Monoamines and the Modulatory Control of Spinal Sensory Processing

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
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Abstract

The descending monoamines serotonin (5-HT), noradrenaline (NA) and dopamine (DA), and acetylcholine (ACh), a spinal monoamine, have been previously shown to exert modulatory control over spinal functions, including nociception. Partly due to the multiple receptor subtypes these transmitters bind to, the mechanisms underlying their actions are not clearly understood. In three interrelated studies, whole-cell patch clamp recordings in a spinal cord slice preparation were conducted to investigate the manner in which the monoamines modify both synaptic responses and cellular properties of deep dorsal horn (DDH) neurons of the neonatal rat.

First, the actions of all four transmitters on individual DDH neurons were compared. The results demonstrated that 5-HT, NA and DA similarly depressed synaptic responses, while ACh produced facilitation in most neurons. Though none of these transmitters altered the passive membrane properties of the neurons, they converted neurons with phasic firing properties, in response to depolarizing current steps, to repetitive.

Secondly, the actions of several selective 5-HT receptor ligands on synaptic responses evoked in two age groups of animals (P3-6, P10-14) were compared. In both age groups, the 5-HT_{1A} receptor agonist produced synaptic depression in most neurons, while the 5-$HT_{3 \text{ and } 7}$ receptor agonists produced synaptic facilitation. Additionally, selective antagonism of the 5-HT_{1A} receptor generally facilitated evoked responses, suggesting that sensory transmission to the DDH is tonically inhibited by endogenous 5-HT.

Thirdly, since long-term potentiation (LTP) of primary afferent synapses in spinal neurons may represent 'memory modules' that produce long-lasting increases in sensory gain, we studied 5-HT's control over the induction and expression of sensory-evoked synaptic plasticity. 5-HT was tested on evoked synaptic responses before, during or after a conditioning stimulation (CS) that produced either LTP or long-term depression (LTD). Overall, 5-HT depressed the evoked responses, before or after CS-induced LTP and LTD. Importantly, CS in the presence of 5-HT significantly increased the incidence of LTD.

These studies suggest that the three descending monoaminergic transmitter systems are organized to exert similar modulatory control over spinal sensory functions. Furthermore, although various mechanisms might be involved, their general action at the cellular level of the spinal cord dorsal horn is depression.

Abbreviations

5-CT; 5-carboxamidotryptamine

5-HT; 5-Hydroxytryptamine; serotonin

8-OH-DPAT; 8-hydroxy-2-(di-n-propylamino) tetralin

ACh; acetylcholine

ACSF; artificial cerebrospinal fluid

AMPA; (±)-α-Amino-3-hydroxy-5-methylisoxasole-4-propionic acid

APV; (±)-2-Amino-5-phosphonopentanoic acid

Ca^{2+}; Calcium

CGS; 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline maleate

CNQX; 6-Cyano-7-nitroquinoxaline-2,3-dione

CPBG; 1-(m-chlorophenyl)-biguanide

CS; conditioning stimulation

DA; dopamine

DDH; deep dorsal horn

DLF; dorsolateral funiculus

DOI; 1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane

EGTA; ethylene glycol-bis(β-amino ethyl ether) N,N,N',N',-tetraacetic acid

EPSC; excitatory postsynaptic current

EPSP; excitatory postsynaptic potential

HEPES; N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

LTD; long-term depression

LTP; long-term potentiation

NA; noradrenaline

NMDA; N-methyl-D-aspartate

P; postnatal day

PAD; primary afferent depolarization

PKC; protein kinase C

QX-314; N-(2,6-Dimethylphenycarbamoylmethyl)triethylammonium bromide

R_{in}; Input resistance

VLF; ventrolateral funiculus

WDR; wide dynamic range

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Introduction

1.0 Overview

In recent years, there have been substantial advances in the understanding of spinal sensory processing. However, because of the clinical relevance of controlling sensory transmission, particularly nociception, there is still a need to elucidate the complex physiological and biochemical processes involved. Some of the hindrances towards understanding spinal sensory processing are related to the enormous heterogeneity of spinal functional populations in the spinal cord, as well as the considerable degree of structural reorganization and functional alteration (plasticity) that can be induced, particularly involving the dorsal horn neurons that integrate then convey incoming sensory information to other locations.

An important observation is that plasticity in spinal cord sensory processing can be induced in response to primary afferent stimulation. For instance, repetitive stimulation of primary afferents at C fibre intensity is often associated with the expression of windup and central sensitization of spinal neurons. In addition, longer-term alterations in synaptic efficacy (LTP and LTD) are also induced following repetitive primary afferent stimulation. A second important observation is that many endogenous transmitters function to modify spinal sensory systems. In particular, one family of transmitters, the biogenic amines, have been shown to exert powerful modulatory control over spinal functions; generally depressing sensory, while facilitating motor systems (Jacobs and Fornal, 1993).

The purpose of this thesis is to explore some of the modulatory properties of spinal dorsal horn neurons, with specific emphasis on neurons found in the deeper laminae of the dorsal horn. Many of these neurons play an essential role in the processing, then rostral or segmental transfer of sensory information, including nociception, and many others are interposed in spinal reflex pathways. The projects comprising this thesis focus on the modifiability of synaptic and cellular properties of deep dorsal horn (DDH) neurons induced by two processes: neuromodulation by bath-application of the monoaminergic transmitters and by conditioning stimulation of primary afferents.

Although, the dorsal horn plays a major role in processing all types of spinal sensory information, in relation to plasticity and neuromodulation, pain is one of the most intensely studied sensory systems. In addition, many of the short- and long-term alterations that occur in the spinal cord dorsal horn may trigger several pain states. Therefore, although this thesis deals with the modification of cellular and synaptic properties of dorsal horn neurons involved in general sensory processing and is not selective to nociception, pain will be the 'model' sensory system discussed in relation to our findings. As a result, the reader should be reminded that our observations are not indicative of the pain system, but of the entire spinal sensory system. For the previously mentioned reasons, it is first preferable to provide an overview of the spinal cord dorsal horn and the sensory system with emphasis on the pain system. I will discuss some of the interesting recent developments in the spinal sensory system. Specifically, I will focus the introduction on an overview of several aspects of the pain system, which will include a brief description of the particular importance of the dorsal horn in sensory processing, the

pain pathway and various pain states. In addition, the endogenous neuromodulatory control of spinal sensory systems by the bulbospinal monoaminergic system and the expression of synaptic plasticity (LTP and LTD) as a cellular mechanism for long-term changes in sensory gain will also be discussed.

1.1 Organization of Thesis

In this thesis, the whole cell 'blind' patch technique in a neonatal rat spinal cord slice preparation was used to record from DDH neurons, while synaptic responses were evoked by electrical stimulation of the dorsal roots. To explore the modification in synaptic and cellular properties of the DDH neurons, three projects were designed. Following a general introduction, each project is presented in individual sections comprising an abstract, introduction, methods, results and discussion for that project. The tables and figures applicable to each project are placed at the end of each specific discussion section. At the end of the third project, a general discussion encompassing all three projects follows.

2.0 Spinal Cord

The spinal cord is a part of the central nervous system (CNS) that has the unique role of conducting impulses between the brain and peripheral nerves; and also serves many important reflex functions (see Heimer, 1983). It consists of the gray matter (containing cell bodies and dendrites of neurons and glial cells) and the white matter (containing mainly axons grouped into fibre tracts). The gray matter, symmetrical across the midline, is divided into the dorsal horn, intermediate gray and ventral horn, each consisting of

several laminae. The dorsal horn serves mainly as an initial CNS site for processing incoming somatosensory information, while the ventral horn is comprised mainly of motor nuclei that innervate skeletal muscle.

Despite the spinal cord primary role as a conductor of impulses, it allows for the immense integration and alteration of both sensory and motor information, prior to their transmission supraspinally or as motor output, respectively. Various types of modifications known as 'plasticity' have been observed in the spinal cord, allowing for amplification or suppression of impulses prior to transmission to the brain (for review see Wilson and Kitchener, 1996). Plasticity is described as "the various properties of neurons, and particularly their synaptic connections that allow changes of functional connectivity to persist long after the termination of the stimulus which initiated the changes" (see Wilson and Kitchener, 1996).

Spinal plasticity includes alterations in termination patterns of primary afferents following peripheral injury (e.g. Woolf et al, 1992). Also, receptive fields of sensory neurons are altered, generally increasing in size following primary afferent stimulation (Cook et al, 1987). This type of plasticity may be associated with the central sensitization (see below) of spinal neurons (Woolf, 1983). One type of plasticity observed in the spinal cord is synaptic plasticity, expressed as both long-term potentiation (LTP) and long-term depression (LTD). This type of plasticity involves long-lasting alterations in synaptic efficacy (for review see: Bliss and Collingridge, 1993; Pockett, 1995b; Randic, 1996; Malenka and Nicoll, 1999).

3.0 Deep dorsal horn

Sensory transmission is initiated by graded stimuli from multiple sensory modalities, many of which converge and interact spatiotemporally on dorsal horn cells. The dorsal horn is sub-divided into superficial (laminae I - II) and deep (laminae III-VI) layers (refer to Willis and Coggeshall, 1991). Here an integrated response is transmitted to relevant brain regions via projection neurons. This sensory transformation is dynamically controlled by many factors and includes; state-dependent gating of reflex pathways by segmental and descending systems (Le Bars et al, 1992; Jankowska et al, 1993; Sandkühler, 1996), neuromodulation of pre- and postsynaptic elements (for review see Randic, 1996) and activity-dependent synaptic plasticity (see references below). While considerable effort has been undertaken to characterize the organization of nociceptive signaling in the superficial dorsal horn, the function of deep dorsal horn (DDH) neurons in nociception is less well understood (e.g. Woolf and King, 1987; Price, 1988; Willis and Coggeshall, 1991; Coderre et al, 1993).

3.1. Properties of the DDH

3.1.a. Cell types

Most of the previous studies classifying the neurons of the deep dorsal horn were conducted in cats and primates (see Willis and Coggeshall, 1991). In contrast, most of the studies classifying neurons of the rat were conducted in the superficial dorsal horn. However, Kobayashi (1998) showed that spinothalamic neurons of the rat DDH exhibit many of the morphological and distribution properties of DDH neurons in other species.

The DDH contains a heterogeneous population of neurons ranging in sizes from small (< 10 μm) in diameter to large (> 40 μms) (see Willis and Coggeshall, 1991). The majority of these neurons are intrinsic interneurons; axons remaining in the spinal gray matter, while others are projection cells; axons entering the spinal white matter (see Willis and Coggeshall, 1991). These interneurons, which include the group Ia, Ib and II interneurons, receiving primary afferent input predominantly from muscle afferents and also from cutaneous afferents, play many roles in mediating several spinal reflexes both at the interneuronal and premotoneuronal level (see Baldissera, 1981; Jankowska, 1992). Projection neurons, comprising a smaller fraction of DDH neurons, are subdivided into propriospinal neurons, whose axons remain in the spinal cord and ascending tract neurons, whose axons project supraspinally. Propriospinal neurons are likely to play an integrative role within the spinal cord in communicating between various segments (Millan, 1999) and may also mediate descending mechanisms of inhibition in the dorsal horn (Sandkühler, 1996). The projection neurons comprise the spinocervical, spinocerebellar, postsynaptic dorsal column, spinothalamic, spinoreticular and spinomesencephalic cell groups (Willis and Coggeshall, 1991). With the exception of the spinocervical cell group, these are all ascending tract neurons, conveying sensory information to the cerebellum, thalamus, reticular formation and brain stem.

3.1.b. Primary afferent input

Neurons in the DDH receive primary afferent input from a variety of sources, such as hair follicle afferents, Pacinian corpuscle afferents and rapidly and slowly adapting

mechanoreceptive afferents (Willis and Coggeshall, 1991). A prominent number of group Ia muscle spindle afferents is also found in lamina VI (Maxwell and Bannatyne, 1983). In addition, many interneurons located in laminae IV, V and VI receive synaptic input from group Ib and II afferents, cutaneous and joint afferents (reviewed in Baldissera et al, 1981; Jankowska, 1992). Some nociceptors, mainly Aδ fibres and primary afferents of visceral origin, project segmentally to laminae V and deeper laminae of the spinal dorsal horn (e.g. Cervero and Connell, 1984). In addition, many neurons of the DDH send their dorsal dendrites superficially (antenna-type cells), thus they receive direct primary afferent input from fibres that terminate superficially (Woolf and King, 1987; Todd, 1989; Willis and Coggeshall, 1991), which includes substance P containing primary afferent fibres, presumably nociceptors (Naim et al, 1997).

3.1.c. Neurochemicals

Many neurally active compounds and transmitters are present in the DDH (for review see Fields et al, 1991; Willis and Coggeshall, 1991; Broman, 1994; Millan, 1999). The presence of these compounds is consistent with the role the DDH plays in sensory processing including nociception. These include the excitatory amino acids (glutamate and perhaps aspartate), which bind to two main classes of glutamate receptors: ionotropic (NMDA and AMPA/kainate) and metabotropic receptors; and the inhibitory amino acids, γ-aminobutyric acid (GABA) and glycine (see Dickenson,1997). Also found in the DDH are the biogenic amines: serotonin, noradrenaline, dopamine and acetylcholine; the neuropeptides, which include the opiates (enkephalin and dynorphin) and the tachykinins (e.g. substance P). In addition, the purines (e.g. adenosine) (Coderre et al, 1993; Reeve

and Dickenson, 1995) and the recently discovered opioid-like peptide, orphanin FQ/nociceptin are also found in the DDH (e.g. Riedl et al, 1996).

3.1.d. Synaptic properties

Primary afferent-evoked synaptic responses to DDH neurons are mainly glutamatergic, comprising primarily of an early fast (short latency) AMPA/kainate and late slow (longer latency) NMDA receptor-mediated components (Gerber and Randic, 1989; Miller and Woolf, 1996). Dorsal horn neurons have been classified into 3 general categories based on their source of peripheral afferent input (Mendell, 1966). Generally, nociceptive specific (NS) neurons receive input only from nociceptors (Aδ and C) while low-threshold (LT) neurons receive input only from low threshold afferent fibres (Aαβ). Wide dynamic range (WDR) or convergent neurons, also referred to as class 2 neurons, receive convergent synaptic input from both low and high threshold afferents (see Chung et al, 1979; Schouenborg et al, 1995). Most DDH neurons fall into this third class of neurons (see below).

3.1.e. Intrinsic membrane properties

The integrative properties of DDH neurons may represent an initial sensory point through which most incoming sensory information must be processed and also provides a critical site for both segmental and descending neuromodulatory control. Some membrane properties of DDH neurons in the rat have been characterized intracellularly both in *in vivo* (e.g. Jiang et al, 1995) and *in vitro* studies (King et al, 1988; Huang, 1989; Lopez-Garcia and King, 1994; Hochman et al, 1997; Morisset and Nagy, 1998 and 1999).

Since the DDH comprises a heterogeneous population of neurons, it is not surprising that measured intrinsic membrane properties were observed to be diverse. Membrane properties such as post-spike afterhyperpolarizations and afterdepolarization, plateau potentials, bursting and intrinsic membrane voltage oscillations have been observed in various subpopulations of DDH neurons (King et al, 1988; Lopez-Garcia and King, 1994; Jiang et al, 1995; Morisset and Nagy, 1996, 1998 and 1999).

The majority of these membrane properties result from the interplay of voltage-dependent conductances, which are under neuromodulatory control (Hille, 1992). Alterations in voltage-dependent membrane properties can even contribute to sensory-evoked synaptic plasticity (Russo and Hounsgaard, 1994). Lopez-Garcia and King (1994) observed that dorsal horn neurons possess differences in their cellular membrane properties that relate to their source of synaptic input. For example, WDR neurons generally fired tonically in response to depolarizing current steps, while nociceptive specific neurons tended to fire a single spike. This observation suggests that the membrane properties of dorsal horn neurons are functionally differentiated (also see Ritz and Greenspan, 1985). Furthermore, Hochman et al (1997) demonstrated that the firing properties of DDH neurons in response to depolarizing current steps are differentiated at an early postnatal age. Differences in intrinsic cellular properties may also affect the neuronal responses to synaptic input. For instance Hounsgaard's group demonstrated an interrelation between the generation of plateau potentials and windup in the turtle dorsal horn.

3.2. Role in spinal integration including reflex pathways and nociception

The deep dorsal horn serves many important functions in the integration of sensory information. DDH interneurons are interposed in many spinal reflex pathways, where they can function at the premotor neuronal level to alter the gain of motor output. For example, interneurons receiving a dominant group Ib afferent input, project to α motoneurons and may function mainly to protect muscles from excessive stretch (reviewed in Jankowska, 1992). Several other dorsal horn interneurons, such as those receiving dominating input from group II afferents, also participate in spinal reflex pathways (reviewed in Jankowska, 1992).

In relation to pain transmission, the deep dorsal horn region of the spinal cord is of particular importance. This is because: (i) Functional activity mapping studies suggest that following noxious input, such as noxious heat, formalin injection or chronic constriction ligation models, the greatest increase and spatial spread of neural activity occurs in deeper regions of the spinal cord dorsal horn (Coghill et al, 1991; Porro et al, 1991; Mao et al, 1993). This increase in neural activity may occur secondarily to activity in the superficial dorsal horn (Mao et al, 1992; Yashpal et al, 1995). (ii) The majority of neurons within the DDH are functionally categorized as WDR neurons (e.g. Mendell, 1966; Chung et al, 1979; Cervero, 1986; Lopez-Garcia and King, 1994; Herrero and Headley, 1995; Schouenborg et al, 1995). These neurons, like nociceptive specific neurons, encode the intensity of noxious stimuli and may also account for the sensory discriminative aspects of pain sensation (see Treede et al, 1992; Coghill et al, 1993; Millan, 1999). (iii) The DDH contains the greatest number of ascending tract cells which

convey pain information of the brain (Willis and Coggeshall, 1991). Interestingly, most ascending tract neurons are characterized as WDR (reviewed in Le Bars et al, 1992; also see Willis and Coggeshall, 1991; Millan, 1999).

4.0 Pain

The various types of plasticity occurring in the spinal cord may contribute to the generation of several behaviourally-defined pain states (refer to Woolf et al, 1992). Thus it is important to discuss the mechanisms underlying the transmission and expression of pain. The Committee for Taxonomy of the International Association for the Study of Pain in Seattle, Washington describes pain as "an unpleasant sensory and emotional experience arising from the exposure of skin or deep tissue to damaging or potentially-damaging 'noxious' stimuli" (Anand and Craig, 1996). Pain may be described as nociceptive - resulting from the activation of nociceptors or neuropathic - resulting from injury to sensory fibres or the CNS itself. Furthermore, pain is sub-divided into (i) acute pain which is biologically useful in that it signals injury or diseases and subsides as healing progresses and (ii) chronic pain which is an on-going unpleasant sensation and serves no useful biologic function (see Markenson, 1996).

The transfer of noxious impulse along its sensory channel involves processing and integration at several CNS sites (nociception) and ultimately perception in the corticolimbic sections of the brain (pain) (refer to Willis and Coggeshall, 1991; Siddall and Cousins, 1997; Millan, 1999). Many structures and substances play key roles in the modulation of noxious impulses as they are conveyed from the periphery to the brain.

Some key players involve ion channels, second messengers, neurotrophic factors, purines, neuropeptides and descending neurotransmitters (for review see Fields and Basbaum, 1978; Hammond, 1986; Fields et al, 1991; Millan, 1999).

4.1. Nociceptors

A noxious stimulus is transmitted to the spinal cord from specialized free nerve ending fibres located in skin and deep tissues. These fibres are called nociceptors and have small diameter axons that are either myelinated (Aδ) or unmyelinated (C). Generally, Aδ fibres give rise to sharp, prickling (fast or first) pain; while C fibres trigger poorly localized burning (slow or second) pain. Nociceptors are slowly conducting (0.5-36 m/s) primary afferents and require high intensity stimulation for activation (reviewed in Markenson 1996; Millan, 1999). They are also sub-classified by their specific sensory modality such as chemonociceptors responding to noxious chemical, while thermo- or mechano-nociceptors respond to noxious heat or noxious mechanical stimuli respectively. Some nociceptors share sensory modalities, which include the polymodal nociceptors, responding to all kinds of noxious stimuli (for review also see: Markenson, 1996; Wilson and Kitchener, 1996; Millan 1999; Treede, 1999).

4.2. Types of pain states

Following nociceptor activation due to tissue or nerve injury, several chronic pain states may develop. These include: (i) allodynia, a reduction in pain threshold (ii) persistent pain (iii) hyperalgesia, an increased responsiveness to stimuli and, (iv) secondary hyperalgesia (mechanical allodynia), the spread of pain to uninjured areas. Experiments

have demonstrated that these sensations are in part controlled by mechanisms contained within the spinal cord (for review see: Willis and Coggeshall, 1991; Coderre et al, 1993; Woolf and Doubell, 1994).

Spinal dorsal horn neurons are sensitized following stimuli that activate nociceptive fibres (Woolf, 1983). This *central sensitization* has been experimentally characterized by an increased excitability in response to afferent (sensory) inputs, a prolonged afterdischarge to repeated stimulation termed 'windup' (Mendell, 1966), and expanded peripheral receptive fields (Cook et al, 1987; Woolf and King, 1990; Coderre et al, 1993). Windup may also lead to characteristics of central sensitization, such as the expansion of receptive fields and enhanced responses to C-fibre stimulation (Li et al, 1999), and is often associated with an increase in response to Aβ-fibre activity, thereby contributing to mechanical allodynia (Woolf and Doubell, 1994).

4.3. Transmission of noxious stimuli

Following activation of peripheral nociceptors, impulses travel along their peripheral axons to their central terminals. Centrally, nociceptors release neurotransmitters such as the excitatory amino acid, glutamate; the purines, adenosine and ATP; and the tachykinin, substance P (for review see Broman, 1994; Millan, 1999). Most nociceptors terminate in the superficial dorsal horn, although some Aδ fibres and visceral nociceptors may terminate in deeper regions (laminae V, VI and X). In the dorsal horn, neurons containing glutamatergic, purinergic and neurokinin receptors are excited. Following integrative and modulatory processing in the dorsal horn, the nociceptive signals are

transmitted supraspinally to the thalamus, reticular formation and midbrain via the spinothalamic, spinoreticular and spinomesencephalic tracts respectively (see Willis and Coggeshall, 1991; Besson, 1999; Millan, 1999). The impulses are then conveyed to the somatosensory cortex, anterior cingulate cortex and limbic sections of the brain (for review see Markenson, 1996; Ingvar, 1999; Millan, 1999) where perception occurs.

4.4. Role of the NMDA receptor

Although this thesis is not directed at exploring the operation of the NMDA receptor, it is necessary to discuss this receptor for the following reasons. First, NMDA receptor function is critically important in many long-term forms of synaptic plasticity that occur during development (see Feldman et al, 1999) and learning and memory formation (Morris et al, 1990; Morris and Davis, 1994). Secondly, this receptor has been similarly implicated in the expression of central sensitization of dorsal horn neurons (Woolf and Thompson, 1991; Coderre and Melzack, 1992), thereby playing a role in the development of various pain states such as allodynia and hyperalgesia (see Millan, 1999). NMDA receptors present on the central terminals of primary afferent fibres may enhance the release of substance P and excitatory amino acids, thereby allowing further sensitization of dorsal horn neurons (reviewed in Millan, 1999).

Nociceptor fibres are thought to release L-glutamate as their principle transmitter, thus primary afferent input to dorsal horn neurons is predominantly glutamatergic (see Miller and Woolf, 1996; Millan, 1999). Glutamate acts on two general post-synaptic ionotropic receptor subtypes termed N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-

methylisoxasole-4-propionic acid (AMPA)/kainate (after their preferred agonists). Experimentally, repetitive nociceptor fibre activation produces a 'windup' of dorsal horn neuronal activity (e.g. Mendell, 1966; Fitzgerald and Wall, 1980; Woolf and King, 1987), which can be blocked by NMDA receptor antagonists (Davies and Lodge, 1987; Dickenson and Sullivan, 1987). Glutamate-induced NMDA receptor activity also appears to be essential for the synaptic plasticity following activation of nociceptive afferents, since NMDA receptor antagonists are capable of blocking both LTP and windup in the spinal cord (Thompson et al, 1990; Randic et al, 1993; Liu and Sandkühler, 1995).

Though both the NMDA and AMPA/kainate glutamatergic receptors are mixed cation channels, allowing the influx of both Ca^{2+} and Na^{+} ions, the importance of the NMDA receptor function is dependent on two of its many unique properties. First, its unusually high permeability to Ca^{2+} compared to the AMPA/kainate receptor and secondly, its non-linear current flow in relation to membrane voltage (due to the voltage-dependent blocking properties of extracellular Mg^{2+}) (reviewed in Zorumski and Thio, 1992).

It is assumed that the trigger for NMDA receptor-mediated synaptic plasticity following activation of nociceptor is identical to that observed at many other CNS synapses. During repetitive high-frequency tetanic stimulation, massive Na^{+} entry through the AMPA/kainate receptor causes sufficient membrane depolarization to repel the extracellular Mg^{2+} from the NMDA receptor ionophore, allowing Ca^{2+} to enter. The increase in cytoplasmic Ca^{2+} is thought to activate metabolic cascades that promote a maintained increase or decrease in synaptic response (reviewed in Coderre et al, 1993).

Additionally, the NMDA receptor is susceptible to phosphorylation by PKC (Xiong et al, 1998; Lu et al, 1999). Therefore, following phosphorylation, the NMDA receptor can now counteract the Mg^{2+}-block and thereby operates at more hyperpolarized potentials.

Recently, many studies have provided information regarding a possible interaction between NMDA receptors and opioid agonists (reviewed in Mao, 1999). Thus, an understanding of the NMDA receptor function during the development and expression of spinal pain mechanisms, in addition to its known interactions with opioid agonists may be central to the discovery of novel therapeutic interventions aimed at reducing hyperalgesia following injury. The presence of numerous ligands for and binding sites on the NMDA receptor also provides for considerable manipulation therapeutically (see Collingridge and Watkins, 1994; Mao, 1999).

5.0 Neuromodulatory control of spinal sensory processing

There are several endogenous systems that exert powerful modulatory control over spinal sensory processing. They may alter both the strength of primary afferent-evoked synaptic responses and integrative cellular properties of spinal neurons (refer to Fields and Basbaum, 1978; Millan, 1999). In addition, many of these systems function as a part of the endogenous analgesic system, serving to reduce the expression of pain (Basbaum and Fields, 1984). Generally, these endogenous pain control systems that include the opioidergic, cannabinoid and monoaminergic systems are part of the supraspinal-spinal circuitry that exerts analgesia by affecting spinal or thalamic nociceptive processing (see Basbaum and Fields, 1984).

One very important family of antinociceptive systems which remain poorly understood, are the bulbospinal monoaminergic systems, which release the biogenic amine (monoamine) transmitters: 5-hydroxytryptamine (5-HT; serotonin), noradrenaline (NA) and dopamine (DA). Since there appears to be no intrinsic monoaminergic neurons in the spinal cord (for review see, Willis and Coggeshall, 1991; Broman, 1994), the modulatory actions of these transmitters within the spinal cord are attributed to several brainstem nuclei that project to and terminate extensively throughout the spinal cord (e.g. Dahlström and Fuxe, 1965; Björklund and Skagerberg, 1979; Steinbusch, 1981; Bregman, 1987; Clark and Proudfit, 1991 and 1993; Holstege et al, 1996; for review see Fields and Basbaum, 1978; Basbaum and Fields, 1984; Fields et al, 1991; Hammond, 1986; Millan, 1995; Lakke, 1997).

5.1. The bulbospinal monoaminergic systems

5.1.a. Anatomy

The serotonergic system originates mainly from the B1-B3 cell groups (nucleus raphe magnus, pallidus and obscurus) and nuclei of the ventral reticulospinal group (nucleus reticularis gigantocellularis pars alpha and paragigantocellularis), and projects to the spinal cord via the dorsolateral (DLF) and ventrolateral funiculi (VLF) (Dahlström and Fuxe, 1965; Millan, 1995; refer to Lakke, 1997). Steinbusch (1981) demonstrated that serotonergic fibres extensively innervate the spinal cord of the rat, with the highest density of innervation found in the ventral horn. However, high to medium density was found at all levels of the dorsal horn. Similarly, Marlier et al (1991a) reported high to

intermediate concentrations of serotonergic fibres innervating the spinal cord dorsal horn of the rat.

Secondly, the noradrenergic system originates from the A5-A7 (which incorporate the locus coeruleus and subcoeruleus) cell groups, projects via the DLF and VLF to innervate the dorsal horn, intermediate cell column and ventral horn of the spinal cord (Dahlström and Fuxe, 1965; Clark and Proudfit, 1991 and 1993; refer to Lakke, 1997). According to Clark and Proudfit (1991, 1993) the A5 and A7 cell groups project primarily to the ipsilateral spinal cord dorsal horn in the rat, while the locus coeruleus (LC) mainly projects to the ventral horn. However, in contrast, Fritschy and Grzanna (1990) reported opposite projections of these nuclei, with the LC projecting most heavily to the dorsal horn and intermediate zone of the rat, while the A5 and A7 cell groups project to the ventral horn.

The third descending monoaminergic system, the dopaminergic system, originates from the A11 cell group found in the diencephalon (which comprises the nucleus parafascicularis prerubralis and subparafascicularis thalami) and also projects to the spinal cord via the DLF (Björklund and Skagerberg, 1979; Hökfelt et al, 1979; Lakke, 1997). Dopaminergic immunoreactivity in the rat dorsal horn appears to be strongest in laminae III and IV (Ridet et al, 1992; Holstege et al, 1996). Similar labeling pattern is seen in the spinal dorsal horn of other species such as the cat and monkey (Holstege et al, 1996).

5.1.b. Development of spinal projections

In the rat, serotonergic, noradrenergic and dopaminergic neurons are generated between embryonic day (E) 11 - 15, E10 - 13 and E14 - 16, respectively (reviewed in Lakke, 1997). Fibres of all three projecting systems are found in the lumbar spinal cord of the rat at periods ranging from E15 to postnatal day (P) 2 (Bregman, 1987; Ziskind-Conhaim, 1993; refer to Lakke, 1997). However, fibres continue to grow beyond P2 and adult projection patterns are not established until P21 for serotonergic (Bregman, 1987) and P26 for noradrenergic and dopaminergic systems (reviewed in Lakke, 1997). In relation to functional synaptic connections, Fitzgerald and Koltzenburg (1986) reported that despite the early anatomical presence of a descending DLF, there is no functional descending inhibition until P10-12. However, several other studies provide evidence for the existence of descending inhibition much earlier than P10 (e.g. Miyata et al, 1987; Wallis et al, 1993a; Magnuson et al, 1995; Magnuson and Trinder, 1997; Brocard et al 1999). Though none of these studies directly investigated the DLF or the descending monoaminergic systems, Wallis et al (1993a) showed that the strong inhibition of the monosynaptic reflex in the newborn rat is mediated by serotonin.

5.1.c. Evidence for a role of bulbospinal amines in nociception

All three descending monoaminergic systems have been implicated in the control of spinal sensory processing, particularly in relation to nociception. 5-HT and NA have been more extensively studied than DA (Dahlström and Fuxe, 1965; Basbaum and Fields, 1984; Fitzgerald, 1986; Jones, 1991; Millan, 1995; Zhang et al, 1995). Consistent with a

role in nociceptive processing, 5-HT and NA are released in the spinal cord following nociceptor activity (e.g. Tyce and Yaksh, 1981; Yaksh and Tyce, 1981; Satoh and Omote, 1996; Omote et al, 1998) supporting nociception-activated recruitment of these bulbospinal systems.

Stimulation of specific supraspinal sites such as the locus coeruleus/subcoeruleus (LC/SC), nucleus raphe magnus (NRM), periaqueductal gray (PAG), rostral ventral medulla (RVM) and the A11 cell group produces antinociception generally associated with the release of these endogenous transmitters in the spinal cord (e.g. Fields et al, 1977; Margalit and Segal, 1979; Fleetwood-Walker et al, 1988; Sorkin et al, 1993; Waters and Lumb, 1997; Cui et al, 1999).

