A 5-HT Locomotor System: Identification, Actions through 5-$HT_{2A/7}$ Receptor Subtypes, and Effects on the Control of Locomotion

By
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ABBREVIATIONS

5-HT:	5- hydroxytryptamine, serotonin
5-HT+/+:	5-HT7 receptor wild type
5-HT-/-:	5-HT receptor knockout
5-HT-ir	5-HT immunoreactive
AC	adenylate cyclase
aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
AVP	adenosine vasopressin
cAMP	cyclic adenosine monophosphate
CNS:	central nervous system
CPG:	central pattern generator
DRN:	dorsal raphe nucleus
EAA:	excitatory amino acid
ENK	enkephalin
EMG:	electromyogram
EYGFP	enhanced yellow fluorescence protein
GABA	gamma-aminobutyric acid
Gi	gigantocellular reticular nucleus
Giα:	gigantoreticular nucleus pars-α
Gi-v:	gigantoreticular nucleus pars-ventralis
GS	gastrocnemius ankle extensor
HRP	horseradish peroxidase
MED	mediolateral medulla
MLR	mesencephalic locomotor region
MRN:	median raphe nucleus
NE:	norepinephrine
NMDA:	N-methyl-D-aspartate
NRM:	nucleus raphe magnus

PCR	polymerase chain reaction
PLC	phospholipase C
PPR:	parpyramidal region
PTCC	peak-to-trough correlation coefficient
RN	reticulospinal neurons
ROb:	nucleus raphe obscurus
RPa:	nucleus raphe pallidus
PGi	paragigantocellular reticular nucleus
PRV	pseudorabies virus
sAHP	slow afterhyperpolarization
SP	Substance P
TA	tibialis anterior ankle flexor
TRH	hyrotropin-releasing hormone
VLM	ventral lateral medulla

E	embryonic
C	cervical
T	Thoracic
L	lumbar
Co	coccygeal
P	postnatal

ABSTRACT

Locomotion is controlled by neural networks in the spinal cord called central pattern generators (CPG). Accumulated evidence suggests that serotonin also known as 5-hydroxytryptamine (5-HT) is effective in the production of locomotor-like activity in neonatal *in vitro* spinal cord preparations of the rodent. It is well known that the serotonergic descending command system(s) play an important role in the control of locomotion. In this study, we first localized a discrete population of 5-HT cells in the parapyramidal region (PPR) of the medulla that when stimulated, evoked locomotor-like activity in the neonatal rat brainstem-spinal cord preparation. To identify 5-HT receptor subtypes responsible for PPR-evoked locomotion, various antagonists of 5-HT_7 receptor subtypes were applied to the upper and lower lumbar segments of the spinal cord. We found that 5-HT_7 receptors most likely affect neurons of CPG in the low thoracic and upper lumbar cord, whereas 5-HT_{2A} receptors are more likely located more caudally involved in the output stage of the pattern generator for locomotion.

To further test the hypothesis that 5-HT_7 receptors are critical for the production of locomotion in mammalians, we first performed experiments on both neonatal and adult mice with a targeted knockout of the 5-HT_7 receptor gene (5-$HT_7^{-/-}$ mice). In the isolated spinal cord, locomotor-like activity induced by 5-HT in wild-type (5-$HT_7^{+/+}$) mice was disrupted by the specific 5-HT_7 receptor antagonist SB269970. In most cases, 5-HT evoked uncoordinated or synchronous rhythmic activity in 5-$HT_7^{-/-}$ mice. Then, we tested the hypothesis that 5-HT_7 receptors are involved in the control of voluntary locomotion in adult mice. Quantitative methods including kinematic

measurements and electromyographic (EMG) recording were utilized in this study. Our data suggest that intrathecal administration of SB269970 at the levels of L1/2 consistently disrupted locomotion in 5-$HT_7^{+/+}$ mice, producing "hyper-extension" of both hindlimbs or synchronous contraction of ipsilateral TA and GS muscle activity, resulting in slowed locomotion and "dragging" of the hindlimbs. 5-$HT_7^{+/+}$ mice walk normally and in most 5-$HT_7^{-/-}$ mice treated with SB-269970, no overt effects on locomotion were observed. These results strongly suggest that 5-HT_7 receptors play an important role in the control of locomotion in adult as well as neonatal animals and parallel descending mechanisms contribute to activation of the CPG for locomotion

GENERAL INTRODUCTION

It is well known that although the spinal cord central pattern generators (CPG) responsible for locomotion can produce phasic motor output independent of sensory or descending drive, sensory inputs and the supraspinal descending control play an important role in the initiation of locomotor activity (Grillner and Zangger 1984; Barbeau and Rossignol 1987; Jordan 1998; McCrea 2001; Whelan 2003). Previous studies have demonstrated that several specific regions in the brainstem are effective in the generation of locomotion by electrical or chemical stimulation in diverse vertebrate species (Atsuta et al. 1988; Grillner 1981; Shefchyk et al. 1984; Shik et al. 1966; Skinner and Garcia-Rill 1984). Among the spinal descending projections from the brainstem, 5-HT containing neurons originating from raphe nuclei and adjacent areas have been shown to project to the all laminae of the spinal cord (Johnson et al. 1993; Kuypers 1981; Steinbusch 1981; Skagerberg and Bjorklund 1985) and are involved in facilitation of the spinal CPG and motoneuron activity (Harris-Warrick et al. 1985; Jacobs and Fornal 1997; Jacobs and Fornal 1993; Schmidt and Jordan 2000). Several lines of evidence have shown that 5-HT agonists or transplantation of embryonic raphe cells improve locomotor movements in both acute and chronic spinal animals (Barbeau and Rossignol 1991; Feraboli-Lohnherr et al. 1997; Landry et al. 2006; Slawinska et al 2000). In the *in vitro* neonatal spinal cord preparation of the rodent, 5-HT appears to be one of the most effective neurochemicals for induction of locomotor-like activity. Therefore, it is critical to determine whether the activation of 5-HT containing neurons in the brainstem is sufficient to evoke locomotor-like activity and what 5-HT receptor

subtypes are involved in the initiation of locomotion evoked by brainstem stimulation and/or serotonin. Using the *in vitro* neonatal rat (wild type) and mouse (wildtype and 5-HT_7 $^{-/-}$), we demonstrated that 5-HT_7 receptor subtypes are essential in both brainstem stimulation and 5-HT induced locomotor-like activity. To further test the hypothesis that 5-HT_7 receptors are activated in the adult rodent during voluntary locomotion, we employed 5-HT_7 $^{-/-}$mice to perform a series of experiments to answer these questions.

