppsala, Sweden.

Microdialysis

Microdialysis is a procedure in which a probe perfused with fluid is inserted into a living tissue. In the extracellular space of the tissue, substances exchange between the fluid in the probe and extracellular tissue fluid. This mechanism has been used for various applications in medical practice as well as experiments in living tissues. Microdialysis has been used to collect and introduce substances in various places such as brain tissue, spinal cord tissue, epidural space, intrathecal space, blood vessels, uterus, and kidneys. In our experimental model, we performed spinal microdialysis in which a small probe was inserted into the spinal cord tissue.

Anaesthesia was induced with 4.5% isoflurane (Abbott Scandinavia AB, Solna, Sweden) in 100% oxygen. The trachea of rats were intubated and connected to a Harvard (Harvard Apparatus Inc., Holliston, MA, USA) ventilator and placed on a heated pad to maintain body temperature (perirectal temperature) at 37.5°C. During surgery, anaesthesia was maintained with about 3% isoflurane in 100% oxygen and the end-tidal pCO₂ was kept at 4 kPa. For insertion of the microdialysis probe, a midline incision was made at the back of the skull. Neck muscles were removed carefully to expose the *cisterna magna*. The *dura* and *pia mater* were cut and a semi-rigid spinal microdialysis probe was inserted dorsally in the spinal tissue. The probe was located longitudinally with the tip at about the C5 level in the superficial dorsal horn.

The probe was constructed from a hollow fibre of 300 µm outer diameter having a cut-off at 11 kDa molecular weight. The dialysis membrane was

bowed to form a U-shaped loop, 12 mm long. The microdialysis probe was perfused at a flow rate of 2.5 $\mu\text{l}/\text{min}$ with Ringer's solution (147 mM NaCl, 2.4 mM CaCl, 4.0 mM KCl) containing 10 μM neostigmine to prevent degradation of acetylcholine (Billard et al., 1995; Höglund et al., 2000; Roth et al., 1996). Neostigmine is an essential component in the Ringer's solution to prevent acetylcholine degradation and to get measurable acetylcholine levels. We have found that the concentration of neostigmine (range of 0.1 μM to 10 μM) does not influence the percentage increase of acetylcholine after nicotine administration.

After insertion of the microdialysis probe, the isoflurane concentration was reduced to 1.5% and rats were allowed to rest for 40 min before starting the sampling of 20 μl spinal microdialysates. ACh was quantified on-line by High Performance Liquid Chromatography (HPLC) as described earlier (Höglund et al., 2000). In each experiment *in vitro*, pre- and post-recovery of the probes was assessed by dialysis of a 0.5 μM standard to ensure that the probes had not been damaged during the experiment. Only data from experiments where the mean post-recovery was within three standard deviations of mean pre-recovery are presented here.

Tissue preparation

Muscarinic receptors were prepared in Sf9 cells grown in glass spinner bottles at 27°C in SE-900 II medium (Life Technologies, Paisley, U.K) supplemented with 100 U/ml penicillin (Sigma, St. Louis, MO, U.S.A) and 80 U/ml streptomycin (Sigma). The cultures were maintained at a density of 1-3 $\times 10^6$ cells/ml. The cells were infected with recombinant baculovirus designed to express human muscarinic receptor m1-m5 (Kukkonen et al., 1996). After 72 h post infection the cells were harvested by centrifugation and washed in phosphate buffer saline solution. Then cells were rapidly frozen in -20°C temperature.

Rats were killed by decapitation, and spinal cords were removed rapidly and immediately placed in -20°C temperature.

For the binding studies, the tissue (Sf9 cells or spinal cords) was homogenized in a volume of cold buffer (50 mM phosphate buffer with 1 mM MgCl₂, pH 7.4) 50 times its wet weight, using a Teflon tissue grinder. The tissue was centrifuged at about 40000 \times G for 10 min at 4°C (Rotor SS34), and resuspended three times. After the second centrifugation the pellet was resuspended in distilled water, and a 0.2 ml sample was taken to determine the protein content of the homogenate using the BCA protein assay kit (PIERCE, Rockford, IL, USA).