The role of the descending monoaminergic projections in PAG-stimulation induced analgesia is of significance. The significance of the PAG in nociceptive processing may be related to the fact that it contains substantial quantities of all families of endogenous opioids and opioid receptors. In addition, it was one of the first CNS regions to be implicated in pain modulation (reviewed in Basbaum and Fields, 1984). Although, it appears that there are no direct spinal projections from the PAG (see Fields and Basbaum, 1978; Mason, 1999), there are extensive spinal (Swett et al, 1985; Mouton and Holstege, 1998) and brainstem projections to the PAG (refer to Basbaum and Fields, 1984). It is hypothesized that PAG stimulation and supraspinal administration of opiates, such as into the PAG produce analgesia by recruiting bulbospinal monoaminergic neurons (Yaksh, 1979; see Fields and Basbaum, 1978). This hypothesis is further

supported by the observations that 5-HT receptor antagonists (Yaksh et al, 1976) or the selective depletion of brainstem 5-HT with 5,6,-dihydroxytryptamine (Vasko et al, 1984) attenuates the antinociceptive effect of morphine. Also, both PAG-stimulation and brainstem microinjection of opiates-induced analgesia are blocked following lesions of the DLF (Basbaum et al, 1976; Basbaum et al, 1977).

5.1.d. Action of the monoamines on spinal neurons

Both *in vitro* and *in vivo* studies have demonstrated numerous modulatory actions of the monoamines on the cellular responsiveness and synaptic properties of spinal neurons, presumably via actions at various metabotropic receptors (e.g. Belcher et al, 1978; Headley et al, 1978; Jordan et al, 1979; Todd and Miller, 1983; Skoog and Noga, 1995; Hori et al, 1996; Lopez-Garcia and King, 1996; Jankowska et al, 1997; Lopez-Garcia, 1998; Gladwell and Coote, 1999; Baba et al, 2000a and b). Though these transmitters can produce both excitatory and inhibitory actions (see Weight and Salmoiraghi, 1966; Belcher et al, 1978; Headley et al, 1978; Jankowska et al, 1997, 2000), it appears from most studies, that 5-HT generally exerts inhibition in the dorsal horn (Jordan et al, 1979; Lopez-Garcia and King, 1996; Randic and Yu, 1976; Garraway and Hochman, 1998; Hochman and Garraway, 1998), but facilitation in the ventral horn (e.g. Yamazaki et al, 1992; MacDonald et al, 1994; Hochman and Schmidt 1998). This observation is consistent with the general hypothesis forwarded by Jacobs and Fornal (1993), that 5-HT inhibits sensory systems while it facilitates motor systems. In support of this hypothesis, Bell and Matsumiya (1981) previously demonstrated that intraspinal microinjections of 5-HT and NA into the dorsal horn depressed C-fibre reflexes, while they both caused

facilitation when injected into the ventral horn. Additionally, inhibitory actions of both NA and DA have been reported in the spinal cord (Headley et al, 1978; Matsumiya et al, 1979; Skoog and Noga, 1995; Jankowska et al, 1997, 2000; Gladwell and Coote, 1999; Wikström et al, 1999).

Jankowska and colleagues demonstrated the comparative actions of the monoamines within the spinal cord, particularly on dorsal horn interneurons. With respect to non-nociceptive afferent input, they showed that the monoamine transmitters NA and 5-HT influenced synaptic responses of dorsal horn neurons in a highly differentiated manner, which was dependent on the source of afferent input and the type of dorsal horn neuron. Specifically, they demonstrated that group Ia and/or Ib muscle afferent input to four groups of spinal interneurons were facilitated by both NA and 5-HT, while group II input to these neurons was generally depressed (Jankowska et al, 2000). In a similar study, they compared the actions of 5-HT and NA on four groups of ascending tract neurons following cutaneous and group II muscle afferent input. Again, they reported highly differentiated actions exerted by the transmitters, with NA generally causing depression, while 5-HT generally produced facilitation (Jankowska et al, 1997). These results indicate that the monoaminergic pathways differentially influence the transfer of different forms of sensory information from the periphery to supraspinal centres. It also suggests that the actions of the monoamines may be adjusted to regulate information transfer during ongoing behaviours.

The excitatory and inhibitory spinal actions of the monoamines may arise partly as a result of the broad range of metabotropic receptors (see below) they bind to in order to mediate spinal actions. Furthermore, the differential distribution and expression of these receptors throughout the spinal cord might also account for some of the differential actions of these transmitters previously reported.

5.1.e. Monoamine receptor subtypes

Despite the vast number of studies suggesting and confirming the antinociceptive actions of the biogenic amine transmitters, their potential analgesic benefits are limited. One major reason for their limited uses arises from the complexity associated with the pharmacology of these systems.

Presently, there are seven families of 5-HT receptors (5-HT_{1-7}), comprising a total of at least 14 structurally and pharmacologically distinct receptor subtypes (for review see Hoyer et al, 1994; Barnes and Sharp, 1999). There are 3 main families of noradrenergic receptors (α_1, α_2 and β) each giving rise to several subtypes (for review see Bylund et al, 1994) and presently, 5 types of dopaminergic receptors (D_1-D_5) (Vallone et al, 2000). These metabotropic receptors are coupled both positively and negatively to adenylate cyclase (AC) and positively to phospholipase C (PLC) via GTP-binding proteins (Gs, Gi and Gq respectively). The coupling of these receptors to signaling pathways permits a broad overall modulatory capacity as demonstrated by numerous investigators (for review on 5-HT see Anwyl, 1990; Barnes and Sharp, 1999). In addition, the 5-HT_3 receptor is ionotropic and leads to the opening of a cation permeable ion channel. Until recently,

there were not very many specific ligands available for the various receptors. Because most of the available ligands share relatively high affinities for more than one class of receptor (see Kennett, 1998), it has been difficult to assign specific functional roles to individual receptor subtypes (see Van-Wijngaarden et al, 1990).

5.2. Acetylcholine

In addition to 5-HT, NA and DA, another monoamine transmitter, acetylcholine (ACh), also mediates modulatory actions in the spinal cord (see references below). However, unlike the bulbospinal biogenic amines, which are not intrinsic to the spinal cord, populations of cholinergic interneurons have been found in the spinal cord, including the dorsal horn and around the central canal (Barber et al, 1984; Todd, 1991; Huang et al, 2000). The presence of descending cholinergic influences has not been confirmed, and to date it appears that there are no descending cholinergic systems in the rat (refer to Willis and Coggeshall, 1991; Broman, 1994). However, cholinergic modulation of both primary afferent input and sensory processing in the dorsal horn has been observed in different species (e.g. Myslinski and Randic, 1977; Urban, et al, 1989; Zhuo and Gebhart, 1991a; Bleazard and Morris, 1993; Travagli, 1996; Baba et al, 1998), suggesting an effect of ACh in spinal nociceptive processing.

Both ionotropic (nicotinic) and metabotropic (muscarinic) ACh receptors are present in the spinal cord dorsal horn, with the highest density in laminae II, III and IX (Gillberg et al, 1988). In addition, both receptors mediate cholinergic-induced modification of cellular and synaptic properties of spinal neurons (e.g. Urban et al, 1989). In relation to

nociception, both antinociceptive and pronociceptive actions of cholinergic agonists have been reported (Khan et al, 1998). Additionally, spinal cholinergic systems may interact with the descending monoaminergic systems in mediating spinal antinociception (e.g. Zhuo and Gebhart, 1992a).

6.0 Synaptic plasticity: LTP and LTD

Repetitive synaptic activity can induce long-term changes in the properties of activated synapses. These changes are manifest as either a maintained increase in efficacy, long-term potentiation (LTP), or a maintained reduction in synaptic efficacy, long-term depression (LTD). LTP was first demonstrated in the dentate gyrus of the hippocampus (Bliss and Lomo, 1973), where it was proposed to be strongly associated with memory formation (see Morris et al, 1990). Most of what is currently known about LTP and LTD results from studies conducted mainly in the mammalian hippocampal tissue. However, in recent years, both LTP and LTD have been observed at many synapses in the brain (e.g. Froc et al, 2000; Castro-Alamancos and Calcagnotto, 1999; Bear and Kirkwood, 1993) and also in the cerebellum, where synaptic plasticity may similarly underlie some of the mechanisms of cerebellar learning (see Ito, 1986).

6.1. General properties

LTP is characterized by three properties: (i) input-specificity - only the input active at the time of the tetanus contributes to the potentiation (ii) cooperativity - requires an intensity threshold for the induction of LTP, since 'weak' tetani do not trigger LTP and (iii) associativity - a weak input can be potentiated if it is active at the same time as a strong

tetanus to a separate but convergent input (Malenka, 1991; Bliss and Collingridge, 1993). Furthermore, results from many studies have suggested that both pre- and post-synaptic elements may be required for the expression of synaptic plasticity (e.g. Laezza et al, 1999; for review see; Bliss and Collingridge, 1993; McNaughton, 1993; Lisman et al, 1997; Malenka and Nicoll, 1999; Nayak and Browning, 1999). Thus, repetitive stimulation of presynaptic fibres may cause an increase in intracellular Ca^{2+} in the presynaptic terminal, which in turn results in an increase in transmitter release (Lynch and Voss, 1991). Meanwhile, the postsynaptic cell may require adequate depolarization to relieve the highly Ca^{2+}-permeable, voltage-dependent NMDA receptor channel of its Mg^{2+} block (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). This glutamate receptor channel has been implicated in the induction and expression of synaptic plasticity (Morris et al, 1990; Malenka and Nicoll, 1993; Malenka and Nicoll, 1999; also see Liu and Sandkühler, 1995).

A feature of the NMDA receptor channel that highly supports its role in the expression of synaptic plasticity is its susceptibility to phosphorylation by protein kinase C (Chen and Huang, 1992; Tokuda and Hatase, 1998; Xiong et al, 1998). However, NMDA-independent forms of synaptic plasticity have also been characterized in central synapses such as the thalamo-amygdala synapse (Weisskopf et al, 1999); CA3-CA1 hippocampal synapse (Stricker et al, 1999) and it appears that voltage-gated Ca^{2+} channels may provide sufficient influxes in intracellular Ca^{2+} at these synapses. Non-NMDA and metabotropic glutamatergic receptors may also be involved in the induction and

expression of synaptic plasticity (for review see Tokuda and Hatase, 1998; Soderling and Derkach, 2000).

Experimentally, repetitive stimulation of presynaptic fibres has been used as a conditioning stimulation-induction paradigm for LTP and LTD. High frequency stimulation, typically 100 Hz, is better suited for the induction of LTP (see Bliss and Collingridge, 1993), while low frequency stimulation, typically 1 Hz, produces LTD (Mulkey and Malenka, 1992). Interestingly, an intermediate tetanus, 10 Hz, has been shown to result in no long-term modification of synaptic efficacy (Dudek and Bear, 1992).

Other modulators implicated in synaptic plasticity include retrograde signaling molecules such as nitric oxide and carbon monoxide (see Bohme et al, 1991; Hawkins et al, 1994; Haley, 1999; Zhuo et al, 1999). Nitric oxide, in particular, may play a role in the potentiation of synaptic responses via the stimulation of guanylyl cyclase and cyclic GMP-dependent protein kinase, which may increase the phosphorylation of the transcription factor, CREB (Lu et al, 1999).

6.2. The Calcium hypothesis

The mechanisms underlying the induction and expression of synaptic plasticity are still debated. The main issue of such debate lies in whether LTP/LTD occurs at a presynaptic or postsynaptic locus (see Malenka and Nicoll, 1999). One leading and highly regarded hypothesis on the expression of synaptic plasticity is the *Calcium Hypothesis*, which

states that the magnitude of the rise in intracellular calcium (Ca^{2+}) during synaptic activity determines whether synapses undergo LTP or LTD (Lisman, 1989; Artola and Singer, 1993; Malenka and Nicoll, 1993; Cummings et al, 1996). A large increase in intracellular calcium produces LTP, while moderate increases will produce LTD.

Various protein kinases and phosphatases have also been implicated in the induction and maintenance of synaptic plasticity (Lisman, 1989; Malenka and Nicoll, 1993; Bliss and Collingridge, 1993; Lisman et al, 1997; Liu et al, 1999; Soderling and Derkach, 2000). For instance, peptide inhibitors of Ca^{2+}-calmodulin kinase II or protein kinase C block the induction of LTP (Malinow et al, 1989), while protein phosphatase inhibitors or calmodulin inhibitors block the induction of LTD (Mulkey et al, 1993). However, since these Ca^{2+}-dependent enzymes have different affinities for Ca^{2+}-calmodulin and hence Ca^{2+}, it is further hypothesized that their activation determines whether LTP or LTD is produced (Lisman, 1989; Artola and Singer, 1993). More specifically, phosphatases have higher affinity for Ca^{2+}, hence they are activated at lower concentration, while kinases with their lower Ca^{2+} affinity, become active at higher intracellular concentration (Kasai, 1993). In addition, activated kinases interact with phosphatases by inhibiting their activity (see Soderling and Derkach, 2000).

It is further hypothesized that during low frequency tetanic stimulation, low to moderate increases in intracellular Ca^{2+} occur, thus favoring the activation of phosphatases and hence producing LTD. In contrast, high frequency tetanic stimulation results in massive Ca^{2+} influx, activates kinases, leading to the induction of LTP. Coussens and Teyler

(1996) demonstrated that LTD could be induced at 10 Hz tetanic stimulation only in the presence of a kinase inhibitor or by reducing the concentration of extracellular Ca^{2+}. Conversely, LTP can be induced if extracellular Ca^{2+} is increased or if phosphatase activity is inhibited.

6.3. LTP, LTD and plasticity of spinal sensory processing

Recently, LTP and LTD of primary afferent input were observed in the neonatal rat spinal cord following repetitive primary afferent stimulation (e.g. Pockett and Figurov, 1993; Randic et al, 1993; Liu and Sandkühler, 1995; Lozier and Kendig, 1995; Pockett, 1995a; Garraway et al, 1997; Svendsen et al, 1997; Liu et al, 1998). An important goal is to understand the synaptic mechanisms producing LTP and LTD since these phenomena may represent a physiological substrate for 'memory traces' and hence become strongly implicated as a storage mechanism for long-term pain sensation (or pain reduction) (for review see, Pockett, 1995b; Randic, 1996). Randic et al (1993) observed that both LTP and LTD could occur in the same neuron (in superficial dorsal horn). This conversion was accomplished by adjusting the membrane potential of neurons with intracellular current injection prior to the conditioning tetanus. The more depolarized membrane potential, (and presumed greater Ca^{2+} load), produced LTP. Furthermore, Pockett (1995a) showed that pharmacological blockade of inhibitory synapses in the neonatal spinal cord transverse slice preparation decreased the incidence of LTD, while correspondingly increasing the incidence of LTP.

6.4. Synaptic plasticity and modulatory descending controls

The powerful modulatory influences the bulbospinal systems exert over spinal functions are perhaps exemplified by the disruption and alterations in spinal neuronal properties following spinal cord transections. For instance, there is an exaggeration of spinal reflex systems characterized as hyperreflexia and spasticity following spinal cord injury. Schouenborg et al (1992) demonstrated an increase in reflex excitability following spinalization in the rat. In addition, Besson et al (1975) reported an increase in spontaneous activity of lamina V neurons, while Hochman and McCrea (1994) reported increases in amplitudes of EPSP in motoneurons of the cat following spinalization, again suggesting that descending neuromodulators may tonically inhibit spinal neurons.

Interestingly, in relation to the expression of synaptic plasticity in the spinal cord, Sandkühler and Liu (1998) demonstrated that natural activation of nociceptors in skin induced LTP of C-fibre evoked field potentials in dorsal horn *but only following spinalization*, suggesting a potent inhibitory control from descending systems (also, see Svendsen et al, 1999). Liu et al (1998) also demonstrated that Aδ-fibre mediated LTD of C-fibre evoked spinal field potentials could be switched to LTP following spinalization. Thus, descending systems appear to be able to control both the induction and direction of the evoked synaptic plasticity, favoring LTD. Hence an identification of the mechanisms that control synaptic plasticity are of considerable interest. For example, Aδ fibre-induced LTD of nociceptor afferents in spinal cord (Liu et al 1998) is blocked with μ-opioid receptor antagonists (Zhong and Randic, 1996) potentially linking LTD to opioid-induced analgesia.

Hypothesis, Goals and Objectives

Most of the earlier mentioned studies have provided information that identifies the monoamine transmitters as key modulators of varying aspects of sensory processing within the spinal cord. Although, the spinal cord dorsal has been demonstrated to be a major site for integration of sensory information, neuromodulation and plasticity (see earlier references), the DDH has been less studied than the superficial dorsal horn. Therefore, this study is designed to explore modifications in the cellular integrative and synaptic properties of DDH neurons in the neonatal rat spinal cord produced by two processes: ***synaptic activity-independent*** (neuromodulation by bath application of the monoaminergic transmitters and selective serotonergic receptor ligands) and ***synaptic activity-dependent*** (alterations resulting from repetitive electrical stimulation of primary afferents).

In all experiments, we employ the whole cell 'blind' patch-clamp technique, which offers some important advantages over conventional approaches (see Hochman et al, 1997). For instance, previous intracellular (sharp) electrophysiological studies in DDH were limited by impalement-induced injury and the preferential impalement of larger cell somas. In contrast, patch recording permits stable recording from both large and small cells with less injury. Furthermore, the low electrode impedances allow greater current injection without electrode rectification. In addition, we utilize the neonatal rat transverse spinal cord slice preparation, which provides a reasonable outline of the spinal cord when viewed microscopically and hence permits reliable targeting of DDH neurons (King et al,

1988). Another advantage of this *in vitro* preparation is the control over drug application and washout. Figure 1; project (I) provides an illustration of the experimental setup.

Hypothesis

The general hypothesis of this thesis is: "Bulbospinal monoamine transmitters interact with a multiplicity of receptor subtypes within the spinal cord to regulate the strength of sensory input, with the predominant action being depression."

Goals

Our specific aims are to:

I. Compare the effects of the bulbospinal monoamine transmitters (5-HT, NA, DA) and ACh on the modifiability of synaptic and cellular properties of DDH neurons.

II. Undertake a pharmacological characterization of the 5-HT receptors responsible for the observed modifications in primary afferent input to DDH neurons and examine developmental changes in the selective ligands effects during the first two weeks of postnatal life.

III. Demonstrate the effects of serotonin on both the induction and maintenance of primary afferent-induced LTP and LTD in neonatal DDH neurons, and identify which 5-HT receptors mediate these actions.

The following experiments were conducted to address our goals and test our hypothesis:

I. *Modulation of sensory integrative properties in spinal cord deep dorsal horn neurons by serotonin, noradrenaline, dopamine and acetylcholine (P10-14).* We compared the actions of at least two and up to all four monoamine transmitters on the intrinsic membrane properties, firing properties during current injection and synaptic responses evoked in DDH neurons.

II. *Pharmacological characterization of serotonin receptor subtypes modulating primary afferent input to deep dorsal horn neurons in the neonatal rat (P3-6 and P10-14).* In these experiments, we bath applied selective 5-HT receptor ligands to identify the roles of individual 5-HT receptors contributing to serotonin-induced modifications of synaptic properties of DDH neurons. In addition, we compared their actions in two age groups of animals to determine whether changes in receptor function occur during the first two weeks of postnatal development.

III. *Serotonin increases the incidence of primary afferent-evoked long-term depression in rat deep dorsal horn neurons (P3-6).* In these experiments, we investigated the effects of 5-HT on (i) the naïve synaptic responses, (ii) synaptic responses which had already undergone LTP or LTD, and (iii) the induction of synaptic plasticity. In addition, we studied the effects of specific receptor ligands on the naïve synaptic responses and on the induction of synaptic plasticity.

Project I - Modulation of sensory integrative properties in spinal cord deep dorsal horn neurons by serotonin, noradrenaline, dopamine and acetylcholine

Abstract

The deep dorsal horn represents a major site for the integration of spinal sensory information. The bulbospinal monoamine transmitters, released from serotonergic, noradrenergic and dopaminergic systems, exert modulatory control over spinal sensory systems as does acetylcholine, an intrinsic spinal cord monoamine transmitter. Whole-cell recordings of deep dorsal horn neurons in the rat spinal cord slice preparation were used to compare the actions of serotonin, noradrenaline, dopamine and acetylcholine on dorsal root stimulation-evoked afferent input and membrane cellular properties. In the majority of neurons, evoked excitatory postsynaptic potentials were depressed by the bulbospinal transmitters serotonin, noradrenaline and dopamine. In contrast, in the same neurons, acetylcholine generally facilitated the evoked responses, particularly the late, presumably NMDA receptor mediated component. None of the transmitters modified neuronal passive membrane properties suggesting that the modulation of evoked synaptic responses occurred at the activated synapses. In contrast, in response to depolarizing current steps, the monoamines converted the firing pattern in a population of neurons that originally fired phasically to repetitive. Together, these results demonstrate that even though the deep dorsal horn contains many functionally distinct subpopulations of neurons, the bulbospinal monoamine transmitters can act at both synaptic and cellular sites to alter neuronal sensory integrative properties in a rather predictable manner, and clearly distinct from the actions exerted by cholinergic neurons originating in the spinal cord.

Introduction

Neurons within the spinal cord represent a primary site for the integration of somatosensory input. Spinal sensory integration is a dynamic process, regulated by factors that include multisensory convergence and pathway selection (Lundberg, 1979; Baldissera et al, 1981; Jankowska, 1992) activity-dependent plasticity (see Millan, 1999), and neuromodulation (see Randic, 1996). Neuromodulatory responses within the spinal cord include actions mediated by monoaminergic systems that originate in the brainstem. These bulbospinal monoaminergic nuclei can be divided into 3 subtypes by their transmitter phenotype, serotonin (5-HT), noradrenaline (NA), or dopamine (DA). Neurons within these nuclei are characterized by their widespread projections throughout the spinal cord (Clark and Proudfit, 1991, 1993; Marlier et al, 1991a; Holstege et al, 1996).

The monoaminergic modulation of two prominent spinal cord functional systems has been examined in some detail. These are the control of motor output and nociception. Generally, the monoamines have been reported to facilitate motor activity and inhibit sensory systems (Basbaum and Fields, 1984; Jacobs and Fornal, 1993; Bell and Matsumiya, 1981; Willis and Coggeshall, 1991; Wallis, 1994), consistent with a general hypothesis on 5-HT function in the CNS forwarded by Jacobs and Fornal, (1993). As serotonergic, noradrenergic and dopaminergic systems have a similarly diffuse distribution in the spinal cord (Rajaofetra et al, 1989; Fritschy and Grzanna, 1990; Clark and Proudfit, 1991, 1993; Marlier et al, 1991a; Rajaofetra et al, 1992; Holstege et al, 1996) and their monoamine transmitters frequently exert similar actions (Weight and Salmoiraghi, 1966; Belcher et al, 1978; Headley et al, 1978; Bell and Matsumiya, 1981),

it is possible that these transmitter systems act at similar spinal sites and by similar mechanisms. For example, descending monoaminergic transmitters powerfully inhibit nociceptive information in neurons by activation of serotonergic 5-$HT_{1A \text{ and } B}$, α_2-adrenergic, and D_2-dopaminergic receptors (Pertovaara, 1993; Kiritsy-Roy, 1994; Zemlan, 1994) all of which are negatively coupled to adenylate cyclase (reviewed in Bylund et al, 1994; Hoyer et al, 1994; Vallone et al, 2000). However, the existence of many bulbospinal monoaminergic systems with heterogeneous transmitter phenotypes (including co-transmitters) that act on a variety of spinal metabotropic receptor subtypes (e.g. Huang and Peroutka, 1987; Marlier et al, 1991b; van Dijken et al, 1996; Stone et al, 1998), suggest that neuromodulation in the spinal cord is a highly differentiated process. Indeed, more recent findings indicate that different noradrenergic or serotonergic nuclei can exert opposing modulatory actions on spinal cord nociceptive function (Calejesan et al, 1998; Zhuo and Gebhart, 1992b; Martin et al, 1999). Further, the actions of 5-HT and NA on the afferent-evoked recruitment of functionally-identified spinal neurons can differ considerably (Bras et al, 1989; Jankowska et al, 1997, 2000). For example, the recruitment of ascending tract neurons following primary afferent stimulation is commonly facilitated by 5-HT yet depressed by NA (Jankowska et al, 1997).

The monoamine acetylcholine (ACh) also modulates spinal sensory processing in the dorsal horn (e.g. Myslinski and Randic, 1977; Urban et al, 1989). As it appears that there are no descending cholinergic systems in the rat (refer to Willis and Coggeshall, 1991), these actions probably arise from a population of intrinsic cholinergic interneurons found in the dorsal horn (Barber et al, 1984; Todd, 1991).

Several studies have compared the actions of these monoamine transmitters on the modulation of sensory input onto spinal neurons (Weight and Salmoiraghi, 1966; Belcher et al, 1978; Headley et al, 1978; Todd and Millar, 1983; Willcockson et al, 1984; Bras et al, 1989; Skoog and Noga, 1995; Jankowska et al, 1997, 2000). However, in these studies, only modifications in extracellular spiking or field potentials were recorded and transmitters were applied by iontophoresis (but see Bras et al, 1989). While it is apparent from these studies that the transmitters have both common and distinct actions on the modulation of spinal sensory input, the effects of monoamines on intrinsic cellular properties and synaptic potentials in individual neurons were not studied. Clearly, additional insight into monoamine transmitter function may be achieved by a more direct examination of their actions with intracellular recordings (e.g. Lopez-Garcia and King, 1996; Khasabov et al, 1999; Lopez-Garcia, 1998; Khasabov et al, 1998).

Therefore, in this study, we compared the effects of bath applied 5-HT, NA, DA, and ACh on cellular properties and primary afferent-evoked synaptic responses in individual deep dorsal horn (DDH) neurons. Parts of these results have been presented in abstract form (Garraway and Hochman, 1999).

Material & Methods

Preparation of spinal cord slices

All experimental procedures complied with the Canadian Council of Animal Care guidelines. Neonatal rats (Sprague-Dawley postnatal days 10-14) were first anesthetized with 10% urethane (2 mg/kg body weight i.p.), decapitated and spinal segments L2 - S1 were removed. The isolated spinal cord was embedded in Agar, 2.5% w/v, (Type E, Sigma) and sliced on a vibrating blade microtome in 500-600 μm transverse sections (Leica VT1000S or Pelco 101) in cooled (< 4°C) oxygenated high sucrose-containing artificial cerebrospinal fluid (ACSF) containing (in mM); sucrose, 250; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 3; glucose, 25; NaH_2PO_4, 1.25; $NaHCO_3$, 26; at a pH of 7.4. Short dorsal rootlets remained attached to the spinal segments to allow for electrical stimulation of primary afferents. Refer to figure 1.

Electrophysiology

Slices were incubated at 32°C for at least 1 hour in normal ACSF containing (in mM); NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 25; NaH_2PO_4, 1.25; $NaHCO_3$, 26; at a pH of 7.4 and oxygenated with 95%O_2-5%CO_2. For experimentation, spinal cord slices were affixed to a recording chamber using platinum U-frames with a parallel array of nylon fibres glued across (Edwards et al, 1989). Patch electrodes were prepared from 1.5 mm outer diameter capillary tubes (Precision Instruments or Warner) pulled in a two-stage process (Narishige PP83) producing resistance values ranging from 4-7 MΩ with recording solution containing (in mM): K-gluconate, 140; EGTA, 0.2; HEPES, 10; Mg-ATP, 4; GTP, 1; pH 7.3. The recording chamber was continuously superfused with

oxygenated normal ACSF at a rate of ~2 ml/minute. The whole-cell 'blind' patch clamp recording technique (Blanton et al, 1989) was undertaken at room temperature (approximately 20°C) using the Axopatch 1D amplifier (Axon Instruments) filtered at 5 kHz (4-pole low-pass Bessel). Voltage and current clamp data were acquired on computer with the pCLAMP acquisition software (v 6.0; Axon Instruments).

Determination of cell membrane properties

Immediately following rupture of the cell membrane (in voltage clamp at –90 mV), the current clamp-recording configuration was used to determine resting membrane potential. Series resistance was subtracted in current clamp mode (bridge balance) and junction potentials were measured and subtracted offline. For the duration of the experiment, leak conductance and bridge balance were monitored; if their values were largely unaltered, the experiments were continued. Mean electrode series resistance was 33 ± 4 MΩ (S.D; n = 37). At an adjusted membrane potential of -70 mV, a series of hyperpolarizing and depolarizing current steps were undertaken to obtain estimates of membrane time constant, cell resistance, rheobase, voltage threshold, action potential height, and action potential duration at half maximal amplitude (half-width).

Primary afferent stimulation

Primary afferents were stimulated electrically with a constant current stimulator (Eide, 1972). In the present comparative study, we used high stimulation intensities in order to recruit the highest threshold unmyelinated afferents, and hence, the majority of afferent fibre types, irrespective of age (typically ≥ 500 μA, 500 μs see Thompson et al, 1990). In

the present sample, 29% of the neurons received synaptic responses at intensities lower than 500μA, 100μs; 49% received synaptic input at 500μA, 100μs, while the remaining 22% of neurons only received input at intensities ≥ 500μA, 500μs. Generally, the evoked synaptic responses were first characterized as excitatory by determining their reversal potential prior to collection of baseline events (refer to project III; Fig. 2D for an example). Neurons with short-latency inhibitory synaptic responses were not included in this study. Excitatory post-synaptic potentials (EPSPs) were evoked at low frequencies (once every 20-60 seconds) by stimulating dorsal rootlets for a baseline period of 10-15 minutes while maintaining the neuron at a holding potential of –90 mV. In all cases membrane potential was carefully monitored, and any alterations in membrane potential were noted, then countered with intracellular current injection in order to maintain a holding potential of –90 mV.

Application of agonists

5-hydroxytryptamine HCl (5-HT, serotonin), norepinephrine bitartrate (NA, noradrenaline), dopamine HCl (DA) and acetylcholine chloride (ACh) were obtained from RBI/Sigma. The solutions were prepared on the day of the experiment from 10 mM frozen stock solutions and bath applied at a final concentration of 10 μM. Ascorbic acid (100 μM), an antioxidant, was added to solutions containing 5-HT, NA and DA to prevent their oxidation. All agonists were dissolved in normal ACSF and bath applied from independent perfusion lines. Generally, each agonist was applied for a period of 10-15 minutes during which time; EPSPs were continually recorded at the baseline parameters described in the section above.

In order to compare the actions of more than one agonist on the primary afferent-evoked synaptic responses and cellular properties of the neurons, we allowed a washout/recovery period of 10-20 minutes before subsequent drug application. Due to the restrictions in recording duration with patch electrodes, we were often unable to observe the effects of all four agonists on a given neuron. However, in all cases, the actions of at least two drugs were compared. The following three combinations of drugs were compared in most cases: (i) 5-HT and ACh, (ii) 5-HT, NA and DA, and (iii) 5-HT, NA, DA and ACh. These transmitters were applied in random order and evoked EPSPs were always recorded at baseline parameters both during drug application and washout. In separate experiments, we compared the magnitude of modulatory actions evoked by independent application of 5-HT and NA to their co-application (5-HT/NA).

Analysis

Recordings were analyzed using pCLAMP (v 6.0, Axon Instruments). Both the maximum amplitude of the synaptic response and the changes in synaptic charge transfer calculated as the integral of the synaptic response (area under the curve) of individual traces were measured. Primary afferent-evoked synaptic responses in dorsal horn neurons are generally glutamatergic consisting of both early and late components; presumably (±)-α-Amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA) -receptor mediated respectively (e.g. Gerber and Randic, 1989). To determine whether, as a first approximation, the drugs differentially modulated these components of the evoked responses, we calculated area under the curve (AUC) at two

time intervals that approximately separate these events: early (< 200 ms) and late (≥ 200 ≤ 750 ms) (personal observations; project III). In a few neurons, the evoked synaptic responses were RC-filtered at 25 Hz to remove overlying contaminating spikes. Control measures indicated that filtering at this frequency did not affect EPSP amplitude or shape, but largely removed the action potential.