1. Serotonin and activation of the spinal locomotor networks

Studies have shown that a wide range of neurotransmitters are able to elicit locomotor-like activity or activate locomotor networks when applied to the isolated spinal cord of a variety of vertebrate species from protovertebrates to mammals (Bonnot et al. 2002; Branchereau et al. 2000; Cohen and Wallen 1979; Jiang et al. 1999; Kudo and Yamada 1987; Nishimaru et al. 2000; Smith and Feldman 1987; Whelan et al. 2000). These neurotransmitters include excitatory amino acids (EAA) such as glutamate, NMDA (Cohen and Wallen 1979; Kudo and Yamada 1987; Cowley and Schmidt 1994), norepinephrine (NE) (Kiehn et al. 1999; Sqalli-Houssaini and Cazalets 2000; Gabbay and Lev-Tov 2004b), 5-HT (Cazalets et al. 1992; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996), dopamine (Smith et al. 1988; Kiehn and Kjaerulff 1996), and acetylcholine (Cowley and Schmidt 1994; Cowley et al. 2005). Neuropeptides have been shown to be modulate spinal CPG for locomotion (Barthe and Clarac 1997; Pearson et al. 2003; Barriere et al. 2005). Spinal locomotor networks could also be facilitated by intrathecal or intraperitonial administration of EAA (e.g. NMDA) and monoaminergic agonists both in the intact

and chronic spinalized animals (Chau et al. 1998; Douglas et al. 1993; Giroux et al. 2003;Guertin and Steuer 2005;Kim et al. 1999). Several lines of evidence have shown that 5-HT is more effective than other monoamines in inducing reliable locomotor-like activity in both the neonatal rat (Cazalets et al 1992; Cowley and Schmidt 1994; Schmidt and Jordan 2000) and neonatal mouse (Nishimaru et al 2000; Branchereau et al 2000; Madriaga et al. 2004).

The location of spinal CPG for locomotion in the rodents has been extensively studied (Bertrand and Cazalets 2002; Bonnot et al 2002; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Nishimaru et al 2000; Schmidt and Jordan 2000). Using compartmentalization of the isolated rat spinal cord, Cazalets et al (1995) proposed that neural networks generating hindlimb locomotion were restricted to the L1/L2 segments. Following this initial report, a consensus has formed suggesting that the mammalian locomotor networks are not restricted to this region and instead exhibit a rostrocaudal gradient of excitability throughout the low thoracic and the lumbar cord (Bertrand and Cazalets 2002; Bonnot, Whelan et al 2002; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997). Effects of 5-HT on locomotor CPG have been extensively examined (Beato and Nistri 1998; Schmidt and Jordan 2000; Pearlstein et al. 2005; Madriaga et al 2004). In a study using mechanical lesion techniques, it was demonstrated that rhythm-generating networks for locomotion were distributed throughout the caudal thoracic and the entire lumbar region in the ventral third of the spinal cord (Kjaerulff and Kiehn 1996). Cowley and Schmidt (1997) showed that in the neonatal rat spinal cord 5-HT-induced locomotor-like activity can only be recorded when at least one low thoracic

segment was kept intact with the lumbosacral cord. Nishimaru et al (2000) reported that in the neonatal mouse after transection of the mid-lumbar cord, the 5-HT induced locomotor rhythm persisted in the upper lumbar cord, but not in the caudal cord. Recently, it has been shown that monoamines (5-HT and dopamine) may establish rostrocaudal gradients of excitability for locomotor networks in the thoracolumbar spinal cord of the mouse (Christie and Whelan 2005). Transplantation of embryonic raphe cells at T11 in the adult chronic spinal rat substantially promoted hindlimb movements, whereas animals transplanted at T9 developed limited and disorganized movements (Gimenez y Ribotta et al. 2000). Improved locomotor activity following grafting of embryonic raphe nuclei cells to the spinal rat can be impaired by systemical administration of a 5-HT_2 receptor antagonist (Majczynski et al. 2005). Taken together, these studies support the idea that 5-HT plays a critical role in initiation of locomotor-like activity and that the 5-HT sensitive networks for locomotion are distributed throughout the low thoracic and lumbar spinal cord, with the low thoracic and upperlumbar cord having a greater rhythmogenic capability than more caudal segments (Schmidt and Jordan 2000).

2. Topography and projections of serotonin neurons in the brainstem

Serotonergic (5-HT) terminals are found throughout the CNS, and the 5-HT cell bodies giving rise to these axons are confined almost exclusively to the brainstem, i.e., raphe nuclei and the adjoining ventral reticular formation. According to their location and axonal projections, 5-HT cells are classically divided into the rostral (superior) and caudal (inferior) groups. Using histofluorescence techniques,

Dahlstrom and Fuxe (1964) were first to describe the distribution of 5-HT containing cells in the rat brainstem and classified these neurons into nine (B1- B9) subpopulations distributed in a caudal to a rostral direction.

Rostral group:

The rostral group is composed of four nuclei: 1) the caudal linear nucleus (CLN, B8), median raphe nucleus (MRN, B8/B5), dorsal raphe nucleus (DRN, B7/B6) and B9 neurons. These clusters of 5-HT neurons are located in the mesencephalon and the rostral pons (See Fig. 1 modified from Jacobs 1992) and send their axons to the forebrain. Several studies using immunocytochemical methods found that 5-HT immunoreactive (5-HT-ir) fibers are detected in virtually all regions of the brain examined except for major fiber tracts, implicating involvement in multiple CNS functions (Azmitia and Gannon 1986; Hornung 2003; Jacobs and Azmitia 1992; Lidov et al. 1978; Steinbusch 1981). Studies making use of anterograde and retrograde tracing techniques have shown that high levels of 5-HT-ir terminals are detected in the neocortex, hippocampus, nucleus accumbens, thalamus, hypothalamus, amygdala, cerebellum and basal ganglia (Azmitia and Gannon 1986; Azmitia and Segal 1978; Dahlstrom and Fuxe 1965; Jacobs and Azmitia 1992; Vertes 1991; Steinbusch 1981; Waterhouse et al. 1986). Studies on the functioning of the ascending serotonergic system suggest that the ascending 5-HT system participates in the modulation of many functions in the brain, such as affective behaviors, mood, circadian rhythms, thermoregulation, sleep, neuroendocrine

response, learning and memory (Barnes and Sharp 1999; Green 2006; Goodwin and Green 1985; Sodhi and Sanders-Bush 2004; Hoyer et al. 2002).