Receptor binding studies

Saturating binding assays were performed using 12 concentrations of [³H]-epibatidine ranging from 1.5 to 3000 pM for spinal cord and using six concentrations of [³H]-NMS ranging from 40 to 4000 pM for each (M1 – M5) receptor preparation. Competition binding assays were performed using [³H]-NMS and ±epibatidine. Homogenates of spinal cords and of each receptor tissue preparation were incubated with 960 pM [³H]-NMS and ±epibatidine in a concentration range from 40 nM to 1000 μM. Non-specific binding was determined in the presence of 4 μM atropin. All incubations were performed in the mentioned buffer. After 2 hrs of incubation, the samples were filtered through Whatman GF/B glass-fiber filters which were pre-soaked in 0.3% polyethylenimine for 2 h, using a 30 sample Brandel cell harvester (Brandel, MD, USA). Radioactivity was counted by using a Philips scintillator. Binding study data was analyzed by using the Graph Pad program.

Results and Comments

Nicotinic acetylcholinergic receptors regulation of spinal acetylcholine release

We investigated the role of nicotinic ACh receptors in the regulation of intraspinal ACh release. Nicotine was administered alone and in combination with the nicotinic antagonists mecamylamine (50 μM), dihydro- β -erythroidine (D β E) (500 μM) and methyllycaconitine (MLA) (40 nM) via the microdialysis probe. Administration of nicotine (1 μM – 1 mM) produced a dose dependent increase of intraspinal ACh release, while 10 mM nicotine resulted in a dramatic increase in ACh release followed by a decrease to baseline. Administration of mecamylamine or D β E also induced an increased ACh release while MLA caused a decreased release. Mecamylamine and D β E, but not MLA pre-treatment attenuated the stimulatory effect of 100 μM nicotine on intraspinal ACh release.

Since nicotine and some of the nicotinic antagonists increased acetylcholine release, it is suggested that spinal ACh release is regulated by different nAChRs. Nicotine and nicotinic antagonists may act on different types of receptors with different affinity to produce the net effect of ACh release. We propose the possibility of an involvement of three nicotinic receptors. Receptor 1 is localised on non-cholinergic neurones that subsequently increase intraspinal ACh release when stimulated by nicotine. Receptor 2 would function as an autoreceptor or reside on inhibitory interneurons that indirectly, tonically, inhibit ACh release. An inhibition of receptor 2 by mecamylamine or D β E would cause an increased ACh release. MLA, contrary to D β E and mecamylamine, decreased ACh release below the basal level. This suggests that receptor 3 which is sensitive to MLA, might be involved in the regulation of basal ACh release. Some of these receptors may be desensitised by high nicotine concentrations leading to a reduction of ACh release.

Effect of the potent nicotinic agonist, epibatidine on spinal acetylcholine receptors

Epibatidine is a potent nicotinic receptor agonist with hundreds of times higher potency than nicotine. We investigated the possibility that part of the

action of (\pm) epibatidine, is mediated through an action on muscarinic receptors. The competition-binding assays showed that epibatidine displaces the muscarinic receptor antagonist from spinal cord homogenates indicating that epibatidine interacts with muscarinic receptors. The subsequent competition-binding studies using Sf9 cells expressing M1-M5 muscarinic receptors showed that epibatidine has some affinity to muscarinic receptors.

The intraspinal administration of 160 μ M epibatidine produced an increase in ACh release that was reduced by pre-treatment with 100 μ M atropine, a muscarinic receptor antagonist. As such a high concentration as 100 μ M of atropine could not fully antagonise the effect of epibatidine, it is likely that only a part of the effect of epibatidine on spinal acetylcholine release is mediated through muscarinic receptors. Contrary to intraspinally administered atropine, subcutaneous administration of atropine did not inhibit epibatidine induced intraspinal ACh release.

It seems that epibatidine, in μ M concentrations, is a partial muscarinic receptor agonist that may interact with spinal muscarinic receptors to increase ACh release. The dual action on both nAChRs and mAChRs may explain the potent analgesic effect observed after intra-spinal epibatidine administration. Epibatidine induced spinal ACh release observed after subcutaneous administration appears not to be mediated via muscarinic receptors, possibly because the effect on ACh release observed after a s.c. administration of epibatidine is a result of an activation of both spinal and supraspinal nicotinic receptors.