The applied transmitters were considered to have a modulatory action if they altered EPSP amplitude ≥10%. Because multiple drugs were added in most experiments, the change in synaptic amplitude was measured as a difference in the mean peak amplitude or AUC during drug application compared to the mean values just prior to drug application (control or washout/recovery). Similarly, the estimated membrane properties and firing properties of the neurons were compared before, during and following washout of the drugs tested. Following analysis, graphs were constructed using Sigma Plot (Jandel Scientific) and imported into CorelDRAW (Corel) for final editing. Unless stated, all values are reported as mean ± S.D. of peak changes (maximum amplitude) of the synaptic response. All statistical analysis was made using Sigma Stat (Jandel Scientific).

Results

A total of 41 deep dorsal horn neurons (laminae III-VI) were recorded. The approximated location of 36 of these neurons is presented in Figure 2A. The membrane properties of the neurons are summarized in Table 1. Neurons can also be grouped by their firing properties in response to current injection. In the present sample, 15 neurons fired spikes repetitively, while the remaining 26 neurons fired phasically, 10 of which fired no more than 2 spikes (1-2 spikes). Examples of neuronal firing patterns are illustrated in Figure 2B. The effects of the monoamines on neuronal membrane properties will be considered later.

Effects of the monoamine transmitters on primary afferent-evoked EPSPs

The effects of bath-applied 5-HT, NA, DA and ACh on the dorsal root stimulation-evoked EPSPs are summarized in Tables 2 and 3 and illustrated in Figure 3. In the majority of neurons, 5-HT, NA and DA depressed evoked EPSPs, while ACh produced synaptic facilitation (Table 2, Fig. 3A). This observation implies that despite the heterogeneous population of neurons found in the DDH, the monoamine neurotransmitters altered afferent-evoked EPSPs rather predictably, with the bulbospinal monoamines having similarly depressant actions. The relative potency of the transmitters at modulating EPSP amplitude was 5-HT>ACh>NA>DA (Table 2). The modulatory actions of 5-HT, NA and ACh on EPSP amplitude were significant (Table 3; $p < 0.05$; paired t-test). Following washout of NA and DA, partial recovery of EPSP amplitudes generally occurred (Fig. 3B). However, in some neurons, during the washout that followed 5-HT-evoked synaptic depression, a rebound potentiation of EPSP amplitude

occurred, usually exceeding control values. In addition, EPSP amplitudes generally remained facilitated following washout of ACh.

Comparison of effects of the agonists on peak amplitude and area under the curve

We compared the differential effects of the agonists on the early versus the late occurring components of the evoked EPSPs by measuring peak amplitude, as well as measuring the area under the curve (AUC) for early and late components of the EPSP (Table 4). The depressant effects of 5-HT, NA and DA on early and late components of the evoked EPSPs were similar, supporting a uniform depression of both AMPA/kainate and NMDA receptor mediated responses. However, ACh preferentially facilitated the late component of the EPSP ($p < 0.01$; paired t-test; Table 4; Fig. 4A). Figure 4B illustrates a representative example of the effects of 5-HT and ACh on evoked EPSPs in a given cell.

Comparison of actions of the monoamines in individual neurons

The modulatory actions of ACh and 5-HT were compared in 12 neurons (Fig. 5). In six of the nine neurons where EPSPs were depressed by 5-HT, ACh facilitated the EPSPs. Thus, 5-HT and ACh have different ($p < 0.01$; ANOVA) and predominantly opposite actions on spinal neurons in the deep dorsal horn.

The generally depressant actions of the three descending transmitters 5-HT, NA and DA were compared in 15 neurons (Fig. 6). With few exceptions, all three transmitters had common modulatory actions on the evoked EPSPs. In 6 of the 15 neurons, all three transmitters produced synaptic depression while in one neuron, all three drugs produced

synaptic facilitation. Otherwise, with two exceptions (2 of 8), whenever the three drugs failed to produce the same action, the opposing action was generally absent (<10%). An example of the common neuromodulatory actions of the brainstem monoamines in a single neuron is presented in Figure 6B. Thus, unlike ACh, which generally supported synaptic facilitation, the three descending monoamines 5-HT, NA and DA commonly exerted similar functions on spinal cord sensory input to a given cell. However, the EPSP depression produced by 5-HT and DA differed ($p < 0.05$; ANOVA).

Co-Application of 5-HT and NA (5-HT/NA)

In 10 neurons, 5-HT and NA were applied individually as well as co-applied. Co-application of 5-HT and NA evoked synaptic depression of a much greater magnitude than 5-HT or NA applied alone in 3 of the 10 neurons tested (Fig. 7A, asterisks). The effects of co-applied 5-HT and NA were not significantly different than the effects of 5-HT alone but significantly greater than effects of NA alone ($p < 0.05$; ANOVA) suggesting a prominent contribution from 5-HT on synaptic depression when transmitters are combined.

Effects of the agonists on cellular properties

As a population, none of the transmitters had significant effects on cell passive membrane properties, rheobase or voltage threshold (Table 1). The effects of the agonists on EPSP amplitude were plotted against cell input resistance (R_{in}) to identify corresponding actions that would support a postsynaptic site of action (Fig. 8A). With the exception of ACh, where a relatively weak relationship existed ($r^2 = 0.18$), no relations were found.

The actions of the monoamines on firing properties were examined in 32 neurons. Neurons were divided into those that fired either phasically (n = 19) or repetitively (n = 13) in response to current injection. In those cells that originally fired phasically, there was a tendency for the monoamines to increase neuronal excitability. In this population, 14 of the 19 cells had increased number of spikes during current injection in the presence of the transmitters (5/6 from neurons initially firing 1-2 spike population) (Fig. 8B, black bars). This increase in spike number was largely attributable to the observation that the cells that originally fired phasically were converted into neurons that fired repetitively during monoamine transmitter application. An example is presented in Figure 8C. In contrast, in cells initially capable of repetitive firing, no such trends were evident (n = 13; Fig. 8B, gray bars). An example of the effects of the monoamines on firing in a cell that was originally capable of repetitive firing is presented in Figure 8D. In this neuron, NA and DA supported increased firing frequencies, while 5-HT decreased cell firing (see boxed region).

Comparison of membrane properties and synaptic actions in the same neurons

No relationship existed between the type of neuronal firing observed in response to current injection (e.g. phasic vs. repetitive) and the effects of the agonists on EPSP amplitude. Figure 9 presents an example of the differentiated modulation of synaptic and membrane properties in one such cell. The modulation of synaptic actions in this cell was generally consistent with those described above, with ACh facilitating and 5-HT, NA,

and DA depressing EPSP amplitude (Fig. 9A). In contrast, only NA modulated membrane firing properties (Fig. 9B).

Discussion

Summary

In this study, we investigated the effect of each monoamine transmitter on primary afferent-evoked synaptic responses and membrane properties in deep dorsal horn neurons. First, we observed that 5-HT, NA and DA generally had common actions on evoked EPSPs in individual neurons, with the dominant action being depression. In contrast, ACh generally increased EPSP amplitude, even in the same neurons where synaptic depression was evoked by the bulbospinal monoamines. The rank order potency of the evoked modulatory actions on EPSPs was 5-HT>ACh>NA>DA. Second, while 5-HT, NA, and DA tended to uniformly depress short and long-latency components of the evoked EPSPs, the ACh-induced facilitatory response was significantly greater for the later, presumably NMDA receptor-mediated component of the EPSP. Third, co-application of 5-HT and NA could produce a much greater synaptic depression than either transmitter applied independently. Finally, while passive membrane and threshold properties of neurons were unaffected by the monoamines, membrane firing properties in the subpopulation of neurons initially expressing a phasic firing pattern were converted to repetitive.

Common actions of 5-HT, NA, and DA on evoked EPSPs in individual neurons

Consistent with previous in vitro studies, we observed that 5-HT generally produced synaptic depression (e.g. Lopez-Garcia and King, 1996; Lopez-Garcia, 1998; Khasabov et al, 1999) although facilitation was observed in a few cells. Like 5-HT, bath application of NA and DA also produced synaptic depression in most cells, consistent with

depressant actions observed in previous studies that monitored alterations in firing frequency (Headley et al, 1978; Willcockson et al, 1984; Fleetwood-Walker et al, 1988; Skoog and Noga, 1995). An important observation in this study is that 5-HT, NA and DA generally produced the same modulatory action on EPSPs when applied independently to the same neuron. Thus, it appears that despite the diverse functional heterogeneity of the spinal cord dorsal horn (Baldissera et al, 1981; Willis and Coggeshall, 1991; Jankowska, 1992), the bulbospinal monoamine transmitters typically produce a widespread depression of sensory synaptic input onto deep dorsal horn neurons.

The bulbospinal monoamine transmitter systems project widely throughout the spinal cord (Marlier et al, 1991a; Rajaofetra et al, 1992; Holstege et al, 1996) and there are many metabotropic serotonergic, noradrenergic and dopaminergic receptors in the dorsal horn (e.g. Huang and Peroutka, 1987; Marlier et al, 1991b; van Dijken et al, 1996; Stone et al, 1998). Hence, the effects of the bath-applied bulbospinal monoamine transmitters observed here probably reflect the actions of these transmitters on their respective families of receptors. That 5-HT, NA and DA had common actions in most cells suggest that receptors for all three transmitters are co-localized on many neurons and/or primary afferent terminals. However, it is possible that part of the observed depression of the longer latency portion of the EPSP is due to the transmitter having a direct voltage-dependent block of the NMDA receptor ionophore (Chesnoy-Marchais and Barthe, 1996).

In contrast to the depressant actions of the monoamines observed here, Jankowska and colleagues (Jankowska et al, 1997, 2000) demonstrated that non-nociceptive afferent input from different afferents to different groups of spinal interneurons or ascending tract cells is modulated by NA and 5-HT in a highly differentiated manner. Depending on the neuron and afferent fibre type, they observed that NA and 5-HT could have common facilitatory, inhibitory or opposing modulatory actions on synaptic input strength as measured using peri-stimulus time histograms measures of extracellular spike latency and frequency. One explanation for the observed differences in their studies and ours is our stimulation at high intensities to also recruit high threshold C and Aδ fibres, which comprise the largest fraction of primary afferent fibres (Willis and Coggeshall, 1991; Snider and McMahon, 1998). Therefore, the strong depressant actions of the monoamines in our study may result from activation of high threshold afferents that mask more subtle differential modulatory actions on the low threshold afferents studied by Jankowska and colleagues. Another explanation for our observed differences may relate to our finding that EPSP amplitude can decrease concomitant with excitability increases postsynaptically (e.g. Fig. 9). For example, the net effect of NA in this neuron with a decreased EPSP amplitude but increased excitability may be a net increase in the numbers of spikes evoked following primary afferent stimulation. Together, these studies support a complex and functionally differentiated modulation of sensory-evoked firing properties in spinal cord neurons.

Differences in the potency of the bulbospinal monoamines in modulating evoked responses

Despite the similar actions of the three transmitters arising from the brainstem, the magnitudes of depression differed in rank order of 5-HT>NA>DA. In addition, the depression evoked by 5-HT was more widespread as a greater proportion of cells underwent synaptic depression by 5-HT. The differences in magnitude of depression may reflect the relative potency of different bulbospinal systems in mediating modulatory actions. The actions of 5-HT and NA on antinociception have been extensively studied (Basbaum and Fields, 1984; Fitzgerald, 1986; Jones, 1991; Millan, 1995). Previous studies have demonstrated that noxious input leads to the release of both NA and 5-HT in the spinal cord (Tyce and Yaksh, 1981; Yaksh and Tyce, 1981; Satoh and Omote, 1996) and these transmitters can also produce antinociception following release after stimulation of specific supraspinal sites (e.g. Sorkin et al, 1993; Cui et al, 1999). In this study, we observed that co-application of 5-HT and NA could produce a synaptic depression of a greater magnitude than 5-HT or NA applied alone. It is possible that both transmitters are co-released physiologically under conditions where a maximal sensory depression is sought. While nociceptive input does not appear to evoke release of dopamine (Satoh and Omote, 1996), both stimulation of the A11 dopaminergic cell group and exogenous application of DA can elicit antinociception (Fleetwood-Walker *et al*, 1988). Overall, the role of DA in mediating antinociception remains poorly studied, and it is possible that DA may play a different role in the modulation of primary afferent input than 5-HT and NA.

Facilitatory actions of ACh

In contrast to the bulbospinal transmitters, ACh generally facilitated primary afferent-evoked responses. Although some studies have reported inhibitory or antinociceptive effects of cholinergic agonists (e.g. Bleazard and Morris, 1993), facilitatory or excitatory actions, consistent with our observations, have also been reported. For instance, Urban et al (1989) reported an increase in excitability of spinal cord dorsal horn neurons by ACh, while Baba et al (1998) reported a muscarinic-induced facilitation of GABA release in substantia gelatinosa neurons of the rat. We also observed that ACh caused significantly greater facilitation of the late, largely NMDA-receptor mediated, component of the evoked EPSP. This is consistent with previous studies demonstrating the facilitatory effects of cholinergic agonists on NMDA-receptor mediated events in various CNS regions including hippocampus (Marino et al, 1998) and striatum (Calabresi et al, 1998) via M_1-like receptor activation. M_1 receptor activation leads to an increase in protein kinase C (PKC) and PKC enhances NMDA receptor activity (e.g. Chen and Huang, 1992; Xiong et al, 1998).

Modulation of neuronal firing properties

The monoamine transmitters did not significantly alter the passive membrane or threshold properties of DDH neurons. However, in neurons that originally fired phasically, the monoamines could reversibly transform firing behaviour from phasic to repetitive. Interestingly, Lopez-Garcia and King (1994) showed that firing patterns in response to current injection are functionally correlated to the source of primary afferent input. For example, wide dynamic range (WDR) neurons receive convergent input from

both low and high threshold afferents, and these cells generally fire repetitively. If we applied this classification to the neuronal firing properties observed following the application of monoamines, the monoamines could convert the functional properties of neurons from those that previously had restricted sensory convergence to a WDR profile. Interestingly, the majority of neurons in the dorsal horn of awake sheep, obviously having normal bulbospinal activity, are WDR (Herrero and Headley, 1995). It is also possible that the monoamines do not alter convergent properties to DDH neurons but rather, the classification scheme developed by Lopez-Garcia and King (1994) does not apply to behaviour of neurons in the presence of monoamine transmitters.

It may seem inconsistent that the changes in neuronal firing properties were unrelated to the observed changes in EPSP amplitude. However, since different receptor subtypes may be found at pre and post-synaptic sites, it is not surprising that the monoamine transmitters exert different actions on the synaptic and cellular properties of spinal neurons. For instance, while one class of receptor may depress sensory input (e.g. Khasabov et al, 1999), another class may increase the excitability of these neurons (e.g. Wallis et al, 1991). Opposing pre- and postsynaptic actions may be a widely employed strategy to alter the network properties of spinal neurons. For example, a depressed sensory input with increased neuronal responsiveness could support a transfer of control from peripheral to descending command systems.

Possible sites of action

Several observations suggest that the depression produced by the bulbospinal monoamines on EPSPs is mediated via presynaptic mechanisms. First, none of these transmitters had effects on the passive membrane properties that would support a reduction in EPSP amplitudes by postsynaptic mechanisms (i.e. a decreased τ_m or R_{in}). In addition the identical percent depression on early, presumable AMPA/kainate and late, presumably NMDA receptor-mediated components can be explained by a reduction in glutamate transmitter release. All three transmitters have been previously shown to exert presynaptic actions consistent with synaptic depression (e.g. Travagli and Williams, 1996; Gajendiran et al, 1996).

In contrast to the bulbospinal monoaminergic transmitters, ACh probably mediates its facilitatory actions predominantly postsynaptically. ACh had a preferential facilitatory action on the late, presumably NMDA receptor mediated, component of the EPSP, consistent with observed modulatory actions of M_1 muscarinic receptor activation on NMDA receptor activity (Marino et al, 1998; Calabresi et al, 1998). If facilitation in synaptic strength involved only presynaptic mechanisms that increase glutamate release, one would expect to see a uniform facilitation of both AMPA/kainate and NMDA receptor mediated responses.

Significance

In conclusion, we provide the first comparative analysis of the actions of the biogenic amine transmitters on synaptic and cellular properties of spinal neurons. 5-HT, NA and

DA are involved in the descending control of spinal sensory integration, and the present observations suggest that the separate brainstem monoaminergic systems can affect spinal sensory integration in a remarkably similar manner. With respect to the control of sensory input, these studies increase our understanding of how these transmitters act, perhaps to maximize antinociceptive actions.

Table 1. Membrane properties of recorded neurons in the presence or absence of a monoamine transmitter. Values reported are mean ± S.D. The range of sample size used to calculate mean values is bracketed in left column. E_{MR}, resting membrane potential; V_{TH}, voltage threshold; R_{in}, cell input resistance; τ_m, membrane time constant; AP height, action potential height; AP ½ width, action potential duration at half maximal amplitude.

	E_{MR} (mV)	R_{in} (MΩ)	τ_m (ms)	Rheobase (pA)	V_{TH} (mV)	AP height (mV)	AP ½-width (ms)
Control (33 - 41)	-59 ± 18	507 ± 250	44 ± 19	57 ± 42	26 ± 7	94 ± 12	3.7 ± 2.0
5-HT (16 - 28)	-58 ± 18	509 ± 187	42 ± 16	57 ± 43	26 ± 8	78 ± 19	4.3 ± 2.0
NA (20- 26)	-59 ± 18	606 ± 288	46 ± 26	60 ± 47	30 ± 12	82 ± 12	4.1 ± 1.3
DA (16 - 20)	-59 ± 19	583 ± 335	46 ± 21	72 ± 56	33 ± 11	82 ± 12	4.8 ± 2.4
ACh (14 - 18)	-57 ± 19	541 ± 246	47 ± 26	69 ± 51	30 ± 6	78 ± 16	4.2 ± 1.4

Table 2. Incidence of neurons having synaptic depression or facilitation evoked by 5-HT, NA, DA and ACh. Values are percentage of sample size indicated in brackets. Shaded boxes indicate predominant action for each transmitter.

Agonist	Increase	Decrease	No Change
5-HT (28)	14%	79%	7%
NA (26)	15%	62%	23%
DA (20)	15%	55%	30%
Ach (20)	70%	15%	15%

Table 3. Effects of 5-HT, NA, DA and ACh on the evoked synaptic responses. Values are mean percentage ± S.D. of change in EPSP peak amplitude. Sample sizes are in brackets.

Agonist	Mean EPSP amplitude ↑	Mean EPSP amplitude ↓	No Change
5-HT (28)*	59 ± 70% (4)	49 ± 20% (22)	(2)
NA (26)*	53 ± 56% (4)	35 ± 24% (16)	(6)
DA (20)	48 ± 30% (3)	32 ± 16% (11)	(6)
ACh (20)*	35 ± 20% (14)	13 ± 2% (3)	(3)

*Overall effect of transmitter on changes in EPSP amplitude is significant ($p < 0.05$).

Table 4. Comparison of changes in peak amplitude, early and late components of the evoked responses in neurons depressed by 5-HT, NA and DA and neurons facilitated by ACh. Values expressed as mean percentage ± S.D. Sample sizes are in brackets.

	Peak amplitude	AUC (<200)	AUC (≥200)
5-HT (22)	-49 ± 20	-53 ± 18	-57 ± 25
NA (16)	-35 ± 24	-42 ± 25	-51 ± 41
DA (11)	-32 ± 16	-30 ± 17	-34 ± 34
ACh (14)	35 ± 20	42 ± 26	121 ± 97*

*Effect of ACh is significantly greater on late verses early AUC ($p < 0.01$) as highlighted with gray shading.

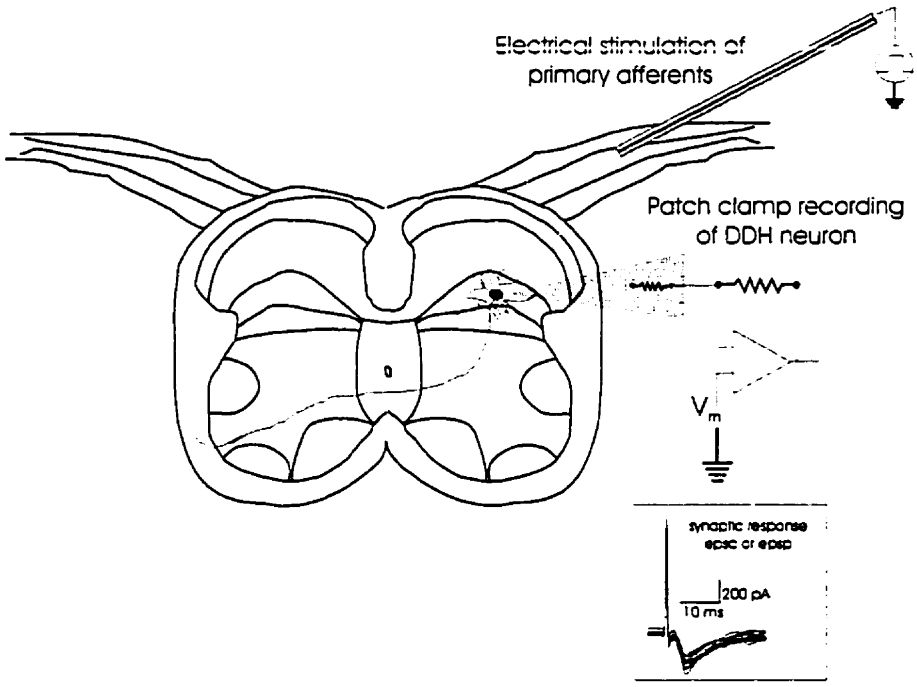

Figure 1: Experimental arrangement. The transverse slice permits an easy targeting of the deeper laminae of the dorsal horn. DDH neurons are recorded using the whole cell 'blind' patch-clamp technique. Electrical stimulation of dorsal rootlets evokes synaptic responses in DDH neurons.

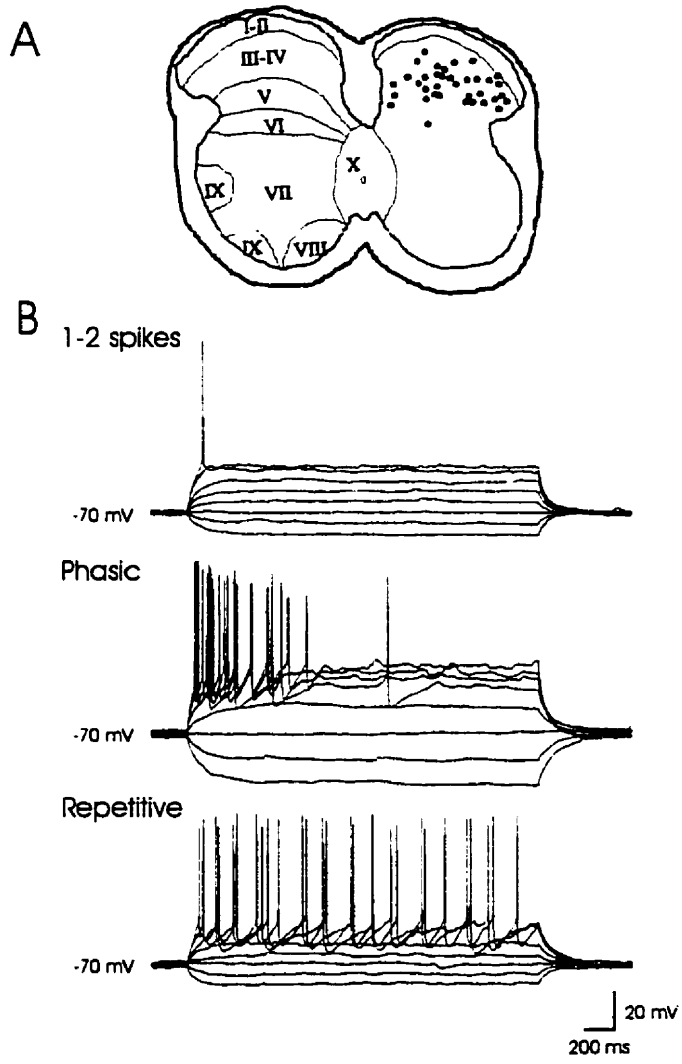

Figure 2: Location and firing properties of recorded neurons. **A.** Approximate topographical distribution of 36 of the 41 neurons used in the present analysis. Left side of cord presents outline of Rexed's laminae for the lumbar enlargement (derived from Kjaerulff et al, 1994). **B.** Firing patterns of DDH neurons in response to current injection. In response to current steps, neurons of the DDH fired 1-2 spikes, phasically, or repetitively. Neurons were held at -70mV and hyperpolarizing and depolarizing current steps were delivered in 5 pA increments.

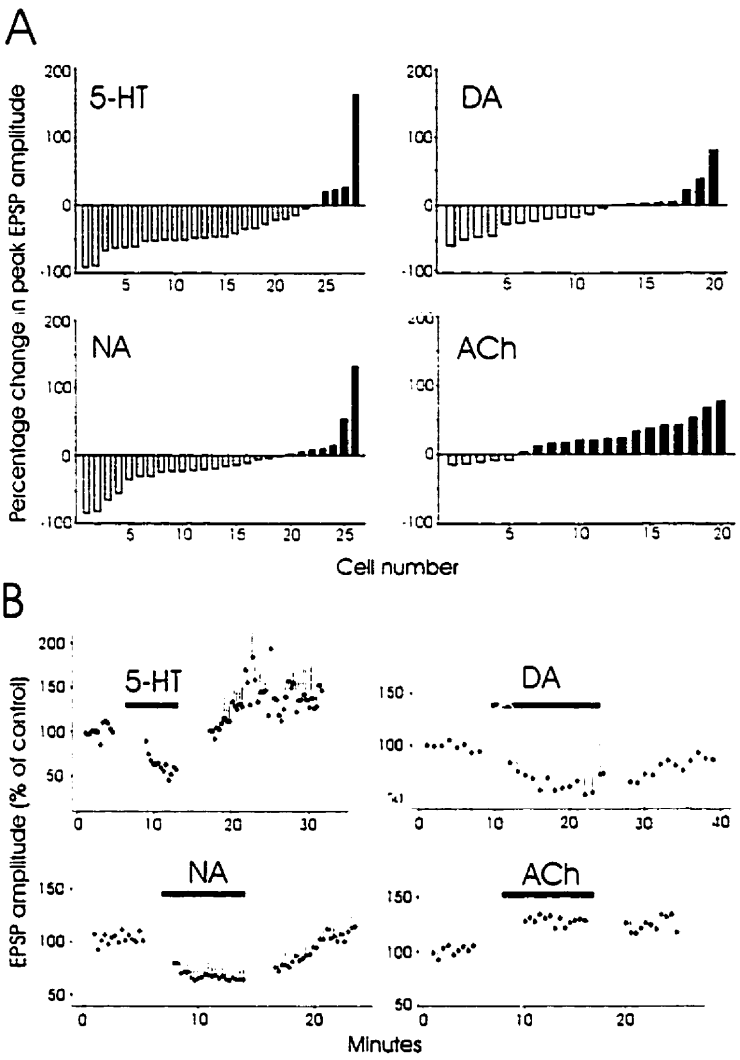

Figure 3: Effects of the monoamine transmitters on EPSP amplitude. **A.** Histograms presenting the modulatory actions of the monoamines on EPSP amplitude in individual cells presented in rank-order from maximal depression to maximal facilitation. Note that the predominant actions of 5-HT, NA and DA were depression while ACh generally caused synaptic facilitation. **B.** Normalized data on the time course of synaptic depression (5-HT, NA, DA) or facilitation (ACh) from 6 representative cells in each. Values are presented as mean + S.E and the duration of drug application is indicated with a horizontal bar.

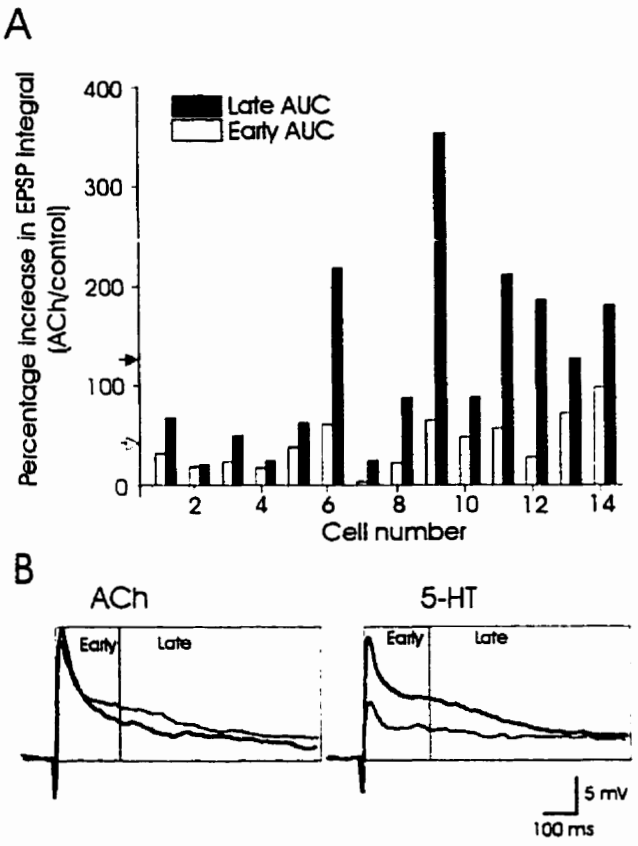

Figure 4: ACh preferentially facilitates the late component of the evoked EPSP. **A.** Histogram showing the effect of ACh on the shorter (0-200ms) versus longer-latency (200-750ms) components of the evoked EPSP (measured as area under the curve: AUC) in the 14 cells facilitated by ACH. Arrows on ordinate present mean percentage increase in AUC for the short (open arrow; 42%↑) and long (closed arrow; 121%↑) latency components. **B.** Average EPSPs from a sample neuron comparing modulatory actions of ACh and 5-HT. While ACh preferentially facilitates the later phase of the EPSP (52%), 5-HT depresses both early and late phases similarly (35% and 34%, respectively). Black traces represent control mean EPSP value prior to application of transmitter.

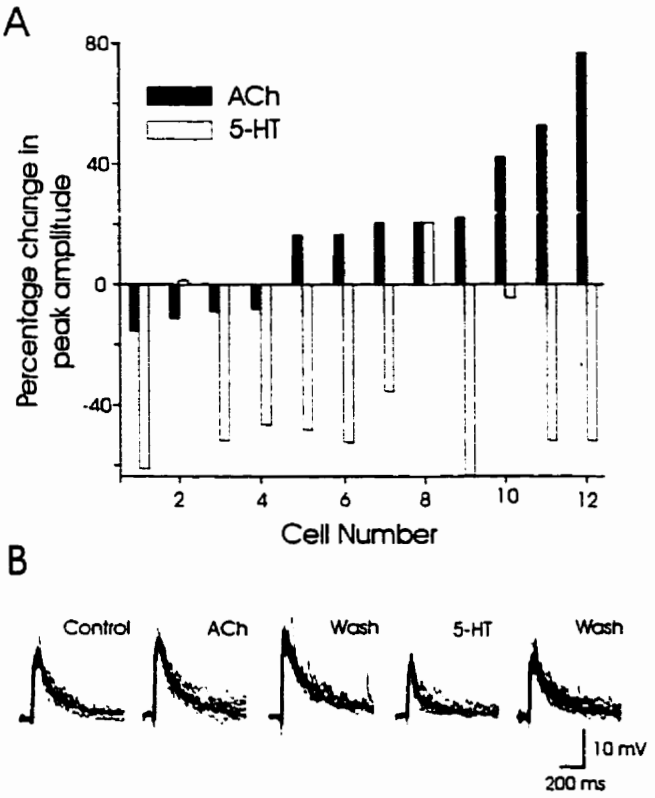

Figure 5: Comparison of the effects of ACh and 5-HT on evoked synaptic responses in individual neurons. **A.** ACh evokes synaptic facilitation, while 5-HT causes synaptic depression in the majority of cells co-tested. In this and the following figures, the shaded area around baseline represents the region of changes in EPSP amplitude ≤ 10%. **B.** Example of the evoked EPSP in a given neuron where 5-HT produces synaptic depression (52%) following ACh-induced facilitation (53%) of the evoked responses. Individual traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace.