***Caudal group*:**

Anatomically, the caudal group of serotonergic neurons comprises four nuclei: raphe magnus (NRM), raphe obscurus (NRo), raphe pallidus (RPa) and ventrolateral medulla, which are located in the medulla and caudal pons (Fig. 1). The caudal medullary raphe nuclei account for approximately 30% of the total 5-HT neurons in the CNS (Bjorklund and Skagerberg 1982). The caudal clusters of 5-HT cells mainly send their axonal collaterals to the brainstem and the spinal cord. Studies in cats have shown that the caudal raphe nuclei and the adjoining reticular formation innervate all laminae of the spinal cord (Holstege et al. 1979; Holstege and Kuypers 1982; Martin et al. 1978). Similar results are also conducted in the monkey (Kneisley et al. 1978), rat (Bowker et al. 1981b; Skagerberg et al. 1985), mouse (VanderHorst and Ulfhake 2006), opossum (Crutcher et al. 1978) and other species (Cabot et al. 1982; Ten Donkelaar 1982). There is a great deal of evidence showing that the serotonergic descending system is closely involved in a variety of spinal activities, such as nociception processing, modulation of autonomic functions and control of motor activity (Arita et al. 1995; Lopez-Garcia 2006; Hermann et al. 2003; Holmes 2005; Millan 2002; Schmidt and Jordan 2000; Wallis et al. 1991).

Nucleus Raphe Magnus

The nucleus raphe magnus (NRM) together with gigantoreticular nucleus pars-α (Gi-α) forms the serotonergic cell group B3. NRM is the largest 5-HT containing nucleus of the caudal group. It extends rostrocaudally from the caudal end of the facial nucleus to the rostral superior olive (Bowker et al 1981b; Dahlstrom and Fuxe 1965; Jones and Light 1992; Skagerberg et al 1985). It is continuous at some points with both the nucleus raphe obscurus (ROb) and raphe pallidus (RPa) (See Fig. 1). The descending fibers of 5-HT cells from NRM travel via the ipsilateral dorsolateral funiculus to all levels of the spinal cord. The densest innervations of 5-HT terminals from NRM are seen in the dorsal horn, laminae I, II and V (Allen and Cechetto 1994; Bernau et al. 1993; Jacobs and Azmitia 1992; Jacobs et al. 2002). Studies using the retrograde labeling technique in the rat have demonstrated that more than 60-80% of the descending fibers from NRM are serotonergic, while nearly 90% of serotonergic neurons in NRM project to the spinal cord (Bowker et al 1981b; Bowker and Abbott 1990). Jones and Light (1992) reported that only about 50% of the spinally projecting neurons in NRM were serotonergic, which represented almost half of 5-HT neurons in NRM. Tracing and functional studies show that neurons in NRM mainly project to the dorsal horn of the spinal cord via dorsolateral pathways (Skagerberg and Bjorklund 1985; Hentall et al. 2001; Holstege and Kuypers 1982; Hornung 2003; Jacobs and Azmitia 1992). 5-HT cell in NRM have direct contact with neuons of the dorsal horn and are proposed to play a critical role in analgesia (Basbaum et al. 1976; Bett and Sandkühler 1995; Gao and Mason 2001b; Jones and Light 1990). There is also evidence that serotonergic

neurons in NRM play a role in modulation of autonomic functions (Gao and Mason 2001a; Zermann et al. 1998).

Nucleus Raphe Pallidus

Nucleus Raphe Pallidus (RPa) contains a cluster of the ventral midline 5-HT cells (B1), which are located between the pyramidal tracts and extend from Nerve XII to the rostral pole of the inferior olive (Fig 1). The descending fibers from RPa course bilaterally through the ventral and ventrolateral funiculi innervating all the levels of the spinal cord. Although RPa largely projects to the intermediolateral area, terminals are also found in the ventral horn (lamina IX, X) both at the cervical and lumbar segments, suggesting its potential involvement in the autonomic and motor functions (Basbaum and Fields 1978; Holstege and Kuypers 1987b; Loewy 1981). Studies using immunocytochemistry combined with retrograde tracers showed that more than 70% of the descending fibers in RPa are serotonergic and mainly project to the intermediate area and the ventral horn of the spinal cord of the rat (Bowker et al 1981b; Bowker et al. 1982; Skagerberg and Bjorklund 1985). Jones and Light (1992) reported that approximately 50% of the cells in RPa have projections to the lumbar cord and almost half of these cells are serotonergic.

Nucleus Raphe Obscurus

Nucleus Raphe Obscurus (ROb, B2) lies dorsal to the pyramids and ventral to the fasciculus longitudinalis medialis. It extends from the caudal end of the pons to the

pyramidal decussation (Fig. 1) (Bowker et al 1981b; Dahlstrom and Fuxe 1965; Steinbusch 1981). Cells in ROb project to the spinal cord bilaterally mainly through the ventral funiculus, and reach all levels of the spinal cord with the predominant axonal collaterals to the intermediolateral area and the ventral horn (Allen and Cechetto 1994; Leong et al. 1984; Loewy 1981; Manaker et al. 1992). For example, descending projection from ROb to the lumbar ventral horn (lamina XI, X) were observed in the cat (Martin et al 1978; Basbaum and Fields 1979), rat (Holstege 1987; Loewy 1981) and monkey (Bowker et al 1982). Similar to RPa, about 50% of cells in ROb project to the spinal cord and half of these are serotonergic (Jones and Light 1992). The consistency of the spinal innervations between RPa and ROb suggests that both of the nuclei may play similar roles in the control of the spinal activities, such as the regulation of autonomic function and motor control (Jacobs et al 2002).