Serotonin receptors regulation of spinal acetylcholine release

We investigated the involvement of 5-HT₁, 5-HT₂, and 5-HT₃ receptor subtypes in the regulation of spinal ACh release. The selective serotonin reuptake inhibitor, citalopram increased ACh release where as 5-HT_{1A}, 5-HT_{1B}, 5-HT₂ receptor agonists increased the ACh release dose dependently. Only the 5-HT_{1A} and 5-HT_{2A} selective antagonists inhibited corresponding agonist induced ACh release.

Since citalopram administration increased the intraspinal ACh release it can be concluded that increased serotonin availability in the dorsal horn stimulates ACh release. Since only 5-HT_{1A}, 5-HT_{1B}, 5-HT₂ receptor agonists increased ACh release dose dependently and only 5-HT_{1A} and 5-HT_{2A} antagonists inhibited corresponding agonist induced ACh release we suggested that only the 5-HT_{1A} and 5-HT_{2A} receptors are involved in the regulation of spinal ACh release. It is likely that 5-HT_{1B} agonist induced ACh release was mediated by 5-HT_{1A} receptors as the 5-HT_{1B} agonist induced ACh release was reduced by administration of the 5-HT_{1A} antagonist.

It is unlikely that 5-HT_{1A} receptors are located on cholinergic neurones as they are not inhibiting ACh release contrary to the inhibitory nature of 5-HT_{1A} receptors. Considering current knowledge, the most probable location of the 5-HT_{1A} receptors is on cell bodies of GABA neurons which inhibit the firing rate of the neurones when activated by serotonin. By inhibiting the release of GABA, one of the inhibitory transmitters in the spinal cord, serotonin indirectly would increase ACh release. On the other hand, 5-HT_{2A} receptors might be located on cholinergic neurones increasing cellular excitability and hence increase ACh release.

GABA receptors regulation of spinal acetylcholine release

GABA_A and GABA_B receptor effects on ACh release were studied since the GABA receptor is one of the main spinal cord receptors involved in pain transmission. Agonists and antagonists for both GABA_A and GABA_B receptors were microdialyzed. We found that the GABA_A agonist, muscimol decreased ACh release whereas the GABA_A antagonist, bicucullin increased ACh release dose dependently. Although the GABA_B agonist decreased ACh release dose dependently, the GABA_B antagonist did not change ACh release.

Decreased ACh release after muscimol administration suggests that GABA_A receptors play an inhibitory role on ACh release in the spinal dorsal horn. Together with the finding that the GABA_A antagonist increased ACh release, this suggests that GABA_A receptors are tonically inhibiting the spinal ACh release. The results suggest that GABA_B receptors also are involved in the regulation of spinal ACh release. However, unlike GABA_A antagonists, GABA_B antagonists did not increase ACh release. This suggests that GABA_B receptors are not tonically regulating the spinal ACh release.

Considering earlier findings together with our results, we suggest that GABA_A receptors might be located on cholinergic neurones, while GABA_B receptors are located on non-cholinergic neurones, possibly glutamatergic neurones.

Conclusion

In the main experimental procedure involved in this thesis, we infused different receptor ligands via microdialysis probes and subsequently spinal ACh release was measured. Limited receptor-ligand binding studies also were performed with spinal cord homogenates as well as receptors expressed in cells.

It is clear that spinal ACh release is regulated by different receptors in the spinal cord. We found evidences for the involvement of three types of nicotinic receptor subtypes, 5HT_{1A} and 5HT_{2A} serotonin receptors and GABA_A and GABA_B receptors. The cholinergic, serotonergic, and GABAergic nervous systems seem to interact with each other and regulate each other by complex mechanisms. This is in consistence with earlier findings by other researchers who report the existence of feed back inhibitory loops as well as excitatory loops in the spinal cord with the involvement of different interneurons and receptors. Since we only measured ACh release, the results do not allow us to explain the exact mechanism by which ACh release is regulated in more details.

Since the receptor systems we investigated modulated ACh release and also are involved in pain transmission, according to earlier behavioural studies, we propose that ACh is one of the key transmitters in the regulation of nociception in the spinal cord.

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