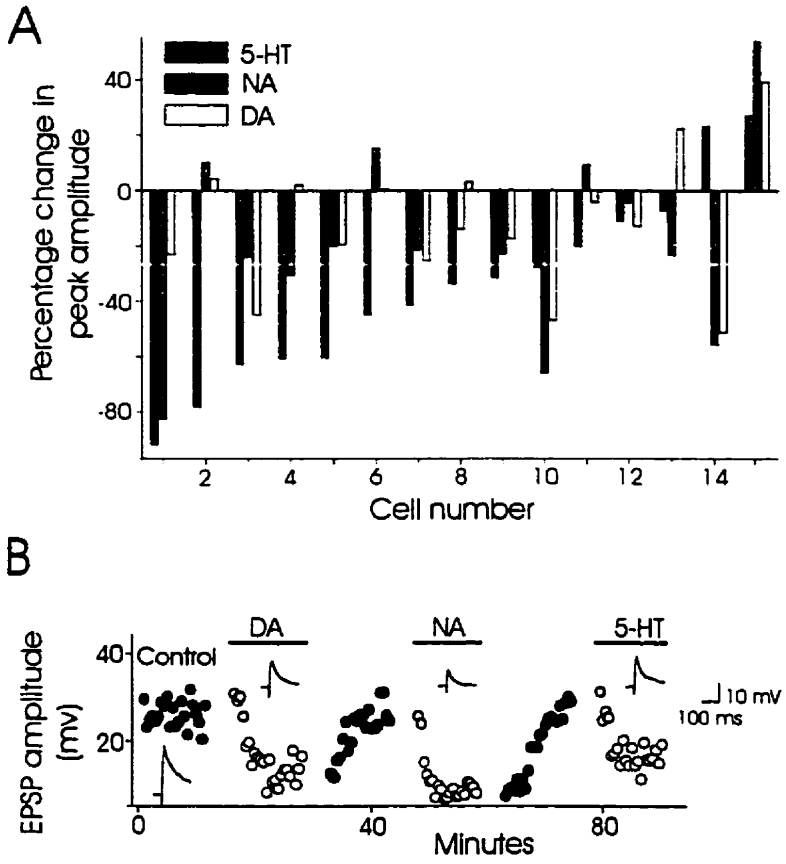

Figure 6: 5-HT, NA and DA generally exert common actions. **A.** Histogram showing the population of cells tested with 5-HT, NA and DA. Excluding changes ≤ 10% (gray shading), these transmitters had similar actions in most cells tested. 5-HT generally produced depression of the greatest magnitude. **B.** Example of transmitter-induced alterations in EPSP amplitude over time in a neuron. Closed circles represent EPSPs evoked during control and washout periods while open circles represent EPSP values obtained during agonist application. The averaged EPSP waveforms obtained during control, DA, NA, and 5-HT are also presented. The magnitudes of depression evoked in this neuron were 47% (in DA), 28% (in 5-HT) and 66% (in NA).

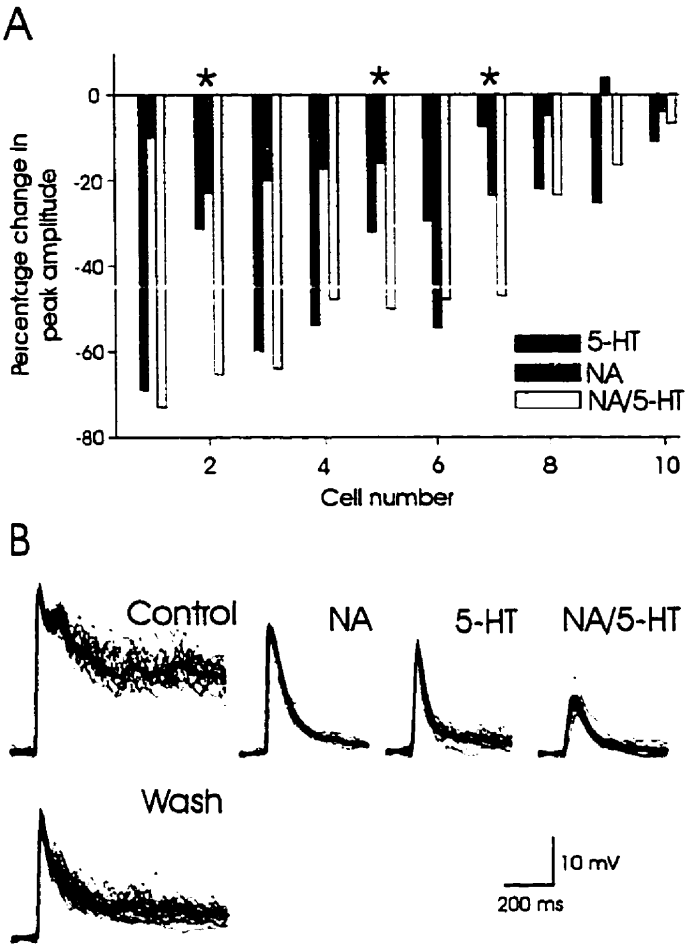

Figure 7: Co-application of NA and 5-HT can produce greater depression than either 5-HT or NA alone. **A.** Histogram showing population of cells tested with NA/5-HT. In 3 of 10 cells (denoted by asterisks), NA/5-HT-induced a synaptic depression considerably greater than either agonist applied alone. **B.** Evoked EPSPs obtained from cell #2 in histogram above. Note that the depression of the EPSP is greater during co-application of 5-HT and NA (65%) than either drug applied alone (31% and 23%, respectively). Individual traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace.

Figure 8

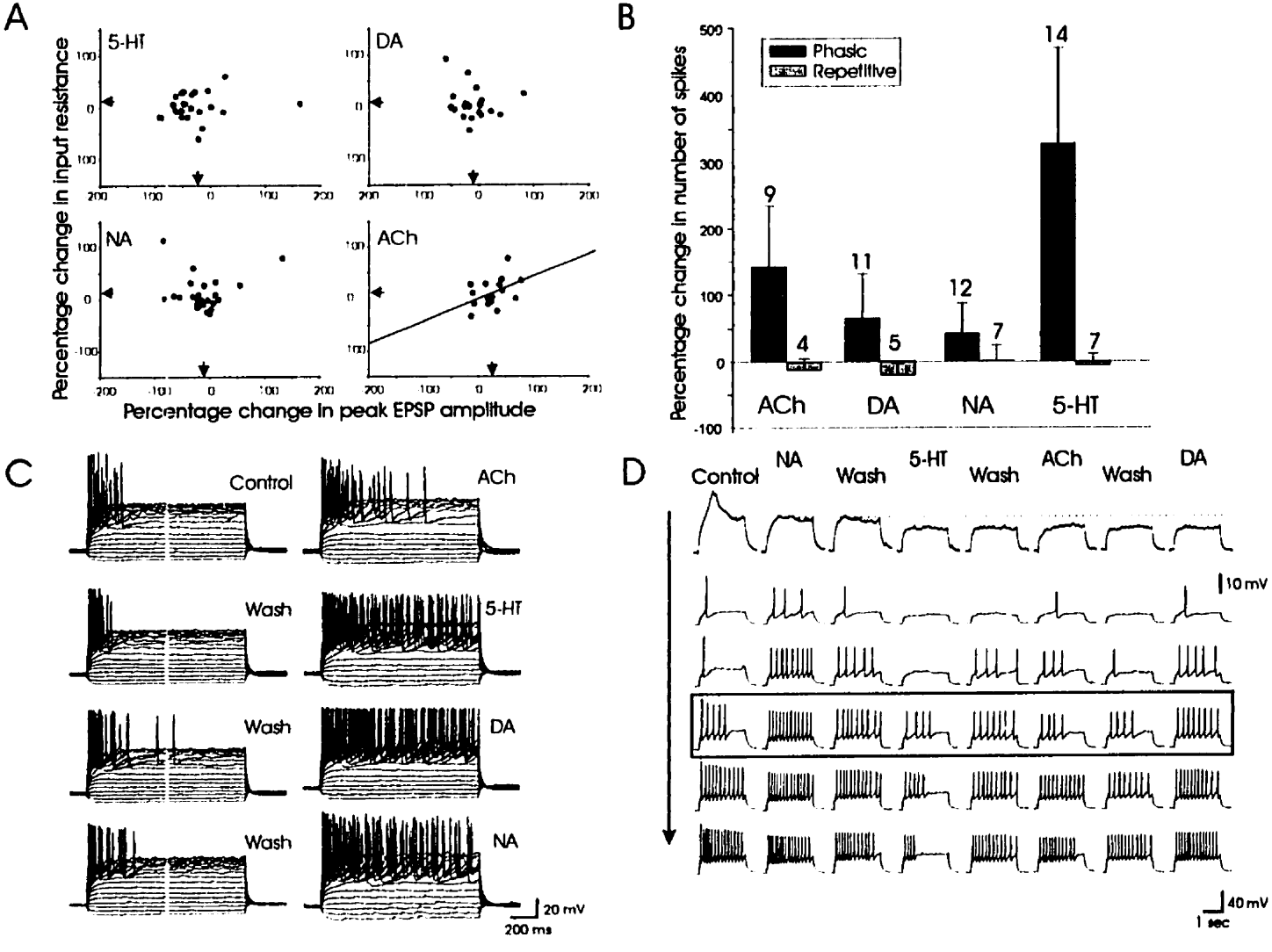
A
5-HT
DA
NA
ACh
Percentage change in input resistance
Percentage change in peak EPSP amplitude
B
Phasic
Repetitive
Percentage change in number of spikes
9
4
11
5
12
7
14
7
ACh
DA
NA
5-HT
C
Control
ACh
Wash
5-HT
Wash
DA
Wash
NA
20 mV
200 ms
D
Control
NA
Wash
5-HT
Wash
ACh
Wash
DA
10 mV
40 mV
1 sec

Figure 8: Effects of the monoamine transmitters on neuronal membrane properties. **A.** Relationship between changes in R_{in} and changes in EPSP peak amplitude. Regression line for ACh is shown ($r^2 = 0.18$; NS). Arrows on axes present mean values. **B.** Several neurons initially having phasic repetitive firing properties in response to current injection preferentially undergo increases in spike numbers. Spike numbers were counted during a 1.2 sec depolarizing current pulses at approximately twice rheobase current intensities. Histograms present percentage change in firing number produced by the monoamine transmitters for neurons originally displaying phasic (black bars) or repetitive firing (gray bars) in response to current injection. Note that neurons originally firing repetitively in response to current injection are unaffected by application of agonist. **C.** Reversible modulation of the monoamine transmitters on membrane firing properties. Current steps were delivered at 20 pA increments and voltage responses are presented superimposed. Note that the bulbospinal monoamines converted firing patterns from phasic to repetitive. **D.** Effects of the monoamines on firing properties (arrow indicates increasing depolarizing current steps). The first row presents membrane depolarization in response to a 20 pA current step and is amplified to allow comparison of voltage responses. The following rows represent successive step increases in current magnitude by 10 pA. Note that 5-HT causes decreased firing while NA and DA increase firing (e.g. responses enclosed in rectangle). 5-HT also appears to cause a lasting decrease in cell resistance (first row, dotted lines).

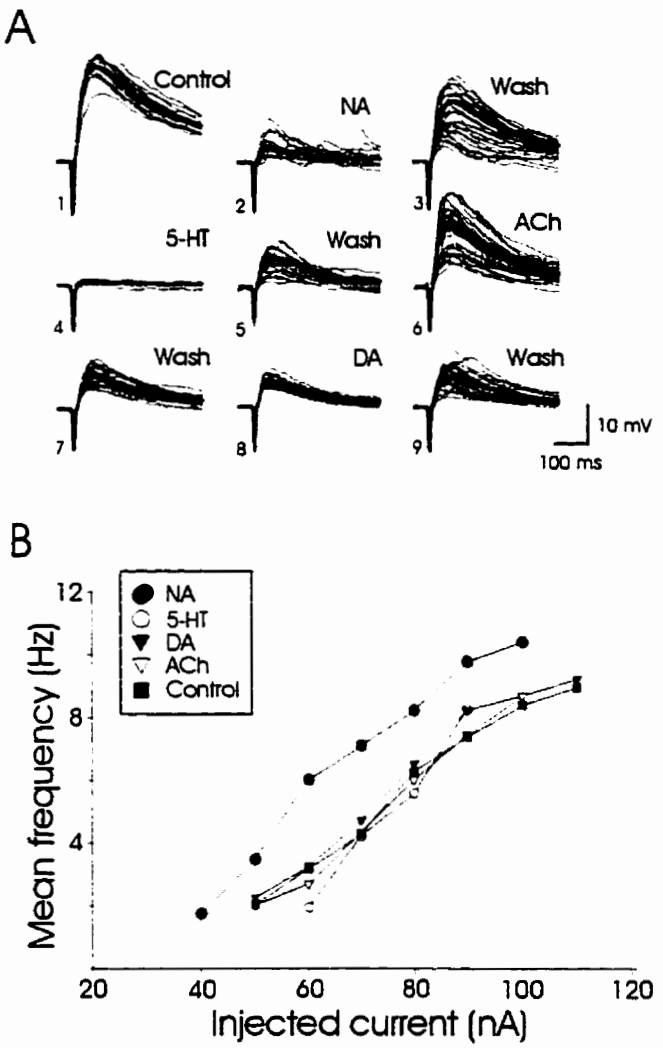

Figure 9: Comparison of the effects of the monoamines on EPSPs and firing properties in an individual neuron. **A.** 5-HT, NA and DA depress while ACh facilitates evoked synaptic responses. Note that EPSPs never fully recover following washout of agonists. Raw traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace. **B.** Modifiability in frequency-current (f-I) relations. Of the monoamines tested, only NA produces a reversible leftward shift in the f-I relation indicative of a reduced rheobase and higher firing frequencies.

Project II - Pharmacological characterization of serotonin receptor subtypes modulating primary afferent input to deep dorsal horn neurons in the neonatal rat

Abstract

Spinal cord slices and whole-cell patch clamp recordings were used to investigate the effects of serotonergic receptor ligands on dorsal root-evoked synaptic responses in deep dorsal horn (DDH) neurons of the neonatal rat at postnatal days (P) 3-6 and P10-14. Bath applied 5-hydroxytryptamine (5-HT) potently depressed synaptic responses in most neurons. Similarly, the 5-$HT_{1A/7}$ receptor agonist, 5-carboxamidotryptamine (5-CT) depressed synaptic responses. This action was probably mediated by 5-HT_{1A} receptor activation, since it occurred in the presence of the 5-HT_7 receptor antagonist, clozapine. In the absence of any agonist, 5-HT_{1A} receptor antagonists often facilitated synaptic responses, suggesting that there is sufficient endogenous 5-HT to tonically activate 5-HT_{1A} receptors. 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), the 5-$HT_{1A/7}$ receptor agonist, facilitated synaptic responses, an action probably mediated by 5-HT_7 receptors, since the facilitation could be reversed by subsequent application of the 5-HT_7 receptor antagonist clozapine. Agonists for the 5-HT_{1B}, 5-HT_2 and 5-HT_3 receptors exerted only modest modulatory actions. A pharmacological analysis of the depression evoked by 5-HT suggested an action partly mediated by 5-HT_{1A} receptor activation, since antagonism of the 5-HT_{1A} receptor with NAN-190 or WAY-100635 partly reversed 5-HT-evoked depression. In comparison, 5-HT_7 receptor activation could account for much of the 5-HT evoked facilitation. We conclude that 5-HT is capable of modulating sensory input onto DDH neurons via several receptor subtypes, producing both

facilitatory and depressant actions. Also, the actions of most receptor ligands on the evoked responses were similar within the first two postnatal weeks.

Introduction

The deep dorsal horn (DDH) region of the spinal cord is a primary site for the integration of somatosensory information, including nociception. Several endogenous systems within the CNS modulate the synaptic and cellular properties of DDH neurons (e.g. Basbaum and Fields, 1984; Hammond, 1986). One family of transmitters known to exert such actions comprises the brainstem monoamines that include several distinct descending serotonergic pathways. For example, serotonergic neurons of the raphe nuclei project widely to modulate spinal cord function via the dorsolateral and ventrolateral funiculi (Dahlström and Fuxe, 1965; for review see Basbaum and Fields, 1984; Fitzgerald, 1986; Hammond, 1986; Millan, 1995).

5-hydroxytryptamine (5-HT; serotonin) is released in the spinal cord following noxious input (e.g. Omote et al, 1998) and has been demonstrated to exert spinal antinociceptive actions (for review see Fitzgerald, 1986; Eide and Hole, 1993; Millan, 1995), implicating brainstem serotonergic systems in the control of spinal nociception. Exogenously applied 5-HT generally depresses, but can also facilitate primary afferent-evoked synaptic responses onto dorsal horn neurons (e.g. Randic and Yu, 1976; Headley et al, 1978; Jordan et al, 1979; Lopez-Garcia and King, 1996; Lopez-Garcia, 1998). The mechanisms underlying 5-HT-evoked modulation of synaptic properties of spinal neurons are not fully understood but it appears from most studies that the 5-HT_1 receptors play an inhibitory, presumably antinociceptive role, while the 5-HT_2 receptors play a facilitatory (pronociceptive) role (e.g. Eide and Hole, 1991; Hori et al, 1996; Lopez-Garcia and King, 1996).

Currently, there are seven families of 5-HT receptors (5-HT_{1-7}), comprising at least 14 distinct receptor subtypes (for review see Hoyer et al, 1994; Barnes and Sharp, 1999). Several receptors, which include the 5-HT_1, 5-HT_2 and 5-HT_3 receptors, have been identified in the spinal cord dorsal horn (e.g. Huang and Peroutka, 1987; Marlier et al, 1991b; Kidd et al, 1993; Pompeiano et al, 1994). With the exception of the ionotropic 5-HT_3 receptor, all serotonergic receptors are G protein-coupled receptors and hence capable of exerting a broad modulatory influence on network and cell behaviour, as most, if not all, ligand- and voltage-gated channels can be modulated by 5-HT (e.g. see reviews by Anwyl, 1990; Barnes and Sharp, 1999).

In this study, we used the spinal cord slice preparation and whole-cell patch recordings of sensory synaptic input onto DDH neurons to characterize and compare the modulatory actions of 5-HT to receptor subtype selective ligands with reported high affinity for 5-HT_{1A}, 5-HT_{1B}, 5-$HT_{2A/2C}$, 5-HT_3 and 5-HT_7 receptors. While several investigators have examined the actions of 5-HT receptor ligands on the control of spinal cord function in vivo (e.g. Ali et al, 1994, 1996; Clarke et al, 1996; Gjerstad et al, 1996, 1997; Ogilvie et al, 1999), only a few studies have examined their actions at the spinal cellular level in vitro (e.g. Lopez-Garcia and King, 1996; Lopez-Garcia, 1998; Khasabov et al, 1999). In vitro studies offer the advantage of applying multiple ligands at known concentrations (e.g. Wallis et al, 1993a; Wallis and Wu, 1993) while intracellular recordings permit a cellular characterization of neuromodulatory mechanisms of action. We examined dorsal root-evoked synaptic responses in two age groups of neonatal rats (P3-6 and P10-14) to

determine whether developmental alterations occur in serotonergic receptor modulation during the first two postnatal weeks. Our results suggest that primary afferent synapses are modulated by several 5-HT receptor subtypes and that most evoked responses can be depressed by 5-CT acting on the 5-HT_{1A} receptor and facilitated by 8-OH-DPAT acting on the 5-HT_7 receptor. These actions were independent of age range examined. Parts of this work have been published previously in abstract form (Hochman and Garraway, 1998).

Material & Methods

Preparation of spinal cord slices

Sprague-Dawley rats, postnatal days (P) 3-6 and P10-14, were used. The older animals (P10-14) were first anesthetized with 10% urethane (2 mg/kg body weight i.p.), decapitated and spinal segments L2 - S1 were removed using cooled (< 4°C) oxygenated (95%O_2-5%CO_2) high sucrose-containing artificial cerebrospinal fluid (ACSF) containing (in mM); sucrose, 250; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 3; glucose, 25; NaH_2PO_4, 1.25; $NaHCO_3$, 26; at a pH of 7.4. The younger animals (P3-6) were decapitated and spinal segments L2 - S1 were removed using cooled oxygenated normal ACSF containing (in mM); NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 25; NaH_2PO_4, 1.25; $NaHCO_3$, 26; at a pH of 7.4. The isolated spinal cord was embedded in Agar, 2.5% w/v, (Type E, Sigma) and sliced on a vibrating blade microtome (Leica VT1000S or Pelco 101) to yield transverse slices (500-600 μm thick), incubated for at least 1 hour prior to experimentation in normal oxygenated aCSF maintained at 32°C.

Electrophysiology

Spinal cord slices were affixed to a recording chamber for experimentation (Edwards et al, 1989). The transverse slice provides a reasonable outline of the spinal cord gray matter when viewed microscopically to permit reliable targeting of the DDH (King et al, 1988). Short dorsal rootlets remained attached to the spinal slices to allow for electrical stimulation of primary afferents. Patch electrodes were prepared from 1.5 mm outer diameter capillary tubes (Precision Instruments or Warner) using a two-stage puller (Narishige PP83) to produce resistance values ranging from 4-7 MΩ. The intracellular

recording solution contained (in mM): K-gluconate, 140; EGTA, 0.2; HEPES, 10; Mg-ATP, 4; GTP, 1; pH 7.3. In most experiments, 2mM QX-314 (RBI) was added to the recording solution to block voltage-dependent Na^+ channels. The recording chamber was continuously superfused with oxygenated normal ACSF at a rate of ~2 ml/minute.

The whole-cell 'blind' patch clamp recording technique (Blanton et al, 1989) was undertaken at room temperature (~20°C) using the Axopatch 1D amplifier (Axon Instruments) filtered at 5 kHz (4-pole low-pass Bessel) to record from DDH neurons (laminae III-VI). Both voltage and current clamp data were acquired on computer with the pCLAMP acquisition software Clampex (v 6.0; Axon Instruments). Immediately following rupture of the cell membrane (in voltage clamp at –90 mV), the current clamp-recording configuration was used to determine resting membrane potential. Series resistance was subtracted in current clamp mode (bridge balance). Most experiments were conducted in the current clamp mode, although in a few cases, voltage clamp recordings were made. In voltage clamp experiments, series resistance remained uncompensated. For all neurons, resting membrane potential, leak conductance and bridge balance were monitored throughout to ensure recording stability, if recordings were unstable, the recording was discontinued and the data rejected. Mean electrode series resistance was 39 ± 10 MΩ.

Dorsal rootlets were electrically stimulated with bipolar tungsten electrodes at intensities that recruit the majority of afferent fibre types (typically $\geq$ 500 μA, $\geq$100 μs) (see Thompson et al, 1990). Stimulation-evoked excitatory postsynaptic potentials or currents

(EPSPs or EPSCs) were collected at low frequencies (usually every 60 seconds in P3-6 and every 20 or 30 seconds in P10-14 rats). Membrane potential was held at –90 mV using injection of bias current for the entire duration of the recording.

Application of ligands

Drugs were prepared on the day of the experiment from frozen stock solutions. All drugs were dissolved in normal ACSF and bath applied using independent perfusion lines connected to a common output. Generally, following a 10-15 minutes period for collection of 'control' evoked synaptic responses, each ligand was then applied for a period of 10-15 minutes, during which time synaptic responses were continually recorded at baseline membrane potential and frequency. If membrane potential was altered by drug application, bias current was injected to return the membrane to –90 mV prior to continued collection of evoked synaptic responses. In order to compare the actions of several 5-HT receptor ligands on the evoked synaptic responses of a given neuron, we allowed a washout/recovery period of 10-20 minutes between subsequent drug applications.

Drugs used

5-HT was applied at 10 μM (in 100 μM ascorbic acid to prevent oxidation). The 5-HT receptor agonists used were: 5-carboxamidotryptamine (5-CT) and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), $5\text{-HT}_{1A/7}$ receptor agonists; 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline (CGS), a 5-HT_{1B} receptor agonist; 1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI), a $5\text{-HT}_{2A/2C}$ receptor agonist;

and, 1-(m-chlorophenyl)-biguanide (CPBG), a 5-HT_3 receptor agonist. The 5-HT receptor antagonists used were; NAN-190 or WAY-100635, 5-HT_{1A} receptor antagonists; ketanserin, a 5-$HT_{2A/2C}$ receptor antagonist; and clozapine, a 5-HT_7 receptor antagonist. 8-OH-DPAT (in 100 μm ascorbic acid), DOI, NAN-190, WAY-100635, ketanserin and clozapine were added at a concentration of 0.1 μM. 5-CT, CGS and CPBG were added at a concentration of 1 μM. Figure 1 provides a summary of the 5-HT receptor subtypes, their identified cellular signaling mechanisms and the relative binding affinities for the pharmacological agents employed in this study. All ligands were obtained from RBI/Sigma (Natick, MA).

Analysis

Recordings were analyzed using Clampfit (v 6.0, Axon Instruments). The maximum amplitude of the synaptic response of individual traces was measured. Comparison of evoked synaptic responses before and after application of 5-HT receptor ligands was made and an effect mediated by the applied ligands was considered modulatory if differences in amplitude of the evoked synaptic responses were ≥10%. Because multiple drugs were added in some experiments and evoked responses did not often return to pre-drug baseline values, the change in synaptic amplitude was measured as the difference in the peak amplitude during drug application compared to the amplitude in the control period <u>just prior</u> to drug application.

Following analysis, graphs were constructed using Sigma Plot (Jandel Scientific) and imported into CorelDRAW (Corel) for final editing. All values in text are reported as

mean ± S.D. whilst figures are reported as mean + S.E. bars. Statistical comparisons were made using the Student's paired t-test.

Results

A total of 101 neurons from laminae III-VI were recorded, having a mean resting membrane potential of –55 ± 9 mV. Since QX-314 was added to the recording solution in most neurons, threshold and firing properties were not systematically examined. However, we previously reported a detailed characterization of membrane properties of DDH neuron in these two age groups of rats (Hochman et al, 1997).

Effects of 5-HT on primary afferent-evoked synaptic responses

Bath applied 5-HT significantly depressed synaptic responses by 52 ± 20% in 34/43 DDH neurons obtained from P3-6 animals ($P < 0.001$; Fig. 2A) and by 49 ± 20% in 22/28 neurons obtained from P10-14 animals ($P < 0.05$; Fig. 2B). 5-HT also facilitated evoked responses in a minority of neurons from both age groups (3/43 in P3-6; 28 ± 10%↑ and 4/28 in P10-14; 59 ± 70%↑).

Effects of the selective 5-HT receptor ligands on primary afferent-evoked responses

Table 1 summarizes the actions of receptor selective ligands in the two age groups examined and should be referred to in the following sections.

<u>5-HT_{1A} receptor mediated actions</u>

Because 5-CT has relatively high affinity for both 5-HT_{1A} and 5-HT_7 receptors, we selectively activated the 5-HT_{1A} receptor by adding 5-CT in the presence of clozapine, a 5-HT_7 receptor antagonist, which has a very low affinity for the 5-HT_{1A} receptor. 5-CT/clozapine evoked a synaptic depression of 58 ± 21% in 12 of 14 neurons obtained

from both younger and older animals (Table 1; Fig. 3). In the remaining two neurons (from younger animals), 5-CT/clozapine was without effect. Following washout of drugs, only a partial recovery of the evoked synaptic responses was observed (also see Lopez-Garcia, 1998), perhaps due to persistent alterations in second messengers.

Interestingly, in the absence of any applied agonist, application of either 5-HT_{1A} receptor antagonist, NAN-190 or WAY-100635, produced synaptic facilitation (62 ± 42%) in 9 of 19 neurons. This action suggests that 5-HT_{1A} receptors are tonically active and inhibiting evoked responses in the spinal slice, presumably via endogenous 5-HT release. In addition, in 5/19 neurons, application of NAN-190 produced a synaptic depression (38 ± 21%). We interpreted this as suggesting that in some neurons, NAN-190 may be acting at another receptor subtype (e.g. 5-HT_7) to block a tonic facilitating action of 5-HT (see discussion). Figure 4 illustrates the facilitatory and inhibitory effects of these antagonists.

5-HT_{1B} receptor agonist

The 5-HT_{1B} receptor agonist CGS depressed the evoked synaptic responses in 6/7 neurons from younger animals, but caused facilitation in 4/7 neurons in the older animals (Table 1). Figure 5A and B(i and ii) demonstrate these effects of CGS. Part of the increased synaptic response in older animals may be attributed to an observed increase in input resistance (Fig. 5B(iii)).

5-$HT_{2A/2C}$ receptor mediated actions

The 5-$HT_{2A/2C}$ receptor agonist DOI facilitated responses in 5/16 neurons (44 ± 43%), and only modestly depressed responses in 3 neurons (20 ± 10%) (Fig. 6A). In half of the neurons (n=8) DOI was without effect. In 5 neurons, application of the 5-$HT_{2A/2C}$ receptor antagonist ketanserin alone did not modify the synaptic response (Fig. 6B), suggesting that, unlike the 5-HT_{1A} receptor, these receptors were not tonically activated in the slice preparation.

5-HT_3 receptor agonist

The 5-HT_3 receptor agonist CPBG had modest actions, facilitating synaptic responses in 8 of 13 neurons (19 ± 7%) (Fig. 7A), while depressing responses in only 2 neurons obtained from older animals. Figure 7B demonstrates examples of both the facilitatory and inhibitory actions of CPBG.

5-HT_7 receptor mediated actions

Like 5-CT, 8-OH-DPAT has high affinity for both the 5-HT_{1A} and 5-HT_7 receptors. In the presence of 8-OH-DPAT alone, synaptic facilitation (50 ± 43%) was observed in the majority of neurons tested (12/14) (Fig. 8A,B(i)). In addition, the 5-HT_7 receptor antagonist clozapine was capable of partially reversing the 8-OH-DPAT-induced facilitation (Fig. 8B(ii)), suggesting that the facilitatory action of 8-OH-DPAT is due to activation of the 5-HT_7 and not the 5-HT_{1A} receptor. We also tested the ability of 5-CT to activate 5-HT_7 receptors by adding 5-CT in the presence of the 5-HT_{1A} receptor antagonists NAN-190 or WAY-100635. We observed a synaptic facilitation of similar

magnitude (52%) to that produced by 8-OH-DPAT (Fig. 8B (ii)), also suggesting that the facilitatory actions of 5-CT and 8-OH-DPAT are mediated by 5-HT_7 and not the 5-HT_{1A} receptor activation.

Receptors mediating 5-HT-evoked depression and facilitation

The 5-HT_{1A} receptor agonist 5-CT, like 5-HT, generally depressed the evoked synaptic responses (refer to Figs. 2 and 3). Therefore, we tested the possibility that the 5-HT_{1A} class of receptor mediates the depression evoked by 5-HT. In some neurons, we added 5-HT to evoke depression, then added the 5-HT_{1A} receptor antagonists NAN-190 or WAY-100635. In three neurons, the depression induced by 5-HT was partly reversed in the presence of these antagonists (Fig. 9A). However, in other neurons (n = 5), the antagonists had minimal effects on 5-HT-evoked depression, while drug washout reversed 5-HT's depressant actions (Fig. 9B). These results suggest that additional receptors contribute to mediating the 5-HT-evoked depression. Interestingly, in one neuron, neither CGS, 8-OH-DPAT, WAY-100635 nor clozapine altered the amplitude of the evoked response, while 5-HT caused a large depression even following co-application with NAN-190, the 5-HT_{1A} receptor antagonist (Fig. 9C), again suggesting that there are serotonergic receptors other than 5-HT_{1A} or 5-HT_{1B} receptors than can mediate the 5-HT-evoked depression. We did not explore the possibility of a contribution from 5-HT_3 receptors to the 5-HT induced depression because the actions of the 5-HT_3 receptor agonist CPBG (at 1 μM) were only modest and usually facilitatory (see discussion).