The parapyramidal region

The parapyramidal region (PPR) or pararaphe region refers to a cluster of neurons in the ventral medulla lateral to the pyramidal tracts (Helke et al. 1989; Jones and Light 1992). The cells in the PPR consist of several separate subgroups of nuclei that caudorostrally include the ventral medullary reticular nucleus (MdV), the gigantocellular reticular nucleus (Gi), the gigantoreticular nucleus pars-α (Gi-α) and –ventralis (Gi-v) and the paragigantocellular reticular nucleus (PGi) (Lakke 1997), containing the second largest population of 5-HT neurons in the medulla (Hornung 2003). Anatomically, the region of PPR includes the ventral lateral medulla (VLM),

located close to the ventral surface of the brainstem. VLM encompasses three zones in a caudal to rostral direction referred to as L (Loeschcke 1970), S (Schlafke 1970), and M (Mitchell 1963) areas, respectively (Loewy and McKellar 1981). Neurons in the PPR project to the intermediolateral cell column of the thoracic spinal cord, sacro-sympathetic nucleus (Hermann et al 2003), nucleus of the solitary tract (NTS) (Helke et al 1989) and dorsal vagal complex (Lynn et al. 1991). Using transneuronal viral tracing techniques, several investigators have reported that virus-positive neurons were found in raphe and the PPR after microinjection of pseudorabies virus (PRV) into the tail of rats (Cano et al. 2003; Smith et al. 1998; Toth et al. 2006). Toth et al (2006) demonstrated that the majority of the small ventrally located PRV+ve neurons also synthesize 5-HT, and these were in the first wave of brainstem neurons labeled after tail injection of virus. These morphological features provide evidence that the PPR may play an important role in autonomic functions (Helke et al 1989). For example, firing was elicited in the motor branch of the pudendal nerve following ipsilateral or contralateral stimulation of the ventral medullary reticular formation (Johnson and Hubscher 2000). Activation of the neurons in the rostral PPR increased mean arterial blood pressure in adult rats (Howe et al. 1983). Recently, Yang et al (2000) reported that microinjection of kainite into the PPR increased gastric acid secretion, an effect through activation of the vagal reflex pathway.

Several lines of evidence have shown that 5-HT neurons in the VLM may play an important role in the regulation of respiration (Haxhiu et al. 2001; Richerson et al. 2001). There is a great deal of evidence derived from experiments both on the

whole animal and *in vitro* brainstem preparations that 5-HT neurons in the VLM are highly sensitive to changes in CO_2 and $[H]^+$ (Lipscomb and Boyarsky 1972; Miles 1983; Wang et al. 2001). Some serotonergic neurons are in close apposition with the large branches of the basilar artery and they are highly pH chemosensitive (Wang et al 2001). These data suggest that 5-HT cells in the VLM may serve as central chemoreceptive neurons (Haxhiu et al 2001; Paterson et al. 2006; Richerson et al 2001). In contrast to the idea that 5-HT containing neurons in VLM function as chemoreceptors, Mulkey et al (2004) reported that glutamatergic neurons rather than serotonergic neurons in VLM of rats were preferentially activated in response to increase of CO_2.

Although there is a large amount of evidence demonstrating that serotonergic and nonserotonergic cells in the PPR have direct projections to the intermediate area and ventral horn of the spinal cord, little is known about the involvement of neurons of the PPR in the control of locomotion. Immnunocytochemical studies have demonstrated that more than 70% of the neurons in the PPR that project to the lumbar cord contain 5-HT immunoreactivity (Bowker et al 1981b; Bowker and Abbott 1990). Moreover, in a study using microinjection of WGA-HRP gold into the lumbar cord of rats in combination with serotonin immnunohistochemistry, Jones and Light (1992) reported that 59% of the serotonergic neurons in the para-raphe zone (i.e., the PPR) have axonal collaterals to the lumbar cord. Kerman et al (2003) reported that neurons in the PPR were trans-synaptically labeled after injection of pseudorabies virus in to the gastrocnemius muscle of the rat. An experiment done in adult rats showed that cells in the PPR expressed c-fos labeling

after a locomotion task (Iwamoto et al. 1996). Preliminary experiments from our lab showed that c-*fos* positive cells in the PPR of the adult rat were significantly increased after locomotion, but only few *c-fos* labeling was detected in RPa, and none in ROb or NRM (Livingston et al, unpublished observation). There is evidence that PPR stimulation results in release of 5-HT, dopamine and noradrenaline in the thoacolumbar spinal cord (Fyda et al. 1997; Jordan and Schmidt 2002). Taken together, all these studies suggest that cells in the PPR may play an important role in the control of locomotion.

3. Putative neurotransmitters in the caudal raphe nuclei and the PPR

Although the majority of 5-HT containing neurons in the CNS are found in the raphe nuclei and the adjacent regions (e.g. the PPR), many nonserotonergic neurons in the above nuclei have projections in parallel with 5-HT fibres to the forebrain regions and the spinal cord (Allen and Cechetto 1994; Dahlstrom and Fuxe 1965; Jones and Light 1992; Loewy 1981; Holstege and Kuypers 1987b; Skagerberg and Bjorklund 1985). There is evidence that several peptides are present in spinal projecting neurons of the caudal raphe nuclei and the PPR (Cullheim and Arvidsson 1995; Tallaksen-Greene et al. 1993). These neuropeptides include substance P (SP) (Wessendorf and Elde 1987), thyrotropin-releasing hormone (TRH), enkephalin (ENK), somatostatin, cholecystostain and galanin etc (Arvidsson et al. 1992a; Bowker et al. 1987) (Mantyh and Hunt 1984; Marson and Loewy 1985; Skofitsch and Jacobowitz 1985; Holstege and Kuypers 1987b). Studies have shown that a large number of 5-HT neurons also contain additional neurotansmitters in these