Since 5-HT_7 receptor activation facilitated synaptic responses, we tested whether the 5-HT-evoked facilitation observed in a small proportion of neurons could be 5-HT_7 receptor mediated. In these experiments, the 5-HT_7 receptor antagonist clozapine was added subsequent to 5-HT. Clozapine reversed the 5-HT-induced facilitation in two of four neurons (Fig. 10A), but had no effect on three neurons where 5-HT produced a synaptic depression (Fig. 10B). Thus, 5-HT_7 receptors appear to play a role in mediating 5-HT-evoked facilitation but not 5-HT-evoked depression. The contribution of 5-HT_2 receptors to a 5-HT induced synaptic facilitation was not examined but may have been responsible for the 5-HT induced facilitation in neurons that were insensitive to clozapine.

Discussion

Summary

This study characterized the actions of 5-HT and several reported receptor-selective ligands on the regulation of sensory synaptic input onto DDH neurons of the neonatal rat. In agreement with earlier studies (e.g. Randic and Yu, 1976; Lopez-Garcia and King, 1996; Lopez-Garcia, 1998), our results demonstrated that 5-HT produced a strong synaptic depression in most neurons. We show that this depression was only partly mediated by 5-HT_{1A} receptor activation, and the modest effects of reportedly selective 5-HT_{1B} and 5-HT_3 receptor agonists could not account for the remaining depression evoked by 5-HT, suggesting a contribution from other, currently untested 5-HT receptor subtypes (however, see later). In contrast, facilitatory actions could be observed in most neurons when 5-HT_7 receptors were selectively activated, suggesting that 5-HT's facilitatory actions are usually masked by a dominating synaptic depression produced by 5-HT from co-activation of other 5-HT receptor subtypes. In addition, with the exception of the 5-HT_{1B} receptor agonist CGS, the general effects of 5-HT receptor ligands in this study were similar within the first two weeks of postnatal development, and also compare to actions reported from earlier studies conducted in adult rats (e.g. Xu et al, 1994; Ali et al, 1996; Gjerstad et al, 1996,1997), suggesting that the function of these 5-HT receptor subtypes in the DDH remains relatively unchanged during postnatal development.

Synaptic Depression

Consistent with previous studies, primary afferent-evoked synaptic responses were usually depressed in the presence of the 5-$HT_{1A/7}$ receptor agonist 5-CT (also see, Lopez-

Garcia and King, 1996; Lopez-Garcia, 1998; Khasabov et al, 1999). Our studies strengthen the notion that the 5-CT-evoked depression is via 5-HT_{1A} receptor activation since the depressant action of 5-CT occurred in the presence of the 5-HT_7 receptor antagonist clozapine. Antagonism of the 5-HT_{1A} receptor with NAN-190 or WAY-100635 partly reversed a 5-HT-evoked depression in some neurons, further demonstrating that 5-HT_{1A} receptors depress synaptic responses. In the younger animals only, we observed that the 5-HT_{1B} receptor agonist, CGS also produced synaptic depression, albeit less potent and widespread than observed for 5-HT_{1A} receptor activation.

Previous studies in the rat indicated that 5-HT_{1A} and 5-HT_{1B} receptors account for 27% and 18% of high affinity 5-HT binding sites in the spinal cord respectively (Huang and Peroutka, 1987). These receptors are present on primary afferent terminals and dorsal horn neurons (Daval et al, 1987; Marlier et al, 1991b; Ridet et al, 1994; Laporte et al, 1995) and may constitute dominant receptor subtypes in the dorsal horn (see Marlier et al, 1991b). Interestingly, Huang and Peroutka (1987) reported that at least 33% of the total 5-HT binding sites in the rat spinal cord are distinct from 5-HT_{1A}, 5-HT_{1B} or 5-HT_{2C}. Since like 5-$HT_{1A\&B}$ receptors, the 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors are negatively coupled to adenylate cyclase (Barnes and Sharp, 1999), they are also likely to produce synaptic depression. 5-HT_{1D} and 5-HT_{1F} receptors are present in spinal cord dorsal horn of humans (Laporte et al, 1996; Castro et al, 1997) and 5-HT_{1D} receptors are present in the dorsal horn of the cat (Mills and Martin, 1995). Though not presently investigated, these receptors may also contribute to the widespread depressant action of 5-HT.

In this study, passive membrane properties of DDH neurons were generally unchanged (except for CGS in P10-14 group; discussed later), suggesting that modulatory actions occurred at the glutamatergic synapse itself, though a pre- and/or postsynaptic locus could not be determined from our studies. Numerous studies in other neurons have shown that 5-HT can depress transmitter release by presynaptic mechanisms (e.g. Sillar and Simmers, 1994; Hori et al, 1996; Travagli and Williams, 1996; El Manira et al, 1997). There is also evidence in support of a postsynaptic site for depressant actions. For example, Lopez-Garcia (1998) demonstrated that 5-HT-induced depression of NMDA responses in dorsal horn neurons is tetrodotoxin-resistant, suggesting a postsynaptic site of action (see also Murase et al, 1990). Thus, the depressant actions of 5-HT on primary afferent input to DDH neurons may involve both pre- and post-synaptic mechanisms.

Synaptic Facilitation

An interesting observation in this study is the facilitation of evoked synaptic responses by 8-OH-DPAT, the $5\text{-HT}_{1A \text{ and } 7}$ receptor agonist (Kennett, 1998). The additional observations that the 5-HT_7 receptor antagonist clozapine partly reversed the actions of 8-OH-DPAT and that facilitation of a similar magnitude was observed using 5-CT in the presence of 5-HT_{1A} receptor antagonists NAN-190 or WAY-100635, further suggest that these actions are mediated at the 5-HT_7 receptor. Moreover clozapine could block the 5-HT induced facilitation observed in some neurons. Thus, it is likely that 5-HT_7 receptors contribute to the facilitatory actions of 5-HT observed in some DDH neurons. Although the 5-HT_7 receptor has been identified in several CNS regions (e.g. Gustafson et al, 1996;

also see Eglen et al, 1997), its presence in the spinal cord has not been examined. Preliminary studies in our laboratory demonstrate 5-HT_7 receptor immunolabeling on neuronal cell bodies in the DDH, intermediate gray region and around the central canal (Hochman and Sawchuk; personal communication).

Our observation that 8-OH-DPAT preferably activates the 5-HT_7 receptor could explain reported inconsistencies between the actions of 5-CT and 8-OH-DPAT. For example, the enhancement of spinal reflexes in the rabbit by 8-OH-DPAT reported by Clarke et al (1997) and Ogilvie et al (1999) was assumed to be a possible combined action mediated at the 5-HT_{1A} and 5-HT_7 receptors.

Activation of the 5-$HT_{2A/2C}$ receptors with DOI facilitated evoked responses in only 5/16 neurons tested. This low frequency may be explained in relation to the relatively small percentage of 5-$HT_{2A/2C}$ receptors present in the dorsal horn of the rat (Pompeiano et al, 1994; Maeshima et al, 1998; Cornea-Hebert et al, 1999). The facilitation observed here is consistent with a facilitation of evoked excitatory synaptic responses in nearly 50% of dorsal horn neurons tested with higher concentrations of DOI (10 μM) reported by Hori et al (1996). 5-HT_2 receptor activation has been previously implicated in pronociceptive actions (e.g. Eide and Hole, 1991).

In addition to the 5-HT_7 and 5-HT_2 receptors, other receptor subtypes, which were not investigated in this study, may also contribute to the facilitatory actions of 5-HT. For instance, the 5-HT_4 and 5-HT_6 are positively coupled to adenylate cyclase and may also

tend to have facilitatory actions. These receptors are localized in various CNS regions (see Verge and Calas, 2000), but only 5-HT_6 receptor expression has been examined in the spinal cord, and its expression is weak (Gerard et al, 1997).

Unlike the metabotropic 5-HT receptors, the 5-HT_3 receptor is ionotropic and leads to the opening of a monovalent cation permeable channel. Reports from previous studies on the events mediated by 5-HT_3 receptor activation are conflicting. For instance, in relation to nociception, both pronociceptive (Ali et al, 1996; Oyama et al, 1996) and antinociceptive effects (Alhaider et al, 1991; Bardin et al, 1997; Giordano, 1997; Bardin et al, 2000) have been reported. Here, we observed that the high affinity 5-HT_3 receptor agonist CPBG (applied at 1 μM), produced facilitation of naïve synaptic response in most neurons, though of small magnitude, an observation consistent with an increase in number of evoked spikes in dorsal horn neurons of adult rats (Ali et al, 1996). This however opposes the attenuation of afferent-evoked neurotransmission observed by Khasabov et al (1999) in similarly aged animals when CPBG was applied at much higher concentrations (10 - 50 μM). Since the vast majority of 5-HT_3 receptors present in the spinal cord dorsal horn are found on primary afferent terminals (Hamon et al, 1989; Kidd et al, 1993; Laporte et al, 1995) where they can mediate a primary afferent depolarization (Khasabov et al, 1999), synaptic depression via presynaptic inhibitory mechanisms is expected. Even though 5-HT_3 receptors are also present on some dorsal horn neurons including GABAergic (Morales et al, 1998) and enkephalinergic interneurons (Tsuchiya et al, 1999), we found no evidence to suggest that CPBG mediated direct excitatory actions on the DDH neurons. We suggest that the lower concentrations of CPBG used here

preferentially activates receptors other than the 5-HT_3 receptor, and so our results using CPBG should be interpreted cautiously. For example, although CPBG is a potent high affinity 5-HT_3 receptor agonist (Kilpatrick et al, 1990), it has also been shown to interact with the catecholamines, facilitating the release of noradrenaline (Schlicker et al, 1994) and inhibiting the reuptake of dopamine (Campbell et al, 1995).

Endogenous release of 5-HT in the spinal slice

In the absence of applied agonists, the 5-HT_{1A} receptor antagonists NAN-190 and WAY-100635 typically produced modest facilitation of the evoked responses. Since there appears to be no serotonergic neurons intrinsic to the spinal cord, this observation suggests that even in the spinal cord slice preparation, the endogenously-released 5-HT from brainstem nuclei can tonically depress primary afferent input to DDH neurons at least partly via 5-HT_{1A} receptor activation (c.f. Wallis et al, 1993a & b). 5-HT has very high affinity for the 5-HT_{1A} receptor (Zifa and Fillion, 1992) and Hadjiconstantinou et al (1984) demonstrated that endogenous 5-HT remains in the spinal cord for more than a week following spinal transection. It is possible that low levels of endogenously released 5-HT serve to tonically regulate synaptic gain of primary afferent input.

Unexpectedly, in a few cases, NAN-190 alone produced a depression of the evoked response. We presume this action is mediated by blockade of tonic activation at the 5-HT_7 receptor, which binds NAN-190 with moderate affinity (see Hoyer et al, 1994). Interestingly, Zhuo and Gebhart (1991b) found that the descending serotonergic facilitation of nociception, abolished by bilateral transection of the dorsolateral funiculus,

was not attenuated by the 5-HT_2 or 5-HT_3 receptor antagonists, but by methysergide, a non-specific 5-HT receptor antagonist, concluding that the facilitation is mediated by a 5-HT_1 receptor. Rather, our findings indicate that tonic descending facilitation may be mediated by 5-HT_7 receptor activation, while tonic descending inhibition is mediated via the 5-HT_{1A} receptor.

Unlike the 5-$HT_{1A/7}$ receptors, there was no evidence to support tonic endogenous activation of the 5-$HT_{2A/2C}$ receptor, since ketanserin applied alone had no effect on naïve synaptic responses. These results are consistent with the higher affinity of 5-HT for 5-HT_{1A} and 5-HT_7 receptors over 5-HT_2 receptors (see Zifa and Fillion, 1992; Hoyer et al, 1994) and also the low expression of 5-$HT_{2A/2C}$ receptors in the dorsal horn (see references above).

A working hypothesis for interpretation of the actions of 5-HT receptor subtypes and ligands

There is considerable evidence to suggest that metabotropic 5-HT receptors positively coupled to signal transduction pathways facilitate synaptic responses, while those inhibiting signaling pathways exert synaptic depression (for review see Anwyl, 1990; Uphouse, 1997). Protein kinase A (PKA) facilitates activity at AMPA/kainate receptors (e.g. Wang et al, 1991; Raymond et al, 1993) and protein kinase C (PKC) facilitates AMPA/kainate and NMDA receptor mediated responses (e.g. Wang et al, 1994; Blank et al, 1996; Lu et al, 1999). If we apply these general findings to 5-HT's actions on glutamatergic sensory synaptic input, we would predict that activation of 5-HT_1 receptors

(↓PKA) should lead to depression whilst activation of 5-HT_2 (↑PKC) and 5-HT_7 receptors (↑PKA) should facilitate glutamatergic synaptic responses. It is therefore useful to interpret the observed actions of 5-HT receptor ligands in relation to their proposed activation of a specific receptor subtype.

Accordingly, the facilitatory action of 8-OH-DPAT is consistent with 5-HT_7 receptor activation and the depression evoked using 5-CT is consistent with activation of 5-HT_{1A} receptors. The actions of DOI were predominantly facilitatory, consistent with 5-HT_2 receptor activation and a link between DOI, the 5-HT_2 receptor and PKC mediated facilitation of glutamatergic responses has been reported previously in dorsal horn neurons (Hori et al, 1996). The CGS-mediated synaptic depression in the young animals is consistent with 5-HT_{1B} receptor activation but the facilitation in the older neonates would not be predicted to be 5-HT_{1B} receptor mediated. Since our CGS-mediated facilitatory actions were due partly to postsynaptic mechanisms (increased cell resistance) that conflict with observations that 5-HT_{1B} receptors only occur presynaptically on axon terminals (Verge and Calas, 2000; see also Wu et al, 1991), it is likely that CGS had its dominant action at a receptor other than 5-HT_{1B} in older animals. Previous studies that have demonstrated inhibitory actions of 5-HT_{1B} receptor agonists in the mature rat did not use CGS as the selective 5-HT_{1B} receptor agonist (e.g. Murphy and Zemlan, 1990; Ali et al, 1994; Xu et al, 1994; Gjerstad et al, 1997).

Relevance

Although 5-HT is implicated in the descending control of nociception, its potential analgesic benefit is limited by its widespread actions and complex pharmacology with different receptor subtypes capable of producing opposing modulatory actions. Our results demonstrate that despite the functional heterogeneity of 5-HT receptor subtypes and neurons within the DDH, pharmacological strategies may be employed to activate selective receptor subtypes with a broad neuromodulatory influence on spinal cord sensory function.

Table 1.

Effects of 5-HT receptor ligands on synaptic responses evoked in younger (P3-6) and older (P10-14) neonates. The actions of the ligands tested are indicated at left with the presumed receptor site of action indicated in brackets (+ prefix for agonists and - for antagonists). Neurons are divided into 3 categories, facilitation (%↑), depression (%↓) and no effect, and reported as mean percentage change in peak amplitude. The predominant action of each drug in each age group is shaded for clarification. Sample sizes are presented in brackets. Of all ligands tested, CGS and CPBG appeared to have different actions between the two age groups.

	P3-6				P10-14			
	% ↑	% ↓	No effect	Total (n)	% ↑	% ↓	No effect	Total (n)
5-CT/clozapine (+ 5-HT$_{1A}$) (n = 14)	-	**61%** (6)	(2)	**(8)**	-	*55%* (6)	-	**(6)**
CGS (+ 5-HT$_{1B}$) (n = 14)	-	**34%** (6)	(1)	**(7)**	**57%** (4)	16% (1)	(2)	**(7)**
DOI (+ 5-HT$_{2A/2C}$) (n = 16	28% (3)	20% (3)	(7)	**(13)**	65% (2)		(1)	**(3)**
CPBG (+ 5-HT$_3$) (n = 13)	**22%** (6)	-	(3)	**(9)**	13% (2)	24% (2)	-	**(4)**
8-OHDPAT (+ 5-HT$_7$) (n = 14)	**49%** (10)	56% (1)	(1)	**(12)**	**58%** (2)	-	-	**(2)**
Nan/Way (- 5-HT$_{1A}$) (n = 19)	**71%** (6)	38% (5)	(5)	**(16)**	**43%** (3)	-	-	**(3)**

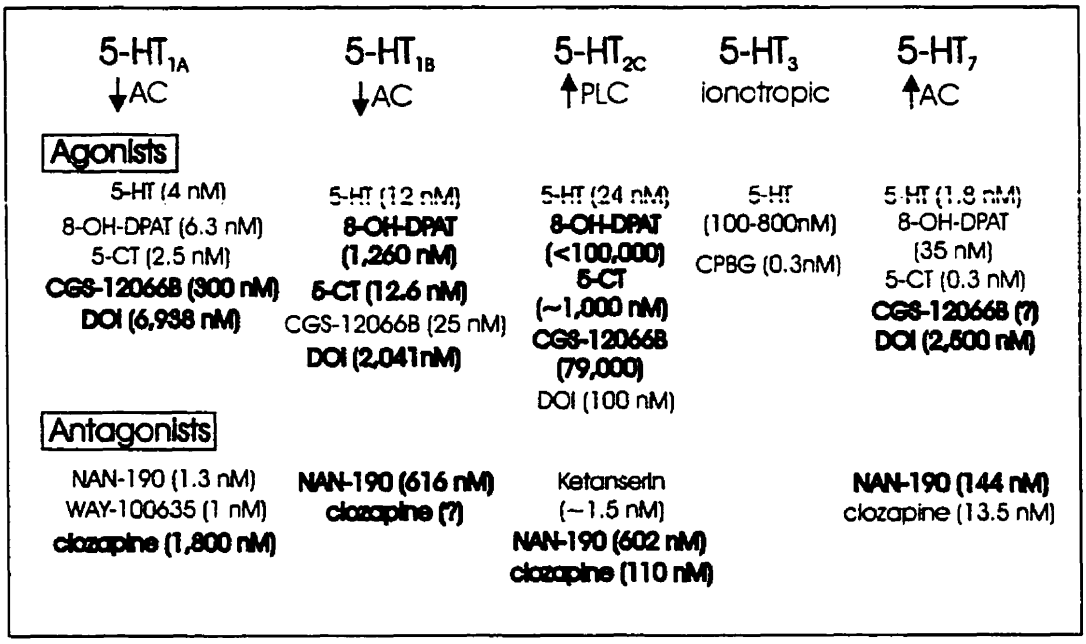

Figure 1: Relative binding affinities of the various 5-HT receptor ligands used in this study. The concentrations sited are the mean values (EC_{50} for agonists and IC_{50} for antagonists) calculated from all values reported in the following references: mentioned below:

Hoyer et al. *Pharmacol.Rev.* 46:157-203, 1994.

Kennet. *TOCRIS review article.* 1998.

Kuoppamaki et al. *Eur. J. Pharmacol.* 245:179-82, 1993.

Zifa & Fillion. *Pharmacol.Rev.* 44:401-458, 1992.

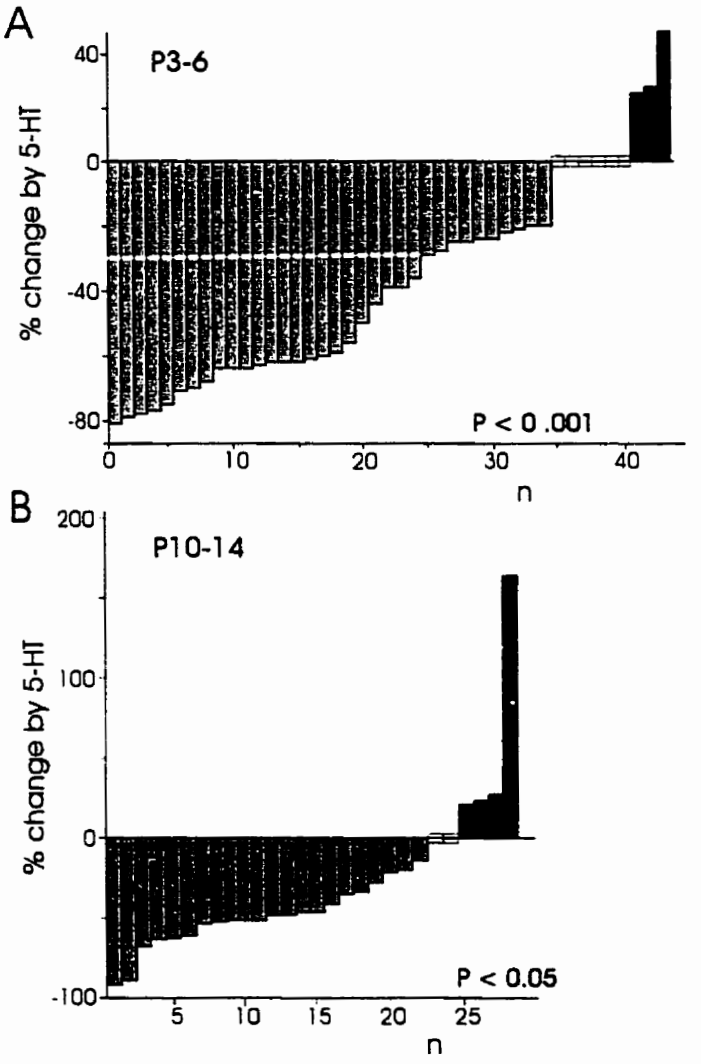

Figure 2: 5-HT generally depresses evoked synaptic responses in DDH neurons in P3-6 **(A)** and P10-14 animals **(B).** Synaptic depression is indicated as a negative % change (gray shaded bars) whilst facilitation is indicated as a positive % change (black bars). Neurons whose synaptic actions were unchanged by 5-HT are indicated by short open bars both (above and below the horizontal line at 0%). The 5-HT-evoked synaptic depression was significant at both age ranges as indicated.

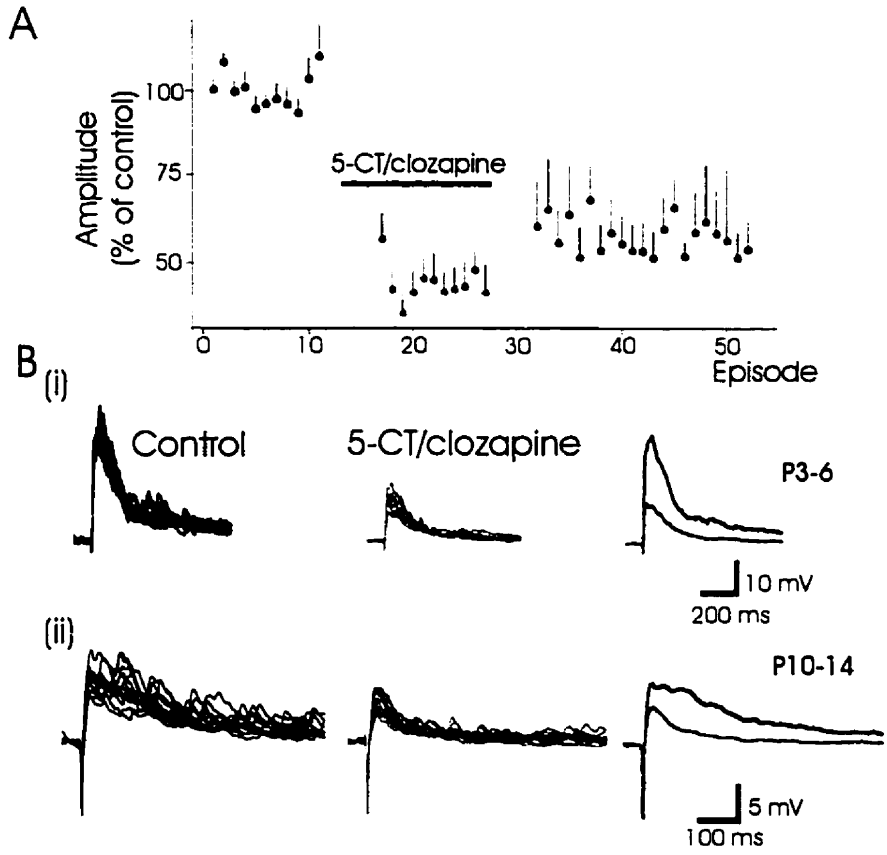

Figure 3: 5-HT_{1A} receptor activation depresses naïve synaptic responses. **A.** Normalized data showing the effect of 5-CT/clozapine on the amplitude of evoked synaptic responses in both older and younger animals combined (n = 12). The horizontal bar indicates the timing of the drug application. Note that the amplitudes of the synaptic responses are only partially reversed following washout of 5-CT/clozapine. The values for normalized data are presented as the mean + S.E. **B.** Examples showing the 5-CT/clozapine-induced depression of EPSPs observed in neurons obtained from P3-6 (63%) and P10-14 (50%) animals respectively. In this and the following similar figures, the individual raw traces of the control synaptic responses are superimposed in black, while the raw traces in the presence of the ligands are superimposed in gray. Averaged synaptic actions are presented to the right.

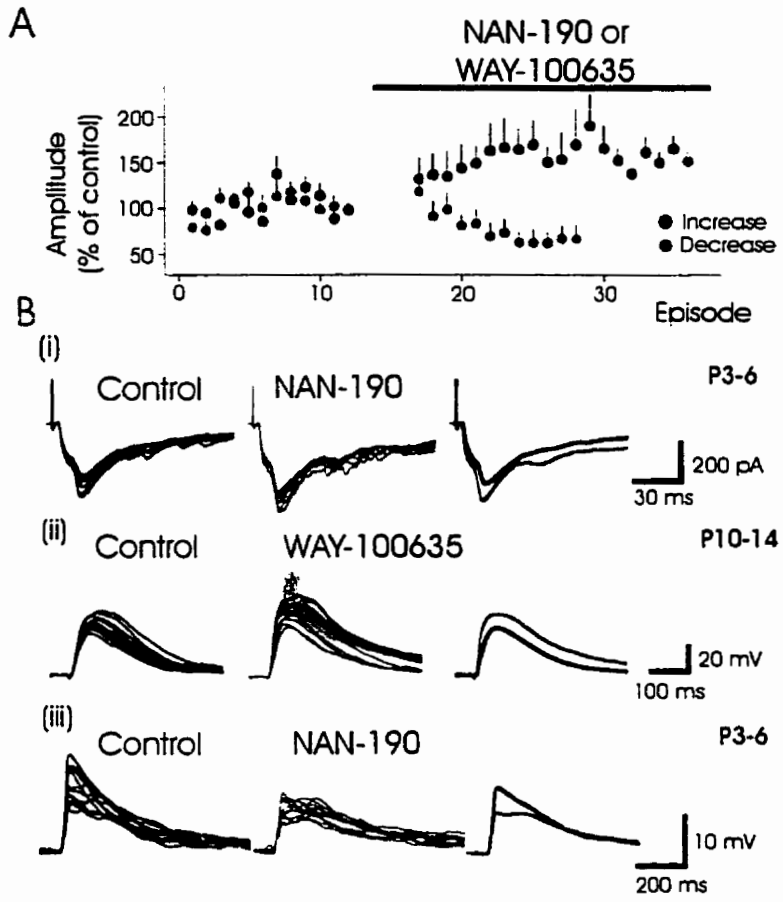

Figure 4: In the absence of agonist, 5-HT_{1A} receptor antagonists can facilitate or depress evoked synaptic responses. **A.** Normalized data showing the effects of NAN-190 or WAY-100635 on evoked synaptic responses. The antagonists could facilitate (black circles) or depress (gray circles) evoked synaptic responses. **B.** Examples showing 5-HT_{1A} receptor antagonist-induced synaptic facilitation in younger **(i)** and older **(ii)** animals. In addition to synaptic facilitation spikes were recruited in the presence of WAY-100635 in **(ii)** and they are presented as lighter shaded events in the raw traces. These events were not included in the analysis of the average EPSP amplitude increase at right. **B(iii).** Example of synaptic depression produced by NAN-190. Percentage change in peak synaptic response in the presence of the ligands were 27%↑, 29%↑ and 37%↓, respectively.

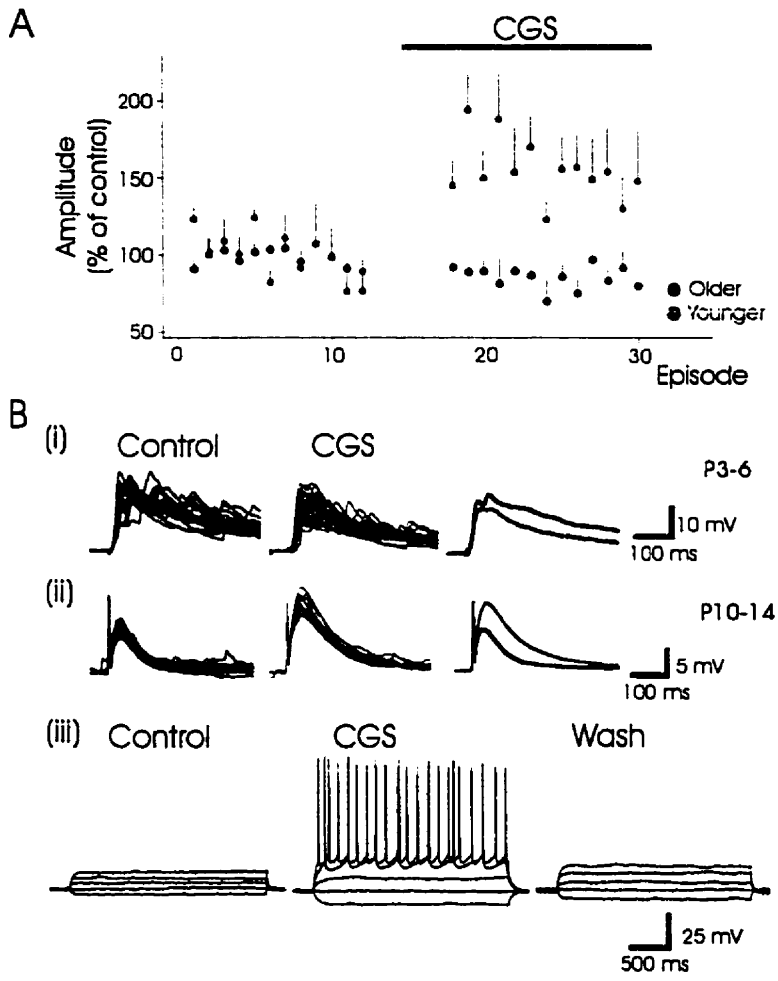

Figure 5: The 5-HT_{1B} receptor agonist CGS produces both depression and facilitation of synaptic responses in DDH neurons. **A.** Normalized data showing a modest CGS-induced EPSP depression in younger animals (black circles; n = 6), while facilitating EPSPs in older animals (gray circles; n = 4). **B(i)** and **(ii)** show examples of CGS-induced depression (24%) and facilitation (67%) of EPSPs respectively. **B(iii).** The CGS-induced EPSP amplitude increase in **(ii)** is associated with a reversible increase in neuronal input resistance (160 MΩ in control; 330 MΩ in CGS) and a decrease in rheobase (70 pA in control; 50 pA in CGS). Responses presented arise from 30 pA depolarizing and hyperpolarizing current steps.

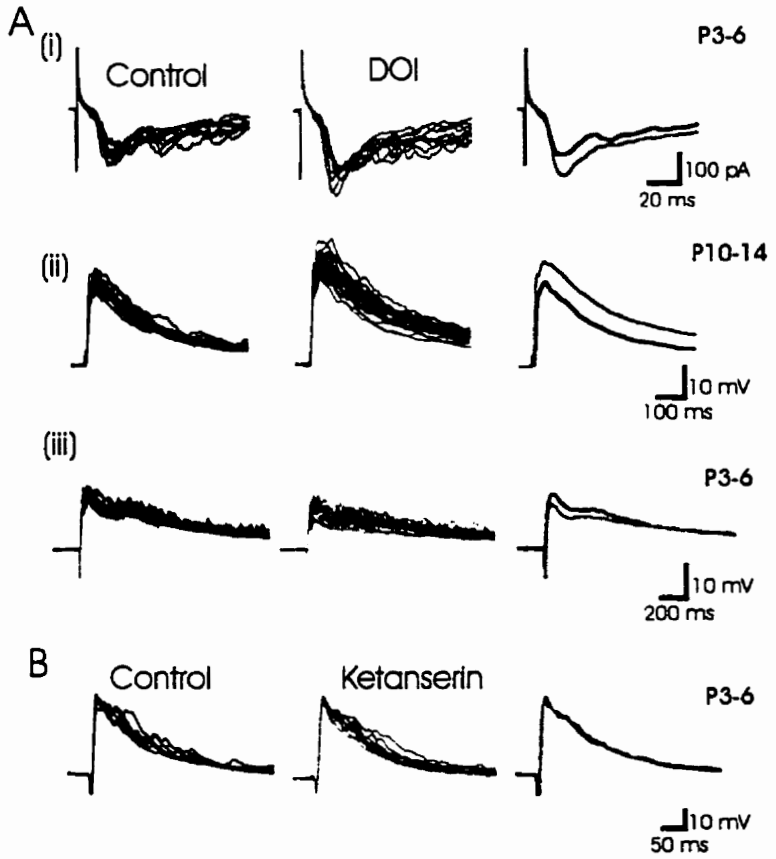

Figure 6: The 5-$HT_{2A/2C}$ receptor agonist DOI facilitates or modestly depresses evoked synaptic responses in DDH neurons. **A.** DOI induces synaptic facilitation in neurons obtained from both P3-6 (**i;** voltage clamp) and P10-14 (**ii;** current clamp) animals. The magnitudes of facilitation are 47% and 18%, respectively. **A(iii).** DOI depresses (20%) the EPSP evoked in a neuron obtained from P3-6 animal. **B.** In the absence of agonist, the 5-$HT_{2A/2C}$ receptor antagonist, ketanserin has no effect on the evoked synaptic responses.

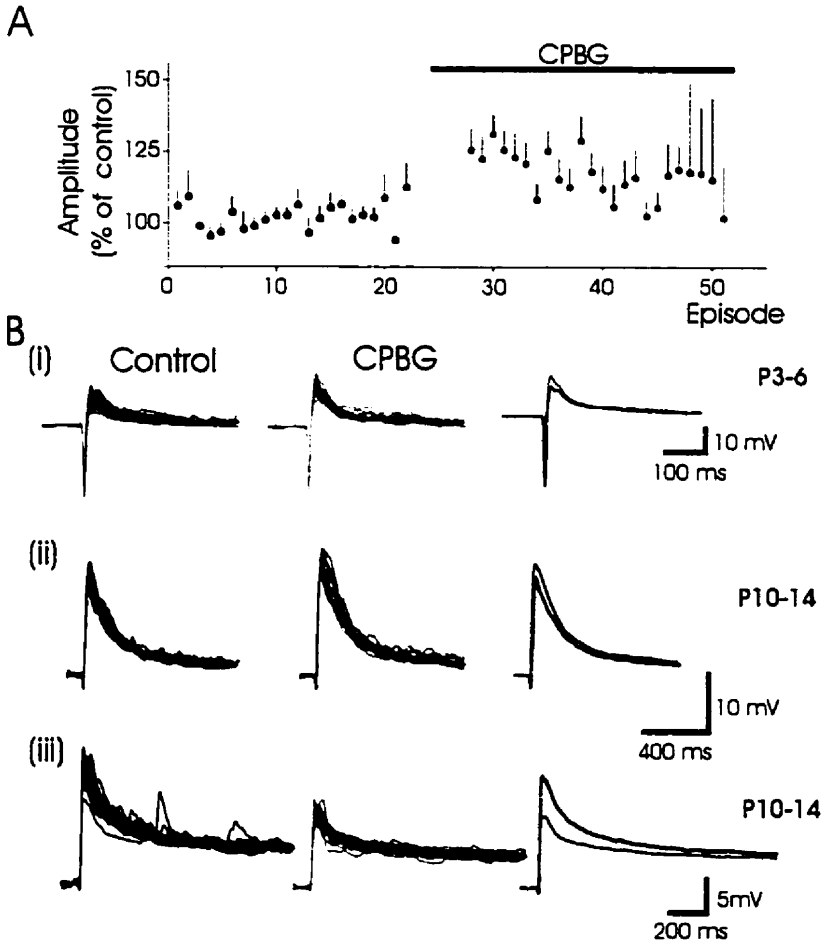

Figure 7: The 5-HT$_3$ receptor agonist CPBG facilitates synaptic responses in the younger animals, but produces both facilitation and depression in neurons obtained from older animals. **A.** Normalized data showing the modest facilitatory actions of CPBG on neurons obtained from both younger (n = 6) and older (n = 2) animals. **B(i)** and **(ii).** Examples showing synaptic facilitation obtained in neurons obtained from both young (29%) and older (15%) animals, respectively. **B(iii)** shows an example of depression of EPSPs (35%) evoked in an older animal.

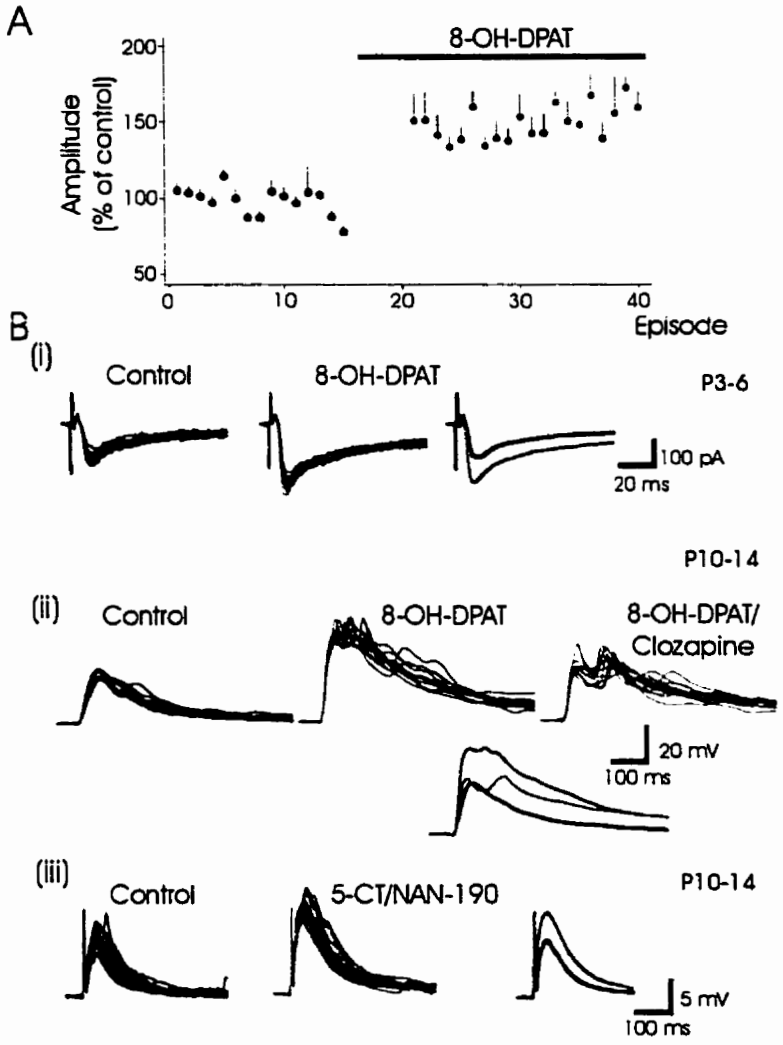

Figure 8: 5-HT, receptor activation mediates synaptic facilitation. **A.** Normalized data demonstrating synaptic facilitation (n = 12) evoked by 8-OH-DPAT in neurons obtained from both younger and older animals. **B(i).** An example of 8-OH-DPAT-induced facilitation (58%) of evoked EPSC. **(ii).** The facilitation of the early EPSP peak produced by 8-OH-DPAT (62%) is almost completely reversed by clozapine, the 5-HT$_7$ receptor antagonist. **B(iii).** Application of the 5-HT$_{1A/7}$ receptor agonist 5-CT in the presence of the 5-HT$_{1A}$ receptor antagonist NAN-190 also facilitated EPSP amplitude (52%).

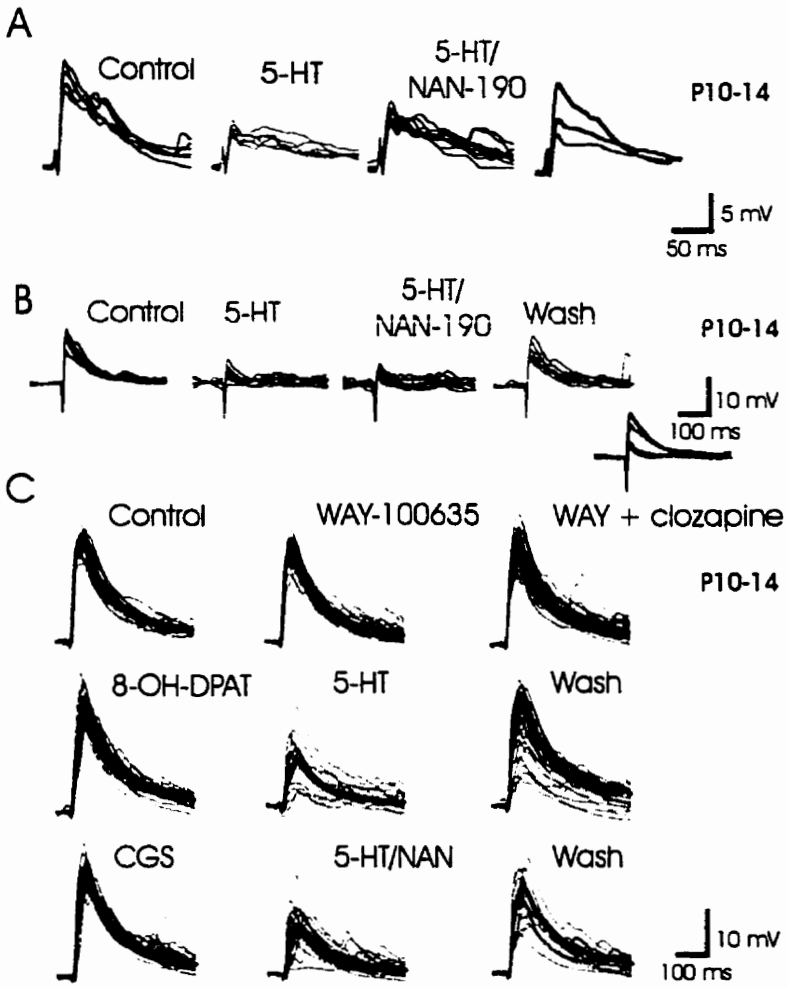

Figure 9: Unidentified receptors mediate part of the depressant actions of 5-HT. **A.** 5-HT-evoked EPSP depression is partly reversed in the presence of the 5-HT_{1A} receptor antagonist NAN-190. **B.** NAN-190 has no effect on 5-HT-evoked depression while washout of 5-HT leads to a near complete recovery of synaptic amplitude. **C.** WAY-100635, clozapine, 8-OH-DPAT, or CGS has no effect on the evoked synaptic responses. However, 5-HT produces potent depression of the EPSP, which remains unchanged by NAN-190, suggesting that 5-HT receptors other than 5-HT_{1A}, 5-HT_{1B} or 5-HT_7 are mediating the depressant action of 5-HT. In **C** only, the individual traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace.

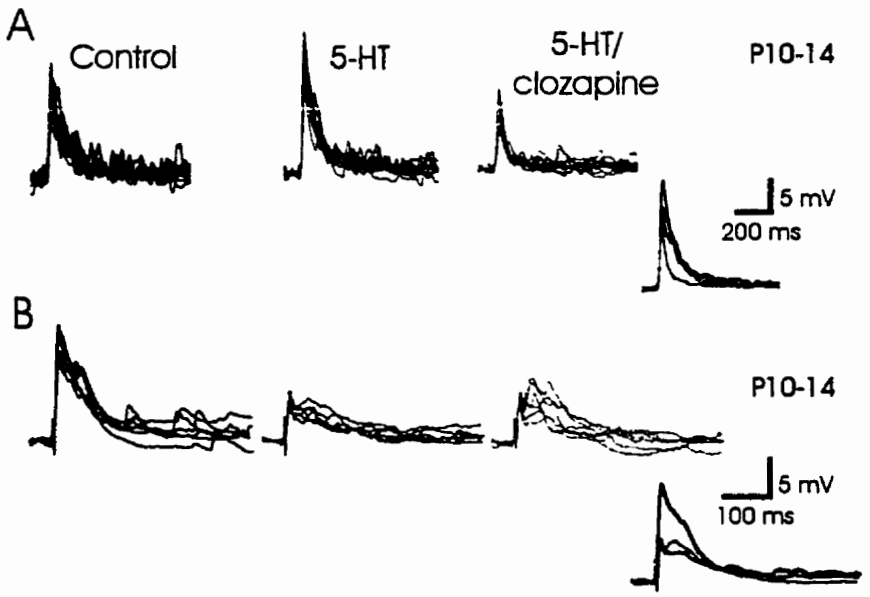

Figure 10: 5-HT, receptor antagonist partly reverses 5-HT-evoked facilitation, but not 5-HT-evoked depression of evoked EPSPs. **A.** The 5-HT-induced facilitation (35%) of EPSP amplitude in a neuron obtained from an older animal is blocked by clozapine, allowing 5-HT to produce synaptic depression, presumably by actions on other receptor subtypes. **B.** Clozapine does not remove the synaptic depression evoked by 5-HT.

Project III - Serotonin increases the incidence of primary afferent-evoked long-term depression in rat deep dorsal horn neurons

Abstract

5-hydroxytryptamine (5-HT) is released in spinal cord by descending systems that modulate somatosensory transmission, and can potently depress primary afferent-evoked synaptic responses in dorsal horn neurons. Since primary afferent activity-induced long-term potentiation (LTP) may contribute to central sensitization of nociception, we studied the effects of 5-HT on the expression of sensory-evoked LTP and long-term depression (LTD) in deep dorsal horn (DDH) neurons.

Whole-cell recordings were obtained from DDH neurons in transverse slices of neonatal rat lumbar spinal cord. High intensity dorsal root stimuli evoked monosynaptic excitatory synaptic responses in most neurons that were blocked by CNQX and APV supporting a glutamatergic synaptic activation.

The effect of 5-HT on the evoked synaptic response was tested before, during or after high frequency conditioning stimulation (CS). In most cells (80%), 5-HT caused a depression of the naïve synaptic response. Even though 5-HT depressed evoked responses, CS in the presence of 5-HT was not only still capable of inducing long-term depression (LTD), but also increased its incidence from 54% in controls to 88% ($p < 0.001$). Selective activation of 5-HT_{1A} and 5-HT_{1B}, but not $5\text{-HT}_{2A/2C}$ or 5-HT_3 receptors, best reproduced these actions. 5-HT also potently depressed post-conditioning synaptic responses, regardless of whether the induced plasticity was LTP or LTD.

Our results demonstrate that, in addition to depressing the amplitude of evoked sensory input, 5-HT can also control the direction of its long-term modifiability, favoring the expression of LTD. These findings demonstrate cellular mechanisms that may contribute to the descending serotonergic control of nociception.

Introduction

The spinal cord dorsal horn represents a nodal point for the integration of sensory information. Many studies have investigated the multi-sensory convergent properties of dorsal horn neurons of various species, particularly in relation to nociceptive input.

High intensity electrical stimulation of primary afferents recruits nociceptors (Aδ and C) and synaptically activates neurons in the dorsal horn (e.g. Jeftinija and Urban, 1994; Miller and Woolf, 1996). Repetitive activation of these afferents can induce alterations in spinal integrative properties that encode persistent changes in nociception. One such change is expressed as increases (LTP) or decreases (LTD) in synaptic strength. LTP and LTD have been observed in the dorsal horn (Garraway et al, 1997; Liu and Sandkühler, 1995; Liu et al, 1998; Pockett, 1995; Randic et al, 1993; Sandkühler and Liu, 1998; Svendsen et al, 1997). These synaptic modifications probably occur at glutamatergic synapses since N-methyl-D-aspartate (NMDA) receptor activation is required for the induction of LTP (Liu and Sandkühler, 1995; Randic et al, 1993; Svendsen et al, 1998).

Sandkühler and Liu (1998) demonstrated that natural activation of nociceptors in skin induced LTP of C-fibre evoked field potentials in dorsal horn, but only following spinalization, suggesting a potent inhibitory control from descending systems. Additionally, Liu et al (1998) demonstrated that Aδ-fibre mediated LTD of C-fibre evoked field potentials could be switched to LTP following spinalization. Thus, descending systems appear to be able to control both the induction and direction of the evoked synaptic plasticity. An identification of the mechanisms controlling synaptic

plasticity is of considerable interest. For instance, the Aδ fibre-induced LTD of nociceptor afferents in spinal cord is blocked with μ-opioid receptor antagonists (Zhong and Randic, 1996) potentially linking LTD to opioid-induced analgesia. It is thus reasonable to hypothesize that synaptic plasticity participates in the physiological encoding of altered nociceptive states (e.g. hyperalgesia and allodynia).

Numerous alterations in spinal synaptic/cellular properties are observed following application of 5-HT. In dorsal horn neurons, 5-HT generally depresses primary afferent-evoked synaptic responses (Headley et al, 1978; Jordan et al, 1979; Khasabov et al, 1999; Lopez-Garcia, 1998; Lopez-Garcia and King, 1996; Randic and, Yu 1976) though facilitation has also been observed (Jordan et al, 1979; Lopez-Garcia and King, 1996). Although many studies have demonstrated that 5-HT exerts antinociceptive actions in the spinal cord, (for reviews see Eide and Hole, 1993; Hammond, 1986; Millan, 1995), details of its mechanism of action and receptor pharmacology remain incomplete.

We hypothesize that one function of descending serotonergic systems is to control the expression of activity-dependent synaptic plasticity within the spinal cord. We sought to determine the effects of 5-HT and its receptor selective ligands on the induction and maintenance of evoked synaptic plasticity in DDH neurons (laminae III-VI). This was undertaken in a spinal cord slice preparation capable of evoking primary afferent-induced LTP and LTD (Garraway et al, 1997). We demonstrate that 5-HT receptor activation, in particular the 5-HT_{1A} and 5-HT_{1B} receptors, promote the induction of LTD in DDH

neurons. Preliminary data was presented previously (Garraway and Hochman, 1997 & 1998).

Material and Methods

Preparation of spinal slices

All experimental procedures complied with the Canadian Council of Animal Care guidelines. Neonatal rats (Sprague-Dawley postnatal days 3-6) were decapitated and spinal segments L2 - S1 were removed. The isolated spinal cord was embedded in Agar, 2.5% w/v, (Type E, Sigma) and sliced on a vibrating blade microtome in 500-600 μm transverse sections (Leica VT1000S or Pelco 101) in cooled (4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM); NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 25; NaH_2PO_4, 1.25; $NaHCO_3$, 26; at a pH of 7.4. Short dorsal rootlets remained attached to the spinal segments to allow for electrical stimulation of primary afferents.

Slices were then incubated at 32°C for at least 1 hour in ACSF. For experimentation, spinal cord slices were affixed to a recording chamber using platinum U-frames with a parallel array of nylon fibres glued across (Edwards et al, 1989). Patch electrodes were prepared from 1.5 mm outer diameter capillary tubes (Precision Instruments or Warner) pulled in a two-stage process (Narishige PP83) producing resistance values ranging from 4-7 MΩ with recording electrodes containing (in mM): K-gluconate, 140; EGTA, 0.2; HEPES, 10; Mg-ATP, 4; GTP, 1; pH 7.3. In most experiments 2mM, QX-314 (RBI) was added to the recording solution to block voltage-dependent Na^+ channels. The recording chamber was continuously superfused with oxygenated ACSF at a rate of ~2 ml/minute.

Electrophysiology

The whole-cell 'blind' patch clamp recording technique (Blanton et al, 1989) was undertaken at room temperature (~20°C) using the Axopatch 1D amplifier (Axon Instruments) filtered at 5 kHz (4-pole low-pass Bessel). Voltage and current clamp data were acquired on computer with the pCLAMP acquisition software (v 6.0; Axon Instruments). Immediately following rupture of the cell membrane (in voltage clamp at – 94 mV), the current clamp-recording configuration was used to determine resting membrane potential. Series resistance was subtracted in current clamp mode (bridge balance) and junction potentials were measured and accounted for. A minority of experiments was undertaken in voltage clamp mode. In these experiments series resistance remained uncompensated. To ensure reliable recording from healthy neurons, for the duration of the experiment, leak conductance and bridge balance were carefully monitored; if their values were largely unaltered, the experiments were continued. Mean electrode series resistance was 45.3 ± 13 MΩ (S.D.).

Primary afferent stimulation

In order to compare the effects of 5-HT and CS on evoked synaptic responses, postnatal day 3-6 neonates were used since both LTP and LTD are evocable in DDH neurons in transverse slices at this age (Garraway et al, 1997). One problem with these early neonates however, is that myelination of many afferent fibres is incomplete (Friede and Samorajski, 1968; see also Fitzgerald, 1985). Hence, we observed that only 13% of cells received synaptic responses at primary afferent stimulation intensities lower than 500μA, 100μs. Sixty nine percent of neurons were observed to have synaptic events recruited at

intensities ranging from 500μA, 100μs to 500μA, 500μs while the remaining 18% of neurons had their first synaptic events recruited at intensities higher than 500μA, 500μs. Thus, we used high stimulation intensities in order to recruit the highest threshold unmyelinated afferents, and hence, the majority of afferent fibre types, irrespective of age (typically ≥ 500 μA, 500 μs; see Thompson et al, 1990).

Generally, the evoked synaptic responses were first characterized as excitatory by determining their reversal potential, prior to collection of baseline events. This was accomplished by recording primary afferent evoked synaptic responses at holding potentials ranging from –90 mV to +30 mV (Fig. 2D). Inhibitory synaptic responses were not included in this study. To further characterize the excitatory synaptic responses, in some cells, the ionotropic glutamate receptor antagonists CNQX (10 – 20 μM) and D,L-APV (50 μM), obtained from RBI/Sigma, were added to determine whether the evoked synaptic responses were mediated by AMPA/kainate and NMDA receptors respectively.

Baseline synaptic responses were recorded for 10-25 minutes by stimulating dorsal rootlets at low frequency (generally every minute) at a holding potential of –94 mV. This was followed by a high frequency CS (five 100 Hz tetani of 1 sec duration at 5 sec intervals), often at a higher intensity stimulation, at a membrane potential of –54 mV, approximately at a cell's resting membrane potential. Unlike the hippocampus, the DDH is heterogeneous in nature thereby making it more difficult to define individual stimulus protocols that reliably elicit LTD or LTP. However, high frequency electrical stimulation of the dorsal roots (Randic et al, 1993) or the dorsomedial white matter (Pockett, 1995)

has been previously demonstrated to induce both LTD and LTP in the spinal cord. Following CS, the synaptic response was then recorded at the pre-conditioning baseline intensity, frequency, and at the same membrane potential. Synaptic plasticity, LTP or LTD, was defined as a ≥ 20% change in amplitude maintained for at least 20 minutes post CS and always for the duration of the recording.

Application of ligands

5-HT was applied at 10 μM (in 100 μM ascorbic acid, an antioxidant). The following 5-HT receptor ligands were used: (i) 5-carboxamidotryptamine (5-CT) in the presence of the 5-HT_7 receptor antagonist clozapine, for selective activation of $5HT_{1A}$ receptors; (ii) 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline maleate (CGS) to activate 5-HT_{1B} receptors; (iii) 1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI) to activate 5-HT_2 receptors, and; (iv) 1-(m-chlorophenyl)-biguanide (CPBG) for activation of 5-HT_3 receptors. Ligands were applied at 1 μM. All drugs were purchased from RBI (Natick, MA).

The relationship between primary afferent-evoked responses and 5-HT application was studied using the three paradigms outlined in Figure 1. These three experimental procedures were used to evaluate the effects of 5-HT on the 'naïve' synaptic response, the post conditioning response, and the induction of synaptic plasticity respectively. To determine the effects of specific activation of 5-HT receptor subtypes on the induction of synaptic plasticity, all experiments involving specific 5-HT receptor ligands were conducted as outlined in Figure 1C.

Recordings were analyzed using pCLAMP (v 6.0, Axon Instruments). The maximum amplitude of the synaptic response of individual traces was measured. Figures were constructed using Sigma Plot (Jandel Scientific) and/or CorelDRAW (Corel). Values are reported as mean + S.E. in Figs. 3-5 and mean ± S.D in text.

Results

A total of 109 DDH (laminae III-VI) neurons were recorded having a mean resting membrane potential of –58 ± 10 mV and input resistance of 635 ± 358 MΩ. The location of a subpopulation of neurons where the topographic location was mapped is presented in Figure 2A.

Characterization of primary afferent-evoked synaptic responses in the DDH

We used an invariable synaptic delay as an indicator of a monosynaptic linkage (Fitzgerald and Wall, 1980). Variability in EPSP latency in a subpopulation of neurons is presented (Fig. 2B(i)) with representative individual values also provided (Fig. 2B(ii)). Generally, synaptic events whose onset occurred before 6 ms following the stimulus artefact had relatively constant latencies. At room temperature, synaptic delay in spinal cord slices from rats in the present age range is ~3 ms (Takahashi, 1992; Jonas et al, 1998) suggesting a minimal value of 6 ms for disynaptic actions. Hence, it is probable that synaptic responses with latencies <6 ms were evoked monosynaptically, originating directly from primary afferents. These were the majority of responses.

Figure 2C depicts representative synaptic responses recorded from DDH neurons. Evoked excitatory postsynaptic potentials (EPSPs) were observed to have 3 general appearances; single peak with slow decay (C(i)), an early and late peak (C(ii)), and EPSPs with fast rate of rise and decay (C(iii)). The longer-latency synaptic responses are APV sensitive (C(i)) and thus due to activation of NMDA receptors while the early response is CNQX-sensitive, due to AMPA/kainate receptor activation (C(ii) and C(iii)).

Application of CNQX and APV largely blocked evoked responses in 8 of 9 cells tested. Thus, primary afferent-evoked responses in the neonatal DDH are predominantly glutamatergic. To further characterize primary afferent evoked responses as excitatory, we determined the reversal potential of the synaptic responses. Evoked responses were generally reversed at membrane potentials close to 0 mV, thereby confirming that they are excitatory (Figure 2D).

Effects of 5-HT on evoked synaptic responses

5-HT did not significantly alter resting membrane potential or cell input resistance. However, 5-HT significantly ($p < 0.01$; Student's t-test) decreased the peak amplitude of evoked excitatory synaptic responses in 37 of 46 cells (Table 1). This depression was largely reversible following drug washout (88 ± 28% of initial amplitude, tested in 17/37 neurons). 5-HT also increased EPSP amplitude in three neurons, while the remaining 6 neurons were relatively unaffected by 5-HT (Table 1). The percent change in peak amplitude corresponded well with changes in synaptic charge transfer calculated as the integral of the synaptic response (area under the curve). For the cells depressed by 5-HT, both the early (presumably AMPA/kainate) and longer latency (presumably NMDA) synaptic components were equally depressed. For example, the area under the curve of the EPSP was depressed identically for synaptic events occurring < 200 ms to those ≥ 200 ms. Hence, hereafter only peak amplitude values were compared.

Relationship between 5-HT's effect on the 'naïve' synaptic response and synaptic plasticity

In 9 of 10 neurons, 5-HT depressed evoked responses which then returned to baseline amplitude following drug withdrawal (to 101 ± 27%; also see Fig. 3). Thereafter, following CS, evoked synaptic responses could be observed to undergo LTD (n = 5; avg. of 45%↓) or LTP (n = 3; avg. of 153%↑) suggesting that the effect of 5-HT in a given cell is independent of the type of synaptic plasticity evoked (Fig. 3A and B respectively). The average magnitude of synaptic depression caused by 5-HT (55%) was very similar to the average magnitude of LTD produced following high frequency CS (58%).

Effects of 5-HT on the post-conditioned synaptic response

In 10 cells, 5-HT was applied after CS, following collection of the post-conditioning baseline response (Fig. 4). Of these cells, 5-HT depressed the amplitude of the conditioned response in 8 of 10 neurons by an average of 62%. This occurred regardless of the conditioning-evoked response in these neurons; two underwent LTP (Fig. 4A), three underwent LTD (Fig. 4B) and 3 were unaffected by CS (not illustrated). Thus, 5-HT can cause depression in addition to CS-induced LTD and can also depress a potentiated synaptic response. After 5-HT washout, evoked responses returned to 76% of their pre-5-HT values (7 cells).

Effects of 5-HT on the induction of synaptic plasticity

In 16 cells, following a 10-15 minute baseline of evoked responses, 5-HT was applied and its action recorded for an additional 10-15 minutes. These cells then underwent CS in

the continued presence of 5-HT and for at least 20 minutes post conditioning (Fig. 5). In the presence of 5-HT, 88% of cells underwent CS-induced LTD. In 11 of the 14 cells undergoing LTD following CS, the naïve synaptic response was already depressed by pre-applied 5-HT (48%↓). These cells underwent an additional 51% depression following CS in the presence of 5-HT. Thus, LTD could be induced on top of, and in addition to, the depression evoked by 5-HT. Table 3 compares the incidence of CS-induced LTP and LTD observed in the absence and presence of 5-HT. Significantly, CS of primary afferents in the presence of 5-HT caused an increased incidence of LTD compared to controls (cells conditioned in the absence of 5-HT) from 54% to 88% (χ^2; $p < 0.001$; see Table 2). The presence of 5-HT however, did not affect the magnitude of the LTD produced. The average CS-induced LTD was 54 ± 24% in the presence of 5-HT and 58 ± 24% in the controls.

Effects of 5-HT receptor agonists on naïve synaptic responses

Serotonin mediates its effect through various classes of receptors, many of which are located in the spinal cord. To determine some of the 5-HT receptors involved in 5-HT-induced alterations of evoked synaptic responses in DDH neurons, we compared the effects of ligands specific to 5-HT_{1A}, 5-HT_{1B}, $5\text{-HT}_{2A/2C}$ and 5-HT_3 receptors on the naïve synaptic responses. Like 5-HT, none of the ligands had any significant effect on the resting membrane potential or measured input resistance of the cells. Table 1 summarizes the effects of these agonists on EPSPs. Briefly, while selective activation of the 5-HT_{1A} receptor produced depressant actions similar to those observed for 5-HT, modulatory actions at 5-HT_{1B}, 5-HT_2 and 5-HT_3 receptors were modest.

Effects of 5-HT receptor agonists on the incidence of synaptic plasticity

The effects of selective 5-HT receptor ligands on the induction of synaptic plasticity was conducted using the experimental paradigm previously outlined in Figure 1C (cf. Fig. 5). The effects of these ligands on the induction and incidence of plasticity are summarized in Table 3. CS of primary afferents during selective 5-HT_{1A} receptor activation (with 5-CT/clozapine) induced only LTD (Fig. 6A). Similarly, CS induced LTD in all three neurons tested during 5-HT_{1B} receptor activation (with CGS; Fig. 6B). In comparison, CS of primary afferents in the presence of agonists at 5-HT_2 or 5-HT_3 receptors (DOI and CPBG respectively) produced both LTP and LTD (Fig. 7).

Discussion

The ability of descending serotonergic systems to depress synaptic transmission in the dorsal horn provides for control of sensory transmission at the first CNS site of synaptic integration. Hence, it is important to elucidate the manner in which sensory synaptic transmission is regulated in the DDH, particularly since this spinal cord region has the greatest longitudinal spread of nociceptor-induced activity (Coghill et al, 1991; Mao et al, 1993; Porro et al, 1991). Our experiments have compared the effects of 5-HT and specific 5-HT receptor ligands on CS-induced synaptic plasticity. Consistent with previous studies, 5-HT generally depressed primary afferent-evoked naïve synaptic response within neurons of the spinal dorsal horn (Jordan et al, 1979; Khasabov et al, 1999; Lopez-Garcia, 1998; Lopez-Garcia and King, 1996; Randic and Yu, 1976). Following washout of 5-HT, CS of primary afferents could induce LTP or LTD indicating that there was no association between the effect of 5-HT in a given neuron, and the direction of induced plasticity. 5-HT also depressed synaptic responses following CS-induced LTP or LTD. Thus, 5-HT can further depress synaptic responses which have already undergone LTD, and can potently depress potentiated synapses. Of particular importance, CS of primary afferents in the presence of 5-HT significantly increased the incidence of LTD, indicating that 5-HT can alter the direction of plasticity, strongly favoring LTD. Given the correspondence of nociceptor activity to the induction of LTP (Liu et al, 1998; Sandkühler and Liu, 1998), these results suggest that 5-HT may prevent the induction of nociceptor-induced LTP as well as depress existing 'sensitized' (potentiated) synapses. In an intact system, descending serotonergic systems may use both methods to attenuate somatosensory input.