regions, including glutamate (Belin et al. 1981; Nicholas et al. 1992), GABA (Millhorn et al. 1988; Kachidian et al. 1991) and peptides (Holstege and Kuypers 1987b; Kachidian et al 1991). All the peptides mentioned peptides have been shown to be co-localized with serotonin (Bowker et al. 1983; Johansson et al. 1981; Nicholas et al 1992; Kachidian et al 1991). The coexistence of 5-HT with both SP and TRH in one neuron of the medullary raphe and adjacent reticular formation was also reported in the rat (Johansson et al 1981) and cat (Dean et al. 1993). In accordance with these observations, serotonergic fibers in the ventral horn of the spinal cord contain collateral peptides, for instance SP, TRH and ENK (Arvidsson et al. 1992b; Bowker et al. 1981a; Wu et al. 1993). There is evidence that bulbospinal 5-HT systems provide direct synaptic contact with midlumbar premotoneurons (Maxwell et al. 2000) and motoneurons in the cat (Alvarez et al. 1998; Holstege and Kuypers 1987b; Okado et al. 1992). Functional effects of the descending serotonergic system on locomotion have been investigated using various experimental designs. In general, 5-HT mediates an excitatory effect on the putative interneurons of the locomotor CPG (Carlin et al. 2006; Jankowska et al. 2000) (Zhong et al. 2006; Parry and Roberts 1980) and modulates the excitability of motoneurons (Alford et al. 2003; Rekling et al. 2000; Wang and Dun 1990; White et al. 1996; White and Fung 1989; Wallis et al 1991). It is quite likely that activation of multiple neuromodulators acting through diverse 2^{nd} messenger pathways act to modulate the locomotor pattern. For example, neuropeptides such as SP, TRH may facilitate the excitatory effect of 5-HT on motoneruons (White 1985; White et al 1996) and modulate locomotor activity in the neonatal rat (Barthe

and Clarac 1997; Barriere et al 2005), suggesting that some peptides play an role in the control of the neural network for locomotion. Studies in the lamprey have shown that neuromodulators interact and are critical for the development of dynamic synaptic and network plasticity involved in locomotion (Svensson et al. 2001).

4. Descending pathways of serotonergic neurons in raphe nuclei and the PPR

Anatomical studies employing retrograde and antegrade transport techniques have shown that the caudal raphe nuclei (NMR, ROb, RPa) and the adjacent parts (the PPR) account for almost all the 5-HT innervation of the spinal cord (Basbaum and Fields 1979; Bowker et al 1981b; Jacobs and Azmitia 1992; VanderHorst and Ulfhake 2006). 5-HT cells in the NMR project mainly to the dorsal horn via the dorsolateral funiculus. There is substantial evidence that serotonergic projections are found in the whole dorsal horn with the most numerous terminals in lamina I and II, where many nociceptive projection neurons are located (Light et al. 1983; Marlier et al. 1991a; Kwiat and Basbaum 1992; Lopez-Garcia 2006). In the dorsal horn, 5-HT terminals appear to synapse directly on spinocerebellar neurons (DSCT), which receive converging sensory information from various sensory receptors (Jankowska et al. 1997; Maxwell and Jankowska 1996). The more caudally located ROb, RPa and the PPR project mainly to the intermediate and ventral horn via the lateral and ventral funiculi (Allen and Cechetto 1994; Azmitia and Gannon 1986; Jacobs and Azmitia 1992; Helke et al. 1997). There is a large body of evidence that serotonergic projections to the intermediate and ventral horn are involved in the

regulation of autonomic function (Cabot et al. 1979; Gilbey et al. 1981; Newton and Hamill 1989) and somatic motor activity (Cao et al. 2006; Commissiong 1981; Jacobs et al 2002; Nicholas et al 1992; Oatway et al. 2005).

The early studies on the distribution of 5-HT terminations in the spinal cord have shown a high density of 5-HT terminals throughout the dorsal horn, intermedio-lateral cell column and in the anterior two-thirds of the ventral horn and central canal (Carlsson et al. 1964; Dahlstrom and Fuxe 1965; Jacobs and Azmitia 1992). Using horseradish peroxidase (HRP) tracing in combination with 5-HT immunofluorescence techniques in the adult rat, 5-HT neurons in the B1 and B2 groups project predominantly to the ventral half of the spinal cord, while the dorsal part of the cord mainly receives 5-HT terminals from serotonin cells of the B3 group (Skagerberg and Bjorklund 1985). Microinjections of WGA-HRP gold into the lumbar cord of rats demonstrated that 53% and 59% of the serotonergic neurons in the raphe nuclei and the PPR project to the lumbar cord (Jones and Light 1992). Alvarez et al (1998) reported that the bulbspinal 5-HT system provides direct synaptic input to spinal motoneurons that innervate hindlimb muscles of the cat. Similar projection pattern of 5-HT descending fibers was also found in the mouse (VanderHorst and Ulfhake 2006). All these studies suggest that the raphe and PPR neurons may play a critical role in modulating spinal cord interneurons and motoneurons, thus affecting the spinal CPG for locomotion and motor output. Nevertheless, the precise projection pattern of 5-HT neurons to motoneurons or other ventral horn neurons is not known.

5. Ontogeny of descending 5-HT projections to the spinal cord

The development of descending 5-HT projections to the spinal cord has been studied in detail in pre and post-natal rats (Bregman 1987; Rajaofetra et al. 1989; Ziskind-Conhaim et al. 1993) and mice (Ballion et al. 2002). In the rat, serotonergic neurons are seen in the brainstem at E11-14 and begin to invade the spinal cord and upper thoracic levels at E15 via the lateral and ventral funiculi. Axons of 5-HT neurons reach lower thoracic and lumbar levels at E16-17 (Ziskind-Conhaim et al 1993). Ingrowth of serotonergic fibers into the gray matter of the spinal cord occurs by collateral branching approximately 1 day after arrival of the first fibers at each level of the spinal cord and the lower thoracic and lumbar gray matter is not invaded until E17-18 (Lakke 1997; Fahn et al. 1995; Rajaofetra et al 1989). A considerable number of serotonergic fibers reach the lumbar cord and have close apposition to motoneuron soma by P2 (Lakke 1997; Ziskind-Conhaim et al 1993). The growth of axons towards the dorsal horn throughout the whole spinal cord is not noticeable until E19. 5-HT axonal collaterals start to proceed diffusely at P5 and ramify profusely in lamina I and II at P7. The adult projection pattern of serotonergic fibers in the spinal cord of the rat is not fully established until P21 (Rajaofetra et al 1989; Lakke 1997). In the mouse, 5-HT immunoreative axons begin to invade the very rostral portion of the cervical cord at E12.5 and reach the thoracic level at E16.5 (Ballion et al 2002). The descending 5-HT fibers are detected in the lumbar cord by E16.5. Similar to rats, 5-HT axonal collaterals start to invade the intermediate and ventral gray matter at E16-17. Ingrowth of axons towards the dorsal horn throughout spinal cord is detectable at P0. By P10, dense 5-

HT innervations are found in all gray matter. Functional studies suggest that changes of 5-HT levels during the developmental stage interefere with the maturation of CPG networks for locomotion (Cazalets et al 2000; Nakajima et al 1998).

In addition to descending 5-HT fibers, a few 5-HT containing neurons have been identified within the spinal cord in many species including the lamprey (Harris-Warrick et al 1985; van Dongen et al. 1985), stingray (Ritchie and Leonard 1982), garfish (Parent and Northcutt 1982), chick (Sako et al. 1986; Niitsu et al. 1995), rat (Newton et al. 1986; Newton and Hamill 1988), monkey (Lamotte et al. 1982) and mouse (Ballion et al 2002; VanderHorst and Ulfhake 2006). In the rat, 5-HT intraspinal cell bodies are not seen until P0, and are found dorsal or lateral to the central canal in laminae VII and X in all spinal segments except cervical levels with the majority (73%) located between T3 and Co1 (Newton et al. 1989). Intraspinal 5-HT neurons are also detected in spinal autonomic areas and may play a role in sympathetic function (Newton et al 1986; Newton and Hamill 1988). Interestingly, 5-HT-ir cell bodies are restricted to the sacral spinal cord from E16.5 to P10 in the mouse (Ballion et al 2002). At postnatal stages, these cells are mainly located in the ventral gray matter and close to the central canal. The physiological function of intraspinal 5-HT cells in mammals is poorly understood. The role of intraspinal 5-HT-ir neurons are proposed to a large extent according to their morphological location. For example, a high density in ventral motor areas of the spinal cord suggests modulation of the CPG for locomotion in the lamprey (Harris-Warrick and Cohen 1985). In mammals such as the rat (Newton et al 1986), monkey (Lamotte et

al 1982) and mouse (Ballion et al 2002), these neurons have been assumed to act as interneurons involved in the regulation of autonomic function.

6. 5-HT receptor subtypes in the spinal cord and locomotor-like activity

So far seven families of 5-HT receptors have been recognized with at least fourteen distinct receptor subtypes, classified based on operational, structural and transductional information (Humphrey et al. 1993; Barnes and Sharp 1999; Hoyer and Martin 1997; Hoyer et al 2002). All 5-HT receptors with the exception of 5-HT_3 belong to G-protein coupled receptor superfamily, producing second messengers that regulate cellular functions via phosphorylation and dephosphorylation of intracellular proteins (Pauwels 2000). As shown in Fig. 2 (reprinted from Hoyer et al 2002), five families of G protein-coupled 5-HT receptors (5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7) regulate two major intracellular second messenger pathways, i.e. adenylate cyclase (AC)/cAMP and phospholipase C (PLC), whereas the 5-HT_3 receptor is a ligand-gated ion channel activated via direct binding of 5-HT.

5-HT_1 receptors

The 5-HT_1 receptor group consists of five receptor subtypes (5-HT_{1A}, 5-HT_{1B}, 5-5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F}) and couple to Gi/o to inhibit AC when they are activated (Fig. 2). 5-HT1 receptor subtypes are believed to be mainly located on primary afferent terminals as well as dorsal horn neurons and play an important role in the inhibition of nociceptive transmission (Barnes and Sharp 1999; Millan 2002). In previous

studies, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} receptors were found in the dorsal horn of the rat (Huang and Peroutka 1987; Marlier et al. 1991b; Thor et al. 1993), with a high concentration of 5-HT_{1A} in superficial layers and the central canal region (Coggeshall and Carlton 1997; Giroux et al. 1999). 5-HT_{1B} receptors are more dense in laminae I, III and IV than in lamina II (Thor et al 1993). Using autoradiographic mapping techniques, Castro et al (1997) reported that 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} are present in the dorsal horn of human beings. 5-HT_1 receptors are found in the ventral horn motoneurons of the rat spinal cord (Ridet et al. 1994). Moreover, 5-HT_{1A} receptor subtypes are diffusely labeled throughout the axonal hillock and the soma of the spinal motoneurons (Azmitia et al. 1996; Kheck et al. 1995). In spinal motoneurons of the neonatal rat, 5-HT facilitates the non-specific cationic current (I_h), activated by hyperpolarization (Takahashi and Berger 1990; Kjaerulff and Kiehn 2001) and inhibits leak K+ conductance mediated by 5-HT_{1A} receptors (Ziskind-Conhaim et al 1993). Gajendiran (2006) reported that 5-HT_{1A} receptors mediate excitability of lumbar Renshaw cells in rats. In cultured mouse spinal neurons, it has been shown that serotonin evoked a decrease in K^+ conductance and an increase in input resistance mediated by activation of a 5-HT_{1A}-like receptor (Legendre et al. 1989).

There is evidence that 5-HT_1 receptors play a role in the control of locomotion. For instance, 5-HT_1 receptor antagonist may block 5-HT-induced locomotion in rats (Cazalets et al 1992). In spinalized rats, activation of 5-HT_{1A} receptor subtypes, but not 5-HT_{1B} facilitates recovery of locomotor activty (Antri et al. 2003; Landry et al 2006). The mechanisms underlying the effect of 5-HT_1 receptors on locomotion remain unclear. Studies in the spinal neurons of the lamprey demonstrated that 5-

HT_{1A} receptors were critical for stabilizing locomotor rhythm (Zhang and Grillner 2000), an effect realized by reducing the conductance of N-type Ca^{2+}-dependent K^+ channels (K_{Ca}) underlying the slow afterhyperpolarization (sAHP) (Wikstrom et al. 1995; Hill et al. 2003). In the turtle, activation of 5-HT_{1A} receptors increases excitability of spinal motorneurons (Perrier et al. 2003). In the neonatal rat, 5-HT slows NMDA-induced locomotor rhythm, an effect mediated by 5-HT_{1A} and 5-$HT_{1B/D}$ receptors (Beato and Nistri 1998). Recently, Schwart et al (2005) demonstrated that 5-HT prolonged the frequency of NMDA-induced ventral root bursting by presynaptically activating 5-HT_{1D} receptors.

5-HT$_2$ receptors

This class is composed of three receptor subtypes, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors, which exhibit a high degree of homology in sequence (46-50%) identity and couple to $G_{q/11}$ to increase the hydrolysis of phospholipids (Fig 2) and elevate intracellular Ca^{2+} concentration. Using polymerase chain reaction (PCR) combined with *in situ* hybridization, all three 5-HT_2 receptor subtypes are detected in the spinal cord of the rat, cat, monkey and human (Helton et al. 1994). 5-HT_{2C} receptors are expressed at high levels in most of the gray matter, except for lamina II, whereas 5-HT_{2A} receptors are densely expressed in lamina IX (Fonseca et al. 2001; Pompeiano et al. 1994). An immunocytochemical study of 5-HT_{2A} receptors in the rat reveals that 5-HT_{2A} receptors are densely labeled in lamina II and IX mainly at postsynaptic sites (Doly et al. 2004). Activation of 5-HT_2 receptors depolarizes the membrane potential and increase excitability of spinal motoneurons (Miller et al. 1996; Rekling et al

2000). 5-HT enhances plateau potentials in turtle spinal motoneurons by facilitating L-type Ca^{2+} channels via 5-HT_2 receptors (Perrier and Hounsgaard 2003). In the *in vitro* neonatal spinal cord of rodents, 5-HT induced locomotor-like activity can be blocked by 5-HT_{2A} and5-HT_{2C} receptor antagonists (Cazalets et al 1992; Bracci et al. 1998; MacLean et al. 1998; Madriaga et al 2004). In the adult mice spinal cord transected at the low thoracic level, intraperitoneal injection of a 5-$HT_{2A/2C}$ receptor agonist, quipazine, but not a 5-$HT_{2B/2C}$ receptor agonist, induces locomotor activity (Landry and Guertin 2004). Facilitation of 5-HT_2 agonists on recovery of hindlimb movements was observed in spinal cats (Barbeau and Rossignol 1990), and in adult and neonatal spinal rats (Kim et al 1999; Antri et al. 2002). Studies in mouse spinal cord preparations show that the 5-HT_2 receptor agonist, α-methyl-5HT, is able to elicit locomotor-like activity which may be disrupted by a 5-HT_{2A} receptor antagonist, ketanserin (Madriaga et al 2004). There is evidence that the 5-HT_2 receptor agonist DOI, which exhibits high affinity for 5-HT_{2A} receptors, depolarizes the resting membrane of spinal motoneurons, an effect that is blocked by the 5-HT_{2A} receptor antagonists ketanserin and spiperone (Wang and Dun 1990) and facilitates sodium persistant inward current (PIC) of spinal motoneurons (Harvey et al. 2006). In the lamprey, 5-HT_2 receptor agonists reduce sAHP in spinal neurons during locomotion, which is counteracted by 5-HT_2 receptor antagonists (Wikstrom et al 1995). Therefore, it is clearly that the 5-HT2 receptor family plays an important role in the control of both motoneurons and CPG networks, but the contribution of specific receptor subtypes to spinal motor control remains to be elucidated.

5-HT$_3$ receptors

As ligand-gated ion channels, 5-HT$_3$ receptors found both in CNS and PNS comprise three receptor subtypes, 5-HT$_{3A}$, 5-HT$_{3B}$ and 5-HT$_{3C}$ (Fig. 2) (Barnes and Sharp 1999;Davies et al. 1999). Activation of these receptors triggers rapid depolarization due to a transient inward current and subsequent opening of nonselective cationic channels (Na^+, Ca^{2+} influx, K^+ efflux) (Hoyer et al 2002; Maricq AV 1991). There is evidence that 5-HT$_3$ receptors are diffusely expressed in the gray matter of the rat spinal cord and show a dorsoventral gradient of expression (Fonseca et al 2001). Following rhizotomy, 5-HT immunostaining is markedly decreased in the superficial layers of the dorsal horn suggesting a preferential location on terminals of primary afferent fibers (Kia et al. 1995). Using immunocytochemical techniques, Morales et al (1998) reported that the intensity of 5-HT$_3$ receptor labeled neurons in the ventral horn and intermediate gray matter is much stronger than that in the dorsal horn of the lumbar spinal cord. However, autoradiographic studies failed to detect 5-HT$_3$ receptor binding sites in the ventral horn of the spinal cord (Laporte et al. 1992). Although the 5-HT$_3$ receptor is involved in mediation of pain tansmission (Holden et al. 2005; Millan 2002), the role of this receptor in the control of locomotion is controversial. Early studies have proposed that 5-HT$_3$ receptor antagonists have no effect on 5-HT-induced locomotor-like activity in the neonatal rat (Cazalets et al 1992) and lamprey (Wikstrom et al 1995). 5-HT$_3$ receptor antagonists have no effect on 5-HT induced excitability in cultured mouse spinal neurons (Legendre et al 1989). However, experiments performed on complete paraplegic mice show that a 5-HT$_3$ receptor

agonist, SR57227A, can induce rhythmic locomotor-like activity, suggesting a possible contribution in locomotor generation (Guertin and Steuer 2005). The differences in 5-HT_3 receptor effect on locomotion may be due to neonatal versus adult preparations. It is possible that the developmental expression of 5-HT_3 receptors is later than other receptor subtypes.

5-HT_{4-6} receptors

These three receptors exert their functions by coupling to adenylate cyclase (Fig. 2). While studies by Northern Blot, *in situ* hybridization or autoradiographic labeling have shown that 5-HT_5 and 5-HT_6 receptors are found in various areas of CNS including the spinal cord of the mouse, there is no report of 5-HT_4 receptors in the spinal cord (Barnes and Sharp 1999; Gerard et al. 1996; Gérard et al. 1997; Hoyer et al 2002; Nelson 2004; Rees et al. 1994). An immunocytochemical study shows that 5-HT_{5A} receptors are found in the dorsal horn, intermediate area and ventral horn of the rat spinal cord (Doly et al. 2004). There is evidence for 5-HT_6 receptor-like immunoreactivity in the CNS, especially in hippocampus, hypothalamus and in the gray matter of the rat cervical spinal cord, but the precise location in the cord was not specified (Gérard et al 1997; Gerard et al 1996). To date there is no evidence for the involvement of these receptor subtypes involved in the modulation of locomotion.

5-HT_7 receptors

5-HT_7 receptors were discovered almost simultaneously by several groups in 1993 (Bard et al. 1993; Lovenberg et al. 1993; Ruat et al. 1993) and are positively linked to cAMP (Fig. 2) (Adham et al. 1998; Hoyer et al 2002). Studies in the brain of numerous species have shown that the 5-HT_7 receptor mRNA levels are highest in the thalamus, hippocampus and hypothalamus. In the spinal cord, 5-HT_7 receptors are found in the dorsal horn and may be involved in pain control (Doly et al. 2005; Gustafson et al. 1996; Meuser et al. 2002). For example, intrathecal adminitration of the 5-HT7 receptor antagonist, SB269970 inhibits antinociceptive effects of systemic morphine (Dogrul and Seyrek 2006). Only recently, involvement of 5-HT_7 receptors in the control of locomotion have been considered significantly important (Alford et al 2003; Cina and Hochman 1998; Hochman et al. 2001; Schmidt and Jordan 2000). Using immunocytochemistry, 5-HT_7 receptors are found in the intermediate area and the ventral horn of the low thoracic and all lumbar segments of the rat, and a large number of locomotor activity-labeled cells (*c-fos* positive) express somatic 5-HT7 receptor immunoreactivity (Hochman et al 2001;Schmidt and Jordan 2000). Intrathecal injection of a 5-HT_7 receptor antagonist, clozapine, abolishes mesencephalic locomotor region (MLR) evoked locomotion in the cat (Schmidt and Jordan 2000). 5-HT or NMDA-induced locomotor-like activity is disrupted by the 5-HT_7 receptor antagonist SB269970 (Madriaga et al 2004; Pearlstein et al 2005). 5-HT_7 receptors also modulate cauda-equina-evoked locomotion in neonatal mice through their activation in the spinal dorsal horn (Gordon and Whelan 2006). In the spinal mouse, activation of5-HT_7 receptors may be involved in 5-HT agonist induced locomotion *in vivo* (Landry et al 2006). The

results in the present study combined with preliminary observations in this lab show that 5-HT_7 receptor antagonists blocks PPR evoked locomotor-like activity in the neonatal rat (Fyda and Jordan 1999; Liu and Jordan 2005). All these data strongly support the hypothesis that 5-HT_7 receptors may play a critical role in rhythmgenesis of the spinal CPG for locomotion.

7. Brainstem control of locomotor activity in the rodent

Skinner and Garcia-Rill (1984) demonstrated that controlled locomotion on a treadmill could be induced by electrical stimulation of an area in the posterior midbrain in the adult rat. This area corresponds to the MLR reported in the cat (Shik et al 1966; Shik et al. 1967). Anatomical evidence has shown that cells in the MLR of the rat give off axonal collaterals to mediolateral medulla (MED) (Garcia-Rill et al. 1983; Garcia-Rill et al. 1986; Garcia-Rill and Skinner 1988). Electrical and chemical stimulation of MED in adult rats can elicit well coordinate locomotor activity (Kinjo et al. 1990; Garcia-Rill and Skinner 1991).

Atsuta et al (1988, 1990) first employed neonatal *in vitro* brainstem-spinal cord preparations to investigate the brainstem control of locomotion. They found that electrical stimulation of two specific regions in the brainstem, i.e. the MLR and the MED (dorsolateral to the pyramids), was able to evoke adult-like step cycle. Using the same preparations, it was demonstrated that a variety of neuroactive agents was effective in inducing locomotion when applied to the brainstem bath (Atsuta et al. 1991; Smith et al 1988), but the morphological identity of the MED in the neonatal rat has not been defined in detail. Since then it has been more than a decade, during

which time few studies were conducted on the brainstem control of locomotor-like activity in *in vitro* neonatal mammalian preparations.

In vitro preparations provide a unique advantage that allows direct exploration of locomotor CPG elements as well as properties of motoneurons in the spinal cord (Clarac et al. 2004). Moreover, locomotor-like activity may be directly elicited by the addition of a variety of neurotransmitters at the level of the spinal cord (Clarac et al 2004; Alford et al 2003; Kiehn and Butt 2003; Schmidt and Jordan 2000). Zaporozhets et al (2004) and Gilmore and Fedirchuk (2004) reported that high intensity electrical stimulation of the surface of the brainstem evoked locomotor-like activity in the neonatal rat. Furthermore, descending serotonergic system(s) exert an excitatory effect on lumbar motoneurons by reducing voltage threshold (Gilmore and Fedirchuk 2004). The purposes of their studies were not to locate specific regions for the initiation of locomotion in the brainstem, thus the anatomical identification of effective stimulation sites was not specified. As described in this thesis, we demonstrated for the first time that chemical or low intensity electrical stimulation in the PPR can sufficiently evoke locomotor-like activity in the *in vitro* neonatal rat, an effect most likely due to activation of 5-HT neurons in the PPR (Liu and Jordan 2004; Liu and Jordan 2005).

8. Purpose of the present study and significance

Previous work in Dr. Jordan's lab has shown that electrical stimulation of the brainstem evokes locomotor-like activity in the neonatal brainstem-spinal cord preparation (Fyda and Jordan 1999). Using dialysis techniques, it was found that

brainstem stimulation produces a substantial release of monoamines in the low thoracic and lumbar spinal cord (Fyda et al 1997; Jordan and Schmidt 2002). Based on the anatomical and tracing studies in the mammalian animals almost all 5-HT in the spinal cord originates from the caudal brainstem. Monoamines are among the first descending connections projecting to the lumbar gray (Lakke 1997). It has become increasingly important to identify a specific region in the brainstem containing a population of serotonergic neurons and to test if locomotor-like activity dependent upon 5-HT receptors can be induced upon its activation.

There is evidence that several 5-HT receptor subtypes are involved in the activation of spinal locomotor networks (Alford et al 2003; Cazalets et al 1992; Guertin and Steuer 2005; Madriaga et al 2004; Landry et al 2006; Pearlstein et al 2005; Schmidt and Jordan 2000). Preliminary data from this lab suggested that 5-HT2 and 5-HT7 may play an important role in the control of locomotion. The aim in Section I of this thesis was to examine this issue and identify the receptor subtypes and their locus of activation in the spinal cord in response to brainstem-induced locomotor-like activity. To this end, various 5-HT receptor subtype antagonists were applied to the different segments of the spinal cord during locomotion