Possible mechanism for 5-HT-induced increases in LTD

A critical trigger underlying virtually all forms of synaptic plasticity is related to changes occurring in the concentration of postsynaptic calcium (Ca^{2+}) (Lisman, 1989; Artola and Singer, 1993). The "calcium hypothesis" proposes that a large postsynaptic Ca^{2+} influx favors LTP whereas moderate increases in postsynaptic Ca^{2+} favor LTD. For example, in the hippocampus, Cummings et al (1996) showed that brief tetanic stimulation (which normally produced LTP) is able to elicit LTD if NMDA channels were partially blocked by moderate concentrations of D-APV or cells were voltage clamped at hyperpolarized potentials, thereby limiting postsynaptic Ca^{2+} influx. The induction of LTP and LTD in the spinal cord may also depend on the magnitude of evoked increases in Ca^{2+} levels (Randic et al, 1993). Since 5-HT did not significantly alter resting membrane potential in our study, we propose that 5-HT is capable of reducing the level of postsynaptic Ca^{2+} influx by depressing voltage-dependent Ca^{2+} channel activity. This in turn causes only moderate increases in Ca^{2+} and hence would tend to induce LTD rather than LTP. There are numerous examples of an inhibition of Ca^{2+} channels by 5-$HT_{1\text{-like}}$ receptors (e.g. Scroggs and Anderson, 1990; for review see Anwyl, 1990).

5-HT receptor subtypes and synaptic plasticity

Pharmacological experiments demonstrated that activation of the 5-HT_{1A} and 5-HT_{1B}, but not the 5-$HT_{2A/2C}$ or 5-HT_3 receptors, best compared to the effects of 5-HT in supporting LTD. LTP was never produced following CS in the presence of these 5-HT_1 receptor ligands. It is not surprising that agonists of both 5-HT_{1A} and $_{1B}$ receptors depress naïve synaptic responses and produce CS-induced LTD. Both subtypes are negatively coupled

to adenylate cyclase activity and have been previously associated with synaptic depression throughout the CNS (see Anwyl, 1990). 5-HT_{1A} and 5-HT_{1B} receptors account for 27% and 18% of high affinity 5-HT binding sites in the spinal cord, respectively (Huang and Peroutka, 1987), and are present on both primary afferent terminals and postsynaptic dorsal horn neurons (see Daval, 1987) suggesting that pre- and/or postsynaptic mechanisms contribute to synaptic depression. Since we failed to observe any significant changes in intrinsic properties of the postsynaptic cell in the presence of 5-HT and receptor selective ligands, depressant actions probably occur at the glutamatergic synapse (see Lopez-Garcia, 1998). Like 5-$HT_{1A \text{ and } 1B}$ receptors, the 5-$HT_{1D\text{-}F}$ receptors also negatively couple to adenylate cyclase and hence, may also mediate synaptic depression. However, details of these receptor subtypes are not well known (Barnes and Sharp, 1999).

The proposed mechanism of 5-HT's depressant action (see above) is consistent with the effects mediated by the 5-HT_{1A} and $_{1B}$ receptors. There are numerous examples of an inhibition of Ca^{2+} channels by 5-$HT_{1\text{-like}}$ receptors (e.g. Scroggs and Anderson, 1990; for review see Anwyl, 1990), which are negatively coupled to AC. Although the NMDA receptor plays a major role in the induction of plasticity, Ca^{2+} entry via non-NMDA receptor channels, such as L-type Ca^{2+} channels can also mediate the induction of synaptic plasticity (e.g. Cummings et al, 1996; Stricker et al, 1999). Therefore the actions of the 5-HT_{1A} and $_{1B}$ receptor ligands in mimicking the actions of 5-HT, by favoring to induction of LTD, may arise from their ability to inhibit voltage-gated Ca^{2+} channels involved in the expression of LTP.

In contrast to the 5-HT_1 receptors, activation of the 5-$HT_{2A/2C}$ receptors with DOI did not appear to favor the expression of LTD. While relatively few 5-$HT_{2A/2C}$ receptors are found in the dorsal horn (Pompeiano et al, 1994; Maeshima et al, 1998; Cornea-Hébert et al, 1999), activation of the 5-$HT_{2A/2C}$ receptors in this region can facilitate glutamatergic responses in some neurons (Hori et al, 1996), and may be involved in pronociceptive processes (e.g. Eide and Hole, 1991).

The 5-HT_3 ionotropic receptor agonist CPBG evoked only a modest facilitation of naïve synaptic responses in 7 of 13 cells tested (at 1 μM), and there was no clear shift towards LTD following CS. The observed EPSP facilitation is consistent with an increase in number of evoked spikes in dorsal horn neurons (Ali et al, 1996), but opposes the attenuation of afferent-evoked neurotransmission observed by Khasabov et al (1999). Khasabov et al (1999) observed that higher concentrations of CPBG (10 – 50 μM) favor synaptic depression. 5-HT_3 receptors are present on primary afferent terminals (Hamon et al, 1989; Kidd et al, 1993) where they can mediate primary afferent depolarization (Khasabov et al, 1999), an indicator of presynaptic inhibition. 5-HT_3 receptors are also found on dorsal horn neurons (see Hamon et al, 1989) where they can cause direct excitation of GABAergic (Morales et al, 1998) and enkephalinergic interneurons (Tsuchiya et al, 1999). Both pronociceptive (Ali et al, 1996; Oyama et al, 1996) and antinociceptive effects (Alhaider et al, 1991; Bardin et al, 1997; Giordano, 1997) have been reported following activation of 5-HT_3 receptors.

Importance of the DDH and synaptic connectivity

Neurons in the DDH represent a functionally heterogeneous population. Most receive convergent input from both low- and high-threshold afferent fibres and hence are classified as wide dynamic range (WDR) neurons, many of which are ascending tract cells conveying nociceptive information to the brain (Chung et al, 1979; Willis and Coggeshall, 1991; Lopez-Garcia and King, 1994; Herrero and Headley, 1995).

DDH neurons project dendrites into superficial laminae and receive direct monosynaptic connections presumably from nociceptive primary afferents in laminae II (Naim et al, 1998; Todd 1989; Willis and Coggeshall, 1991). On the other hand, low-threshold A fibres project monosynaptically onto DDH neurons via collaterals located in laminae III-V (Fitzgerald et al, 1994; Willis and Coggeshall, 1991; see also Miller and Woolf, 1996). Since our study was undertaken in neonates at a period when myelination is incomplete (Friede and Samorajski, 1968), we did not determine the relative contribution of low- and high-threshold afferents to our evoked synaptic responses. However the observed effects of 5-HT must be partly produced in WDR neurons since even at postnatal days 3-6, neuronal firing in response to depolarizing current injection is functionally differentiated (Hochman et al, 1997) and corresponds predominantly to WDR neurons which tend to fire repetitively in response to current injection (Lopez-Garcia and King, 1994).

Descending monoaminergic transmission, antinociception and development

Descending serotonergic systems exert a critical inhibitory control on spinal cord nociceptive transmission (for reviews see Fields and Basbaum, 1978; Basbaum and

Fields, 1984; Fitzgerald, 1986; Hammond, 1986; Millan, 1995). For example, serotonergic fibres originating from brainstem raphe nuclei (Dahlström and Fuxe, 1965) innervate neurons of the dorsal horn and comprise the best-described descending anti-nociceptive pathway. The inhibition of dorsal horn neurons caused by stimulation of brainstem regions is antagonized by the administration of 5-HT receptor antagonists, implicating 5-HT in mediating these antinociceptive effects (e.g. Chitour et al, 1982; Yaksh and Wilson, 1978). Thus, the prevention of LTP in spinal sensory systems with 5-HT is consistent with antinociceptive actions of some serotonergic descending systems.

Although bulbospinal serotonergic axon terminals are abundant in the rat spinal cord at birth (Steinbusch, 1981), modifications in the pattern and density occur postnatally (Bregman, 1987). In relation to functional synaptic connections, Fitzgerald and Koltzenburg (1986) reported that despite the early anatomical presence of a descending DLF, there is no functional descending inhibition until P10-12. However, several other studies provide evidence for the existence of descending inhibition much earlier than P10. For instance, Miyata et al (1987), Wallis and Wu (1993) and Wallis et al (1993a) demonstrated that stimulation of the lateral or latero-ventral thoracic cord resulted in strong inhibition of the segmental monosynaptic reflex (MSR) in neonatal rats (P1-9), an effect mediated by serotonin (Wallis et al, 1993a). Similarly, Brocard et al (1999) demonstrated that in the newborn rat, motoneurons are excited and/or inhibited by stimulating the ventral funiculus, while Magnuson et al (1995) and Magnuson and Trinder (1997) showed that ventral root reflexes are evoked following stimulation of the VLF in the neonatal rat (P1 - 8). Though none of these studies directly investigated the

function of the DLF, clearly, bulbospinal systems including the serotonergic innervations of the spinal cord are present and functional at birth, though presumed to be immature. In addition, many 5-HT receptor subtypes are clearly present and functional in the spinal cord of embryonic and newborn rats (e.g. Hentall and Fields, 1983; Hochman and Garraway, 1998; Ziskind-Conhaim et al, 1993; Wallis et al, 1993b) and there is evidence of endogenous release of serotonin at this stage (Wallis and Wu, 1993). Therefore, the use of 5-HT receptor agonists, even in neonates, may be effective in mediating anti-nociception.

Conclusion

In conclusion, we demonstrate that 5-HT acting at least partly via the 5-HT_{1A} and $_{1B}$ receptors can influence the induction of afferent-evoked synaptic plasticity in spinal cord, favoring depression. Currently, little is known of the modulatory properties of descending monoamine transmitters on the control of the spinal sensory integrative apparatus (see Jankowska et al, 1997). However, the emergence of syndromes following spinal cord injury that involves abnormally high-gain sensory processing (spasticity and chronic pain) attest to the importance of descending inhibitory control on spinal cord function (Ashby and McCrea, 1987; Schouenbourg et al, 1992). Clearly, a better understanding of the serotonergic modulation on spinal activity is required, including an identification of the actions of specific receptor subtypes on sensorimotor integration.

Table 1.

Effect of 5-HT and specific 5-HT receptor ligands on evoked naïve synaptic responses. Values (mean ± S.D.) are percentage changes in the amplitude of evoked synaptic responses in the presence of the agonists. The sample sizes are in brackets.

	Decrease ↓	**Increase ↑**	**No change**	**Total**
5-HT	55 ± 21 (37)*	28 ± 10 (3)	6	46
5-HT_{1A} **(5-CT/clozapine)**	56 ± 16 (5)	_	2	7
5-HT_{1B} **(CGS)**	22 ± 16 (3)	_	2	5
5-HT_2 **(DOI)**	22 ± 8 (4)	_	4	8
5-HT_3 **(CPBG)**	24 ± 16 (2)	20 ± 7 (7)	4	13

* 5-HT significantly depressed the evoked synaptic responses in most neurons ($p < 0.01$).

Table 2.

Effect of 5-HT on the incidence of evoked synaptic plasticity. Values are mean percentage of neurons undergoing LTP, LTD, or no change with sample size in brackets.

	Control	**5-HT**
LTD	54% (30)	88% (14)
LTP	20% (11)	6% (1)
No change	26% (15)	6% (1)

$\chi^2 = 28.2$ ($p < 0.001$)

Table 3.

Effect of 5-HT and specific receptor ligands on CS-induced synaptic plasticity. Values (mean ± S.D.) are percentage changes in EPSP amplitude following CS, in the presence of drug. The sample sizes, representing the relative incidence of LTP, LTD or no change, are presented in brackets.

	LTD	LTP	No change	Total
5-HT	54 ± 24 (14)	62 (1)	(1)	16
5-HT_{1A} (5-CT/clozapine)	20 ± 3 (5)	_	(2)	7
5-HT_{1B} (CGS)	46 ± 23 (3)	_	_	3
5-HT_2 (DOI)	49 ± 10 (3)	52 ± 22 (4)	(1)	8
5-HT_3 (CPBG)	24 ± 16 (2)	21 (1)	(2)	5

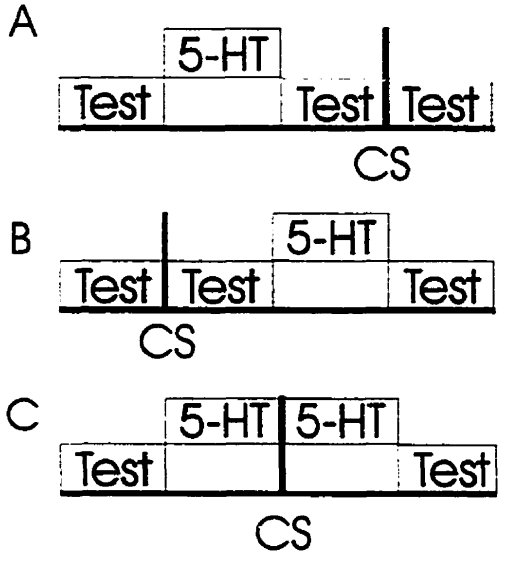

Figure 1: Three experimental paradigms for relating the action of 5-HT to CS-induced synaptic plasticity. The sequential ordering of events for each paradigm are outlined below. **A.** Baseline of evoked test responses → second baseline in the presence of 5-HT (for at least 10 minutes) → 5-HT washed out then test responses collected again → CS → post-conditioned test response. **B.** Baseline test response → CS → post conditioning test response (for at least 20 minutes) → test response in presence of 5-HT (for at least 10 minutes) → drug wash out. **C.** Baseline test response → second baseline in the presence of 5-HT (for at least 10 minutes) → CS in the presence of 5-HT → post conditioning response still in the presence of 5-HT (for at least 20 minutes) → drug wash out.

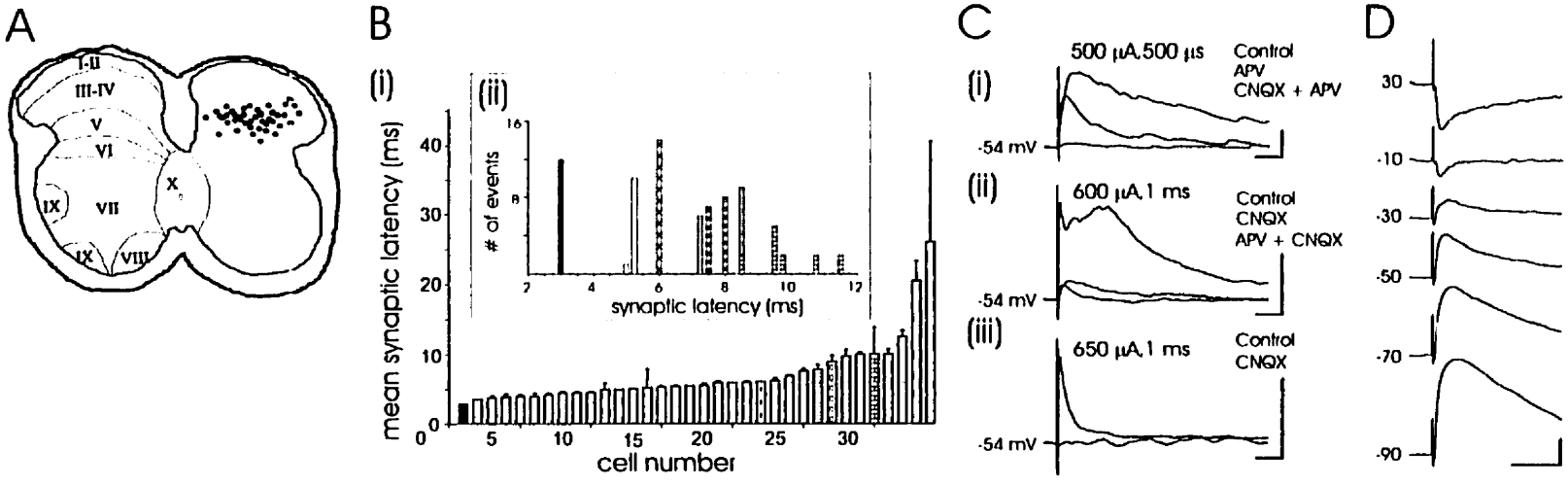

Figure 2: Characteristics of dorsal horn neurons. **A.** Approximate location of 42 of the 109 neurons recorded in the present study. **B.** Mean synaptic latency of evoked responses. Representative selection of 34 neurons. **B(i).** Histogram of mean synaptic latency ± S.D. (usually derived from 12 separate evoked EPSPs). Cells whose mean synaptic latency was below the horizontal dashed bar at 6 ms probably receive monosynaptic excitation from primary afferents (see Results). **B(ii).** Distribution of synaptic latency of the individual evoked events from 6 neurons coded in B(i). Note that the 3 neurons presented, whose mean synaptic latency was ≤6 ms, have little or no variability in latency of synaptic response. **C.** Evoked EPSPs are glutamatergic. Examples of variability of evoked synaptic responses. Each trace is an average of 6 episodes acquired every 10 seconds. **C(i).** APV reduces peak amplitude and hastens EPSP decay. Further, addition of CNQX largely abolishes synaptic response. **C(ii).** Synaptic response contains both early and late peaks. Following addition of CNQX, only a small-amplitude slow-decaying response remains. The decay of the EPSP is then hastened by the addition of APV, leaving a residual PSP. **C(iii).** EPSP with an early peak and rapid decay is completely blocked by CNQX. **D.** In most cells, synaptic responses were verified as being excitatory by determining their reversal potential, which, in the present example, was near -10 mV. Scale bars in **C** and **D** are 10 mV, 200 ms.

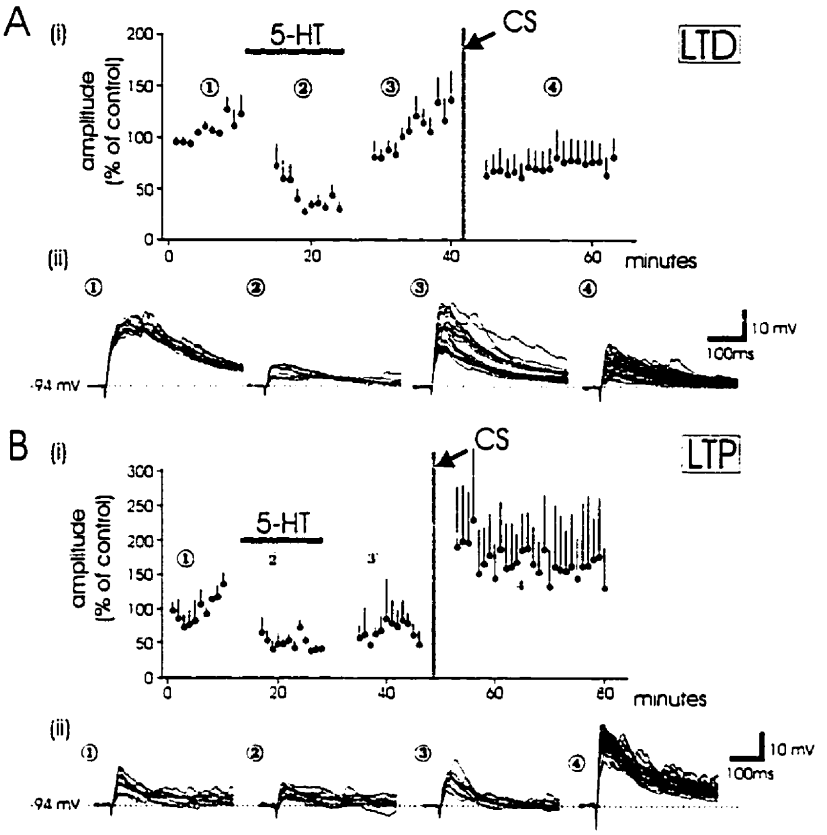

Figure 3: 5-HT can depress the naïve synaptic response irrespective of whether synapse will undergo CS-induced potentiation or depression. Figure **3A(i)** and **3B(i)** demonstrate 5-HT-evoked depression of synaptic responses prior to CS-induced LTD (n= 5) and CS-induced LTP (n= 3), respectively. **A(ii).** An example of 5-HT-evoked depression of the raw synaptic responses (74%↓). The evoked response, which returns to pre-5-HT values following washout, underwent CS induced LTD (66%↓). **B(ii).** Following washout of the synaptic depression evoked by 5-HT (42%↓), CS induced LTP (178%↑) in this cell. In this and the following figures; the timing of 5-HT application is indicated with a horizontal bar and conditioning stimulation (CS) is indicated with a vertical bar, the circled numbers in graphs coincide with the panel of raw superimposed traces presented below them, graphs present normalized response amplitude + S.E., and, cells were held at -94 mV during collection of EPSPs.

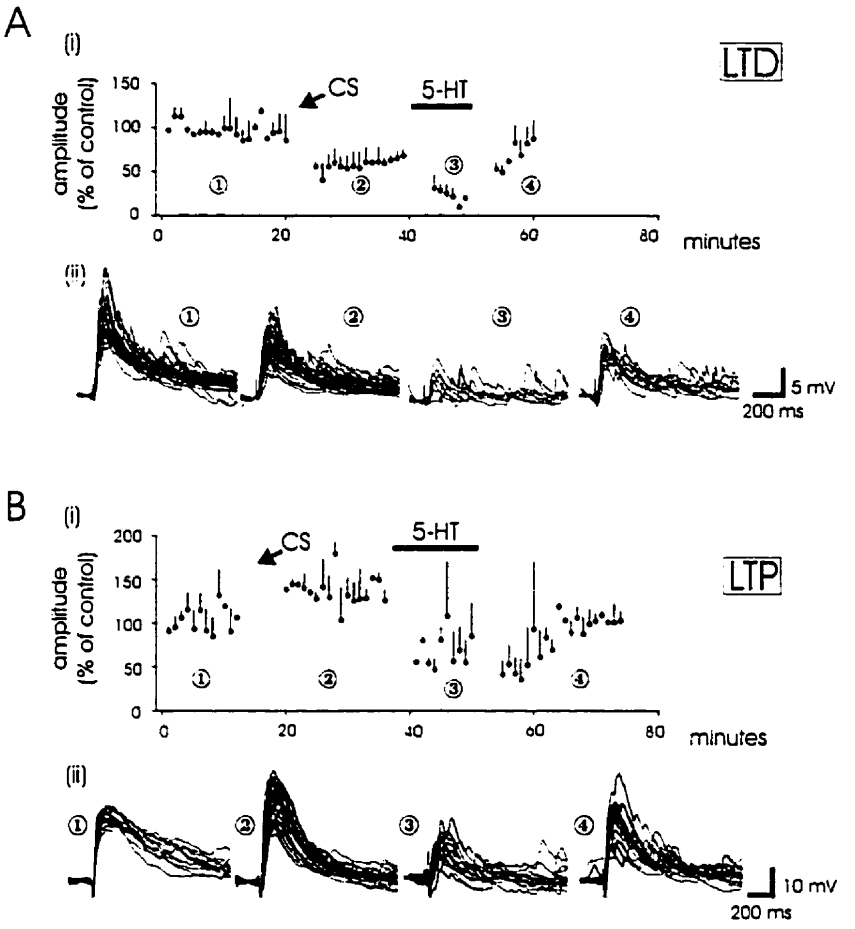

Figure 4: 5-HT depressed the post conditioning synaptic response regardless of whether LTP or LTD was induced. 5-HT evoked depression following CS-induced LTD (**A(i)**) and CS-induced LTP (**B(i)**) respectively. **A(ii).** 5-HT can evoke an additional depression following LTD already induced by CS. **B(ii).** Following CS-induced LTP, application of 5-HT also causes a depression of the synaptic response. In both examples, the effects of 5-HT are at least partly reversed following washout (91 and 77% respectively). Stimulus artefact is truncated in **B(ii)**.

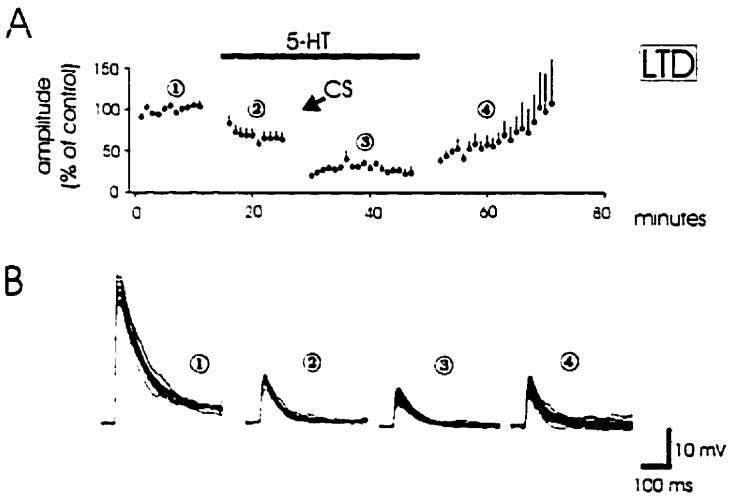

Figure 5: 5-HT favors the induction of LTD. **A** Normalized data of cells undergoing CS-induced LTD in addition to 5-HT evoked depression (n=14). **B.** An example where LTD (29%) is induced in addition to 5-HT-evoked depression (69%). Following washout of 5-HT, the EPSP amplitude increases slightly but still remains strongly depressed (LTD) compared to the pre-5-HT baseline response.

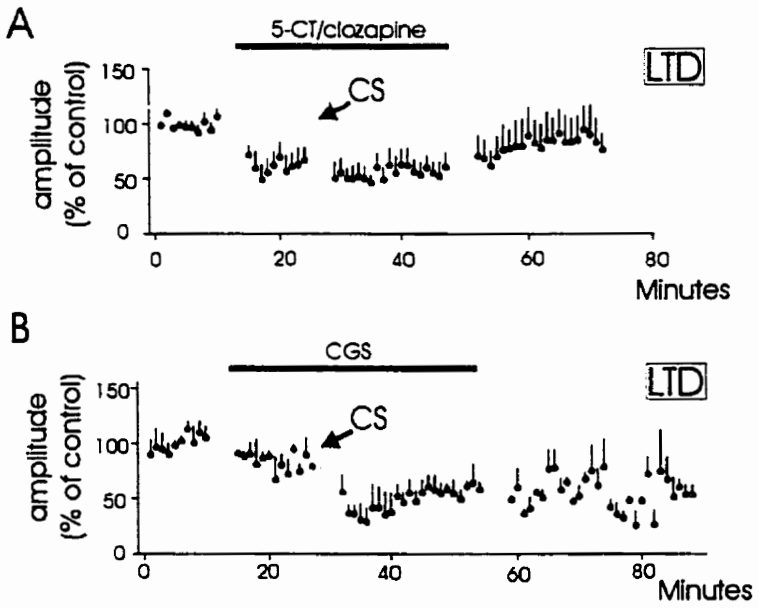

Figure 6: 5-HT_1 receptor agonists favor the induction of LTD. Normalized data showing the effects of 5-CT/clozapine (**A**, n=5) and CGS (**B**, n= 3) on CS-induced synaptic plasticity. CS-induced LTD is produced in the presence of these ligands.

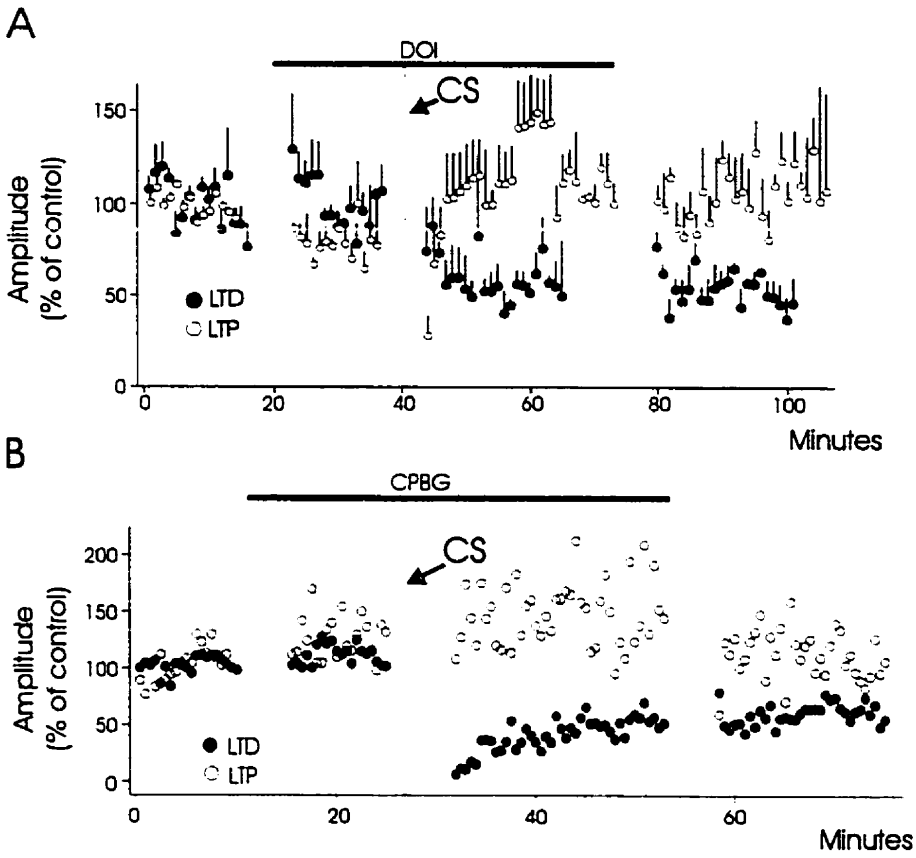

Figure 7: 5-$HT_{2\text{ and }3}$ receptor agonists do not favor the induction of LTD. **A.** CS in the presence of the 5-$HT_{2A/2C}$ receptor agonist, DOI, induced both LTP (open circle, n = 4) and LTD (closed circle, n = 3). **B.** Two neurons; one undergoing LTP (open circle; 21%↑), the other undergoing LTD (closed circle; 62%↓) following CS in the presence of CPBG, the 5-HT_3 receptor agonist.

General discussion

1.0 Summary

The three general studies comprising this thesis explored the modulation of synaptic responses and neuronal properties of deep dorsal horn (DDH) neurons of the neonatal rat following bath application of the monoamine transmitters and also following repetitive stimulation of primary afferents. Our major findings are: (i) The three descending monoamines, 5-HT, NA and DA, generally depress EPSP amplitudes in P10-14 day old animals. In contrast, ACh, a monoamine intrinsic to the spinal cord, generally facilitates the synaptic responses and in addition preferentially facilitates the longer-lasting component of the responses, presumably NMDA receptor mediated. (ii) The monoamines do not alter the passive membrane properties of the DDH neurons, but are capable of altering their firing patterns in response to depolarizing current injection, generally converting phasic firing neurons to repetitive. (iii) Several selective 5-HT receptor ligands modify primary afferent evoked synaptic responses in DDH neurons, generally producing consistent actions within the first two postnatal weeks. (iv) Synaptic depression, like that produced by 5-HT, is partly mediated by the 5-HT_{1A} receptor agonist, while synaptic facilitation is generally mediated by activation of the 5-HT_{7} receptor. (v) Endogenously-released 5-HT may tonically depress synaptic responses via activation of the 5-HT_{1A} receptors. (vi) 5-HT depresses both naïve synaptic responses and responses undergoing LTP or LTD following high frequency conditioning stimulation. (vii) Conditioning stimulation of primary afferents in the presence of 5-HT increases the incidence of LTD. (viii) These actions of 5-HT are mimicked by the 5-HT_{1A} and $_{1B}$ receptor agonists.

1.1. Importance of the DDH and synaptic connectivity

These studies have provided additional information on the modifiability of both synaptic response and the cellular properties of DDH neurons by the biogenic amine transmitters. Clearly, the ability of these bulbospinal monoaminergic and intrinsic cholinergic systems to modify synaptic transmission in the dorsal horn supports them playing a critical role in the control of sensory transmission at the first CNS site of synaptic integration.

Most incoming sensory information must be processed and integrated in the DDH, before they can be transmitted supraspinally, since the majority of ascending tract neurons are located in the DDH (Willis and Coggeshall, 1991). In relation to nociceptive information, the role the DDH plays must be functionally important as this region of the spinal cord undergoes the greatest longitudinal spread of nociceptor-induced activity (Coghill et al, 1991; Porro et al, 1991; Mao et al, 1993). Furthermore, many of the DDH neurons are classified as WDR, which encode much of the psychophysical components of pain sensation. In addition, most of the WDR neurons are also ascending tract cells that encode and convey nociceptive and non-nociceptive information supraspinally. Hence, it is important to elucidate the manner in which sensory synaptic transmission is regulated in the DDH.

Because these studies were undertaken in neonates at a period when myelination of afferent fibres is incomplete (Friede and Samorajski, 1968), we were unable to determine the relative contribution of low- and high-threshold afferents, thereby making it

impossible to functionally identify the DDH neurons based on their synaptic input. However, the effects of the agonists must be at least partly produced in WDR neurons, which are the predominant class of neurons in the DDH (e.g. Herrero and Headley, 1995). Furthermore, WDR neurons have been previously shown to fire repetitively in response to depolarizing current injection (Lopez-Garcia and King, 1994) and these firing patterns of neurons in the DDH are differentiated at early postnatal ages (Hochman et al, 1997).

1.2. General actions of the monoaminergic transmitters

Interestingly, the spinal cord dorsal horn contains a functionally heterogeneous population of neurons, with diverse dendritic arborizations, axonal projections, sizes and functions (Willis and Coggeshall, 1991). Yet, in these studies the global actions of the monoamines are very consistent, with the descending transmitters generally causing synaptic depression, and ACh facilitating the evoked synaptic responses. This observation is surprising since these transmitters mediate their spinal actions via several receptors, ionotropic and metabotropic, present at both pre- and postsynaptic sites; which include the central terminals of primary afferents, terminals of bulbospinal fibres and on intrinsic interneurons. With respect to 5-HT, it appears that facilitation and/or moderate depression of the evoked synaptic responses mediated by non-5-HT_{1A} receptors are generally masked by the more dominant synaptic depression mediated in part by the 5-HT_{1A} receptor.

1.3. Monoaminergic receptors and innervations in the dorsal horn

The monoaminergic receptors are distributed on spinal neurons and on primary afferent terminals (e.g. Daval et al, 1987; Hamon et al, 1989; Kidd et al, 1993; Ridet et al, 1994; Stone et al, 1998). For instance, the 5-HT_1 receptor, which is the dominant class of serotonergic receptor present in the spinal cord dorsal horn (e.g. Marlier et al, 1991b), is concentrated on dorsal horn neurons, although about 20% must be present on primary afferent terminals (Daval et al, 1987; Laporte et al, 1995; reviewed in Millan, 1995). The postsynaptic location of 5-HT_1 receptors may be primarily due to the 5-HT_{1A} subclass of receptors, since it is reported that 5-HT_{1B} receptors are generally presynaptic, on primary afferent terminals or on the terminals of descending serotonergic fibres (see Laporte et al, 1995; Verge and Calas, 2000; reviewed in Millan, 1995), where they can function as autoreceptors. The potent depression evoked by both 5-HT and 5-HT_{1A} receptor agonist is consistent with the 5-HT_{1A} receptor, which is negatively coupled to signal transduction pathway, being the dominant class of serotonergic receptors present in the dorsal horn (e.g. Huang and Peroutka, 1989; Marlier et al, 1991b). The activation of a receptor that is negatively coupled to signal transduction pathway will be expected to produce depression of evoked synaptic responses (refer to project II, discussion).

In contrast to the 5-HT_1 receptors, the 5-HT_2 receptors which are concentrated in the ventral horn, exhibit a low density in the dorsal horn (Marlier et al, 1991b; reviewed in Millan, 1995), while the vast majority of 5-HT_3 binding sites in the spinal cord may be present on primary afferent terminals (Hamon et al, 1989; Laporte et al, 1995). Therefore, the modest action of DOI, the 5-$HT_{2A/2C}$ receptor agonist, is consistent with a

low proportion of receptors in the DDH. However, the facilitatory actions of the 5-HT_3 receptor agonist observed in these studies cannot be explained by the predominance of these receptors on primary afferent fibres, where they have been shown to produce primary afferent depolarization (see below). The presence of the 5-HT_7 receptors in the spinal cord dorsal horn is not yet confirmed, although the potent facilitatory actions of the 5-$HT_{1A/7}$ receptor agonists, suggest the presence of 5-HT_7 receptors on primary afferent or DDH neurons (refer to project II).

It appears that the distribution and expression of the dopaminergic and noradrenergic receptors within the spinal cord dorsal horn are less explored (see Seybold, 1986). However, these receptors may be similarly distributed on primary afferent terminals and dorsal horn neurons. For instance, in the rat spinal cord the α_{2A} receptors are primarily located on capsaicin sensitive primary afferents, while α_{2C} receptors may be located mainly on spinal interneurons (Stone et al, 1998). Neurons throughout the dorsal horn were immunolabeled for the D_2 dopaminergic receptor (van Dijken et al, 1996). Like 5-HT, the depression of evoked synaptic responses by NA and DA may be due to actions at the α_2 and D_2 receptors respectively, both of which are present in the dorsal horn and are negatively coupled to adenylate cyclase.

The cholinergic receptors, muscarinic and nicotinic receptors, are similarly located on both primary afferents and spinal neurons. However, it appears that the percentage of cholinergic receptors located on primary afferents is small, since dorsal rhizotomy in the adult rat non-significantly decreased muscarinic and nicotinic binding sites in the dorsal

horn (Gillberg and Ashmark, 1991). The dominant postsynaptic localization of cholinergic receptors in the spinal cord dorsal horn may explain the proposed postsynaptic actions of ACh observed in this study. However, since ACh did not induce depolarization in any of the DDH neurons studied, it may be assumed that the modulatory actions ACh exerted on evoked EPSPs were mediated at the muscarinic metabotropic receptors, which are expressed in the spinal cord about 2-3 times higher than the nicotinic receptors (Gillberg et al, 1988).

In addition to the multiple classes of monoaminergic receptors present in the spinal cord, the spinal projections of the bulbospinal monoaminergic systems are also diverse. Specifically, with the exception of dopamine, in which case only the A11 cell group projects to the spinal cord, several supraspinal serotonergic and noradrenergic nuclei project to the spinal cord of the rat (see earlier references; also see Lakke, 1997). Therefore, one would expect that distinct bulbospinal projections would also make specific synaptic contacts, resulting in distinct actions. However, further evaluation of the descending monoaminergic systems revealed that despite the dense termination of these fibres in the dorsal horn, only a fraction, sometimes less than half, of these fibres make classical synaptic contacts (e.g. Marlier et al, 1991a; Ridet et al, 1992). Hence, the spinal action of the monoamines is thought to occur mainly by 'volume transmission', which is a diffuse release of the transmitters with the capability of mediating effects at sites distant from the terminals (refer to Ridet et al, 1992; Millan, 1995). As a result, it is not entirely surprising that when bath applied, these transmitters exert global depressant actions.

Accordingly, it appears that the actions of the descending monoamines in the spinal cord dorsal horn are more dependent on the class of receptor activated than on the properties of the bulbospinal neurons. Obviously, the differential expression of these various monoaminergic receptors within the spinal cord would account for the complexity associated with the pharmacology and mechanisms underlying the antinociceptive roles of these transmitters.

1.4. Possible sites of depressant actions

While this study does not conclusively demonstrate the site of the transmitters' actions, it appears that the three descending biogenic amine transmitters (5-HT, NA and DA) depressed EPSP amplitudes by presynaptic mechanisms. This assumption was made based on the following observations: First, none of these transmitters affected the passive properties of the neurons (e.g. a decreased τ_m or R_{in}) that corresponded to their potent actions on EPSP amplitudes (refer to Table 1; project I). Secondly, when changes in EPSP amplitude were compared to changes in R_{in}, no relationship existed (Fig 8A; project I). Thirdly, in contrast to ACh, the three bulbospinal transmitters produced identical percent depression on early, presumably AMPA/kainate and late, presumably NMDA receptor-mediated components of the EPSPs. Additionally, most of the selective serotonergic ligands used also failed to induce direct postsynaptic actions such as on E_{MR} or R_{in}.

Consistent with presynaptic actions, all three transmitters, 5-HT, NA and DA, can depress synaptic responses as a result of their ability to increase potassium conductance (e.g. North and Yoshimura, 1984; for review see Barnes and Sharp, 1999) or by inhibiting both N and L type calcium channels (e.g. Wikström et al, 1999), presynaptically. In addition, previous studies suggested that 5-HT and NA may mediate presynaptic inhibition of glutamate release from primary afferents in the guinea pig (Travagli and Williams, 1996). These actions are likely to be mediated by the receptors that are negatively coupled to signal transduction pathways such as the 5-HT_1 and α_2 receptors. Similarly, presynaptic D_2 dopamine receptor mediates depression of spinal reflexes (Gajendiran et al, 1996) in the adult rat.

Presynaptic inhibitory actions of 5-HT can also involve primary afferent depolarization (PAD). 5-HT has been shown to mediate PAD, although the receptors mediating such actions have not been fully characterized (Lopez-Garcia and King, 1996; Khasabov et al, 1998, 1999). In the neonatal rat (P10-14), the 5-HT_{1A} receptor agonist 5-CT potently depressed dorsal root-evoked EPSPs in dorsal horn neurons, but failed to consistently induce PAD (Lopez-Garcia and King, 1996). However, the 5-HT_3 receptor agonist CPBG induced PAD, though of smaller magnitude than 5-HT (Khasabov et al, 1999) in a similar *in vitro* hemisected spinal cord preparation. In addition, Khasabov et al (1998) demonstrated that capsaicin treatment, which selectively destroys unmyelinated primary afferents, significantly reduced 5-HT-induced PAD in the neonatal rat (Khasabov et al, 1998). Interestingly, the 5-HT_3 receptors are predominantly located on primary afferent fibres. These observations suggest that 5-HT may mediate spinal inhibitory actions,

particularly antinociception, by reducing transmitter release from nociceptors, via the production of PAD. Surprisingly, in this study, CPBG did not produce synaptic depression.

Unlike the serotonergic system, there are no ionotropic dopaminergic or noradrenergic receptors. However, this does not discount the possibility of these transmitters mediating presynaptic inhibition, in the absence of PAD. In this study, we did not record dorsal root potentials or determine the presence of PAD, and so cannot state whether the depressant actions of 5-HT were mediated in part by PAD-induced presynaptic inhibition.

The ability of the monoamines to reduce neurotransmitter release, particularly from nociceptors, at the initial CNS sensory/nociceptive site greatly supports their critical roles in sensory/nociceptive processing, such as being able to alter synaptic gain at the spinal level, prior to transfer of sensory information to the supraspinal relay stations.

In addition to presynaptic actions on primary afferents, the monoamines can mediate their spinal actions via interneurons, which in turn project to DDH neurons. It has been previously hypothesized that the descending aminergic systems interact with enkephalinergic or GABAergic inhibitory interneurons, which in turn inhibit projection neurons, thus mediating spinal antinociception (Basbaum and Fields, 1984). The concept of inhibitory actions in the superficial dorsal horn (substantia gelatinosa; SG) is of considerable importance. The SG was historically referred to as a closed system (see Willis and Coggeshall, 1991), mainly because most of the neurons in that region of the

cord are local interneurons, many of which are inhibitory neurons containing GABA, enkephalins, ACh and glycine (also see Millan, 1999). As a result, output from the SG to the DDH is likely to be inhibitory. Consequently, the activation of inhibitory interneurons in the SG may result in an increase in inhibitory output to the DDH, thereby producing a reduction in the amplitude of excitatory synaptic responses.

Recently, Baba et al (2000a) demonstrated NA-induced facilitation of inhibitory responses in substantia gelatinosa neurons, suggesting that an increase in pre-synaptic GABA or glycine release may contribute to the depressant actions in the DDH. They also showed that this facilitation of inhibitory responses might in turn lead to a depression of excitatory polysynaptic responses evoked in DDH neurons. Furthermore, DDH neurons contain dorsally projecting dendrites and thus may be directly modulated by inhibitory actions exerted by the monoamines occurring in the superficial dorsal horn. Therefore in this study, bath application of the biogenic amine transmitters may similarly increase the inhibitory drive to DDH neurons by activating spinal inhibitory interneurons.

Despite the lack of evidence to suggest a potent postsynaptic mechanism of inhibitory actions, the monoamine transmitters can produce direct hyperpolarization of CNS neurons, by increasing potassium conductances to mediate overall synaptic depression (e.g. North and Yoshimura, 1984; for review see Barnes and Sharp, 1999; also see Hwang and Dun, 1998, 1999). However, since this does not appear to be the mechanism of inhibitory action in these experiments, we suggest that the inhibitory actions might be

mediated by the presynaptic mechanisms, involving primary afferent fibres, mentioned above.

In contrast to their lack of effect on the passive properties of these neurons, the transmitters were capable of altering the firing properties of the neurons suggesting that although a postsynaptic mechanism may not account for the changes in EPSPs' amplitude, the monoamines may mediate postsynaptic actions associated with the alteration of neuronal output properties. As mentioned earlier, monoamine transmitters exert their actions via a multitude of metabotropic (and ionotropic) receptors, many of which are located on postsynaptic dorsal horn neurons (e.g. Laporte et al, 1995; van Dikjen et al, 1996; Stone et al, 1998). Thus, it is not surprising that these transmitters exert direct postsynaptic actions on DDH neurons. However we did not identify which class of receptor is responsible for the alteration in firing.

Though a postsynaptic increase in firing, and hence an increase in neuronal output of DDH neurons by these transmitters, appears to be inconsistent with our general hypothesis of a general depressant action, a concomitant action of the monoamines on both post synaptic properties and presynaptic neurotransmitter release may result in overall inhibition. These different actions of the biogenic amines may function as a 'gating' mechanism. For instance, while the transmitters may be inhibiting the release of neurotransmitter from primary afferents, via the activation of one class of receptors, they may simultaneously, increase the neuron' responsiveness to input from other sources, such as descending inhibitory commands or spinal interneurons. Therefore, as the result,

the overall action of the monoamines might be potent and selective suppression of nociceptive sensory information being transmitted to the brain.

Interestingly, in contrast to the bulbospinal transmitters, and most of the serotonergic ligands, the facilitatory actions of ACh and the 5-HT_{1B} receptor agonist, CGS, may be partly mediated by a postsynaptic mechanism. In the presence of ACh, there was a weak relationship between changes in EPSP amplitude and changes in R_{in}, while the facilitation of synaptic responses evoked by CGS was often associated with an increase in R_{in}. Also, unlike the three descending transmitters that produced relatively uniform depression of the EPSPs, ACh preferentially facilitated the longer-lasting component of the EPSP, suggesting a differential action at postsynaptic glutamatergic receptors. A preferential facilitatory action on the late, presumably NMDA receptor mediated, component of the EPSP, is consistent with observed modulatory actions of M_1 muscarinic receptor activation on NMDA receptor activity (Marino et al, 1998; Calabresi et al, 1998). If facilitation in synaptic strength involved only presynaptic mechanisms that increase glutamate release, one would expect to see a uniform facilitation of both AMPA/kainate and NMDA receptor mediated responses.

Overall, the monoamine transmitters are capable of exerting diverse actions, involving both post and presynaptic loci. However, the underlying mechanisms are still not clearly understood. Since different monoaminergic receptors are located at pre and post-synaptic sites and bath application of the transmitters is likely to activate receptors simultaneously, it is not uncommon to observe differing actions in the presence of these transmitters. For

example, Belcher et al (1978) reported that while 5-HT reduced synaptic responses evoked by noxious stimuli, it increased spontaneous firing and firing evoked by DL-homocysteic acid in dorsal horn neurons.

The actions of the biogenic amine transmitters and selective serotonergic receptor ligands on evoked synaptic responses in these studies attest to the roles the monoaminergic systems play in sensory integration. It has been previously established that the bulbospinal monoamines, part of the endogenous analgesic circuitry, exert part of their antinociceptive actions at the level of the spinal cord dorsal horn (Fields and Basbaum, 1978), where they can mediate both pre- and postsynaptic actions. However, a comparison of the actions of all three descending transmitters and ACh on synaptic responses and cellular properties of individual DDH neurons has not been previously demonstrated. Here, we are reporting that the monoamines are capable of exerting similar and distinct actions on individual DDH neurons.

1.5. Development of bulbospinal systems

One potential weakness of these studies is whether the observed actions of the monoamine transmitters reflect their actions in a mature animal, since clearly many systems in the rat are not fully mature even at P14. Specifically, although bulbospinal monoaminergic axon terminals are present in the rat spinal cord at birth, modifications in the pattern and density occur postnatally (see Lakke, 1997), with serotonergic innervation maturing around P21 (Bregman, 1987; Lakke, 1997), while noradrenergic and dopaminergic projections mature around P26 (Lakke, 1997). However, several studies

suggest that descending inhibition is present in the rat before the maturation of these monoaminergic systems. For instance, Miyata et al (1987), Wallis and Wu (1993) and Wallis et al (1993a) demonstrated that stimulation of the lateral or latero-ventral thoracic cord resulted in strong inhibition of the segmental monosynaptic reflex (MSR) in neonatal rats (P1-9), an effect mediated by serotonin (Wallis et al, 1993a). Similarly, Brocard et al (1999) demonstrated that in the newborn rat, motoneurons are excited and/or inhibited by stimulating the ventral funiculus, while Magnuson et al (1995) and Magnuson and Trinder (1997) showed that ventral root reflexes are evoked following stimulation of the VLF in the neonatal rat (P1 - 8). Though none of these studies directly investigated the function of the DLF, clearly, bulbospinal systems including the serotonergic innervations of the spinal cord are present and functional at birth, though presumed to be immature.

Moreover, many 5-HT receptor subtypes are present and functional in the spinal cord of the embryonic (Ziskind-Conhaim et al, 1993) and newborn rat (e.g. Hentall and Fields, 1983; Wallis and Wu, 1993; Hochman and Garraway, 1998) and there is evidence of endogenous release of serotonin in the newborn (Wallis and Wu, 1993). Similarly, spinal actions mediated by dopaminergic, noradrenergic and cholinergic agonists (e.g. Gladwell and Coote, 1999; Urban et al, 1989; Bleazard and Morris, 1993; Miyazaki et al, 1998) have been observed in neonatal rats, suggesting the presence and functions of these receptors in the spinal cord prior to the maturation of the descending innervation.

Interestingly, in our study, the occurrence and magnitude of the synaptic depression evoked by 5-HT in the P3-6 animals (project III) were comparable to the effects observed in the P10-14 animals (project I), an age at which the descending projections are expected to be physiologically functional (refer to Fitzgerald and Koltzenburg 1986). Furthermore, perhaps with the exception of the 5-HT_{1B} receptor, there was no evidence that suggests the serotonergic and presumably, the other bulbospinal monoaminergic systems undergo functional reconfiguration within the first two weeks of life. Instead, it appears that the functional integrity of monoaminergic receptors is relatively conserved over the first two weeks of postnatal development. Additionally, the actions of these transmitters, and more interestingly, the selective serotonergic receptor ligands are consistent with reported actions observed in adult (e.g. Xu et al, 1994; Ali et al, 1996; Gjerstad et al, 1996, 1997), suggesting that the role of these 5-HT receptor subtypes in the DDH remains relatively unchanged during postnatal development and may mediate similar functions even in the adult.

Since the descending terminals are present at birth in the rat and obviously functional receptors are present in the newborn, the effects of the monoamine transmitters and 5-HT ligands reported here might be consistent with the actions these endogenous transmitters exert on sensory processing in a mature, intact system.

1.6. Relevance

An understanding of the actions of the monoamine transmitters at the spinal cord dorsal horn is an important step towards understanding their analgesic/antinociceptive roles.

Currently, though well established that these transmitters exert analgesic actions, the mechanisms involved are not clearly understood. One obvious obstacle to understanding the mechanisms underlying the spinal actions of the monoamines is related to the multiplicity of receptors they bind to. This thesis provides information on the spinal actions of these transmitters on evoked synaptic responses, the actions of serotonin on primary afferent evoked synaptic plasticity and a pharmacological characterization of several serotonin receptors mediating these spinal actions. Specifically, we showed that while the bulbospinal monoaminergic transmitters can mediate both pre- and postsynaptic actions, the overall effect of bath application of these transmitters to neurons in the spinal cord DDH, is synaptic depression. Therefore, one manner in which these systems may function to effectively provide antinociception may be through the depression of sensory information, including nociceptive input.

In this study, we also showed that the prototypical monoamine, serotonin, exerted potent modulatory actions on primary afferent-induced synaptic plasticity, by promoting the induction of long-term depression (LTD). This observation is relevant, since the ability of spinal synapses to undergo synaptic plasticity, namely LTP, may contribute to memory traces associated with the expression of chronic pain. The ability of serotonin to prevent synapses from undergoing LTP, particularly in response to noxious input, but rather to suppress the synaptic strength at the spinal cord level, may be a critical method employed by the bulbospinal serotonergic systems functioning to produce analgesia. In addition, we were able to show that the 5-$HT_{1A \text{ and } 1B}$ receptors accounted for these actions of 5-HT. This control 5-HT exerts over the induction of primary afferent-evoked synaptic

plasticity is not surprising, since Liu et al (1998) and Sandkühler and Liu (1998) demonstrated that supraspinal systems clearly control the induction and expression of spinal synaptic plasticity. However, with respect to Sandkühler and colleagues study, transection of the spinal cord leads to a non-specific disruption of descending systems and thus the effects of the lesions cannot be entirely attributed to the monoaminergic systems. In contrast, the result of project (III) clearly suggests that serotonin, acting at spinal 5-$HT_{1A \text{ and } 1B}$ receptors, is capable of controlling the direction of synaptic plasticity occurring in the spinal cord deep dorsal horn, favoring LTD. Though not tested, it would be interesting to demonstrate that both NA and DA are similarly capable of controlling the expression of synaptic plasticity in the spinal cord.

Another important observation arising from these projects is related to the various serotonergic receptors mediating the spinal actions of 5-HT. The actions mediated by spinal serotonergic receptors are so often disputed mainly due to the lack of selective ligands. In this study, we used highly selective combinations of ligands to classify some receptors involved in mediating serotonin actions in dorsal horn. First we showed that several 5-HT receptors mediate modulatory actions on synaptic responses evoked in DDH neurons and in addition these actions were consistent in early neonates (P3-6) and young rats (P10-14). Despite the diverse actions observed, the potent depressant action of 5-HT was mimicked by the activation of the 5-HT_{1A} receptors, while the actions mediated by the other tested receptor ligands were generally less potent or widespread. This suggests that the dominant action of 5-HT in the spinal cord dorsal horn is synaptic depression at the 5-HT_{1A} receptors, which might in turn mask the more modest actions,

mediated by non-5-HT_{1A} receptors. This study also revealed that the actions of 5-HT and the selective ligands were generally similar in both age groups of animals tested, and compared to results observed in adult rat (see earlier reference), suggesting the conservation of receptor function and/or role of the monoaminergic systems in the spinal cord during postnatal development. Overall, these studies suggest that even the neonatal rat spinal cord slice preparation can provide critical information on the spinal actions of the monoamines associated with antinociception.

1.7. Clinical significance

The monoamine transmitters have been previously shown to powerfully reduce transmitter release, including substance P from primary afferent terminals in addition to suppressing the activity of WDR neurons (see Yaksh and Malmberg, 1994). Though many experimental studies have demonstrated the spinal antinociceptive actions of the monoamines, for instance, foot shock induced antinociception in the rat is partly reversed by serotonergic and noradrenergic antagonists (see Yaksh and Malmberg, 1994), there has also been conflicting report on the spinal actions of these transmitters, and occasionally, no effects. These conflicting reports on the antinociceptive actions of the monoaminergic transmitters may arise mainly from their complex pharmacology and the lack of selective ligands, previously (Van Wijngaarden et al, 1990). Thus, there is still a need for more investigations on the spinal antinociceptive actions of these transmitters such as on tail flick response latencies and formalin-induced nociception. In this study, we provide information that confirms the potent depressant actions of the bulbospinal biogenic amines and have characterized specific serotonergic receptors mediating spinal

actions. It appears therefore, that some of the previous behavioural studies may h been confounded as a result of inadequate administrations of the ligands, since ther less control over drug administration in *in vivo* preparations as compared to *in v* studies.

In relation to clinical implication, many monoaminergic ligands are currently being u therapeutically. The analgesic and antidepressant actions of monoamine oxic inhibitors are due to their actions on central neurotransmitter functions (Monks, 19 Increased levels of monoamines can inhibit nociception at the spinal, thalamic brainstem levels (Monks, 1994). Several other agents are being used clinica Experimentally, the administration of amine uptake blockers or monoamine oxic inhibitors caused increases in nociceptive response latencies and threshold in anin (Yaksh and Malmberg, 1994). Clinically, selective serotonin reuptake inhibitors are a used as antidepressants (e.g. Fluoxetine HCl; Prozac) and serotonin receptor ligand the treatment of chronic pain syndromes including cancer pain (reviewed in Breitba al, 1994) and migraines (e.g. the 5-HT_{1D} receptor agonist, sumatriptan) (reviewec Hargreaves and Shepheard, 1999). Some of these antidepressants may also potent the analgesic effects of opioids. The α_2 noradrenergic receptor agonist clonidine, wh has been shown to block the release of transmitters and peptides from primary affere has also been compared experimentally to morphine and being reported to be equ effective (Boivie, 1994). Other therapeutic benefits of the monoamines involve the of adrenal medullary transplants, which have been shown to increase the level of b

catecholamines and opioid peptides into the cerebrospinal fluid, and to also cause a reduction in pain sensitivity (refer to Sagen et al, 1991).

Despite the advances in clinical use of the monoamines, many of the drugs have proven disappointing in clinical studies of neuropathic pain and in addition, their mechanisms of actions are not universally agreed upon (Breitbart et al, 1994). Clearly, a lot of effort has been placed in understanding the potential analgesic benefits of the monoamine transmitters. However, since the mechanisms of actions and their roles, including specific receptor subtypes are not fully characterized, there is still a need of active pursuit in this area so that the physiological and clinical benefits of these transmitters can be maximized.

2.0 Limitations

Despite the important observations reported here, there are several limitations to this study. First, bath application of the transmitters is likely to activate all receptors simultaneously, whereas physiologically, it is probable that under some conditions, there is some degree of preferential activation of receptor subtypes by separate descending serotonergic (and presumably other monoaminergic) systems (Wei et al, 1999). Hence, physiological conditions may exist where synaptic facilitation dominates over the strong depression observed presently (see below).

Second, this study used high-intensity electrical stimulation to recruit the majority of afferents within the stimulated dorsal root, and this could mask weaker opposing actions

occurring in some specific afferent fibre populations. For example, Jankowska and co-workers have demonstrated that 5-HT and raphe-spinal stimulation can exert a differential control over primary afferent-evoked responses of different modalities (e.g. Riddell et al, 1993; Jankowska et al, 1997; Jankowska et al, 2000).

Third, since none of the neurons were labeled, we were unable to classify neurons based on their morphology, dendritic arborization or axonal projections. As a result, though the DDH is functionally heterogeneous, all dorsal horn neurons were treated here as a single population. Jankowska and co-workers have demonstrated that the actions of 5-HT may depend on the functional identity of the spinal neuron studied (Jankowska et al, 1997; Jankowska et al, 2000). We however, cannot equate our finding to a selective functional group of interneurons or projection neurons.

Fourth, with the exception of 5-HT, pharmacological studies were not undertaken to determine the specific receptors involved in mediating the observed actions. However, consistent with our hypothesis for project (II), we suggest that receptors negatively coupled to signaling transduction pathways are more likely to produce inhibition, while those positively coupled to transduction pathways produce facilitation. Thus, the 5-HT_1, the α_2 adrenergic and the D_2 dopaminergic receptors all present in the spinal cord dorsal horn (Seybold, 1986; Huang and Peroutka, 1987; van Dijken et al, 1986; Stone et al, 1998), are negatively coupled to adenylate cyclase and may account for the observed inhibitory actions of 5-HT, NA and DA, respectively. In contrast, the M_1 cholinergic receptor positively linked to PLC-PKC may be responsible for the effects of ACh.

Fifth, as mentioned above, we were unable to conclusively demonstrate the site where these agonists are mediating their effects, though we assume the depressant actions on EPSP amplitude by the three bulbospinal amines are mediated by presynaptic mechanisms. In contrast the facilitatory action of ACh might be partly accounted for by postsynaptic actions.

Finally, due to the difficulty associated with long-duration patch clamp recording in the deep dorsal horn, we were usually unable to investigate the effects of all four agonists on the properties of all DDH neurons recorded. In addition, since second messenger systems can initiate cascades that may in turn trigger long-term alterations in cellular properties some of which can be for hours, we may not have always recorded long-lasting changes, prior to applying a subsequent drug. This may partly account for the incomplete recovery/washout of synaptic responses following drug washout.

These limitations notwithstanding, the present observations demonstrate that the monoaminergic systems function to exert modulatory control over spinal sensory, which includes both diffuse and general actions.

3.0 Conclusion

In conclusion, in this thesis we first demonstrated the neuromodulatory interaction of all four biogenic amine transmitters on both synaptic and cellular properties of spinal dorsal horn neurons. In addition, we provide the first demonstration of 5-HT's control over

long-term alterations in synaptic efficacy. 5-HT acting at least partly via the 5-HT_{1A} and $_{1B}$ receptors can depress primary afferent input to dorsal horn neurons and in addition, influence the induction of afferent-evoked synaptic plasticity in spinal cord, favoring depression. Currently, little is known of the modulatory properties of descending monoamine transmitters on the control of the spinal sensory integrative apparatus (see Jankowska et al, 1997). However, the emergence of syndromes following spinal cord injury that involves abnormally high-gain sensory processing (spasticity and chronic pain) attest to the importance of descending inhibitory control on spinal cord function (Ashby and McCrea, 1987; Schouenbourg et al, 1992).

Despite these important observations on the role of monoaminergic transmitters in sensory processing, there are still many unanswered questions as it relates to their function in providing antinociception. For instance, in relation to 5-HT, NA and DA generally producing synaptic depression, are all three systems recruited following nociceptive input or are specific signals required to recruit specific pathways independently? Secondly, what are the physiological advantages of 5-HT, partly via the 5-HT_{1A} and $_{1B}$ receptor favoring the induction of LTD; will targeting these 5-HT receptors prove therapeutically beneficial as in pre-emptive analgesia? Though these studies do not attempt to provide answers to all these questions, we have provided novel information on the interactions of the bulbospinal monoaminergic transmitters. 5-HT, NA and possibly DA, transmitters implicated in the endogenous analgesic circuitry, may interact at the synaptic and possibly cellular level to produce depression of sensory, including 'pain' input to the spinal cord dorsal horn.

An interesting observation arising from this thesis is the ability of 5-HT to control the induction and expression of synaptic plasticity within the DDH of the spinal cord. Because the induction of LTP may underlie the 'memory traces' associated with chronic pain, it appears that 5-HT, by reducing the induction of LTP, may provide some therapeutic benefit in the control of chronic pain. The results of this study also suggest that separate brainstem monoaminergic systems can affect spinal sensory functions in a similar manner and in addition, demonstrate the importance of multiple modulatory systems in spinal sensory integration, including the control of nociception. This is an important step towards understanding how these transmitters interact to produce spinal inhibitory actions such as providing maximum antinociceptive effects.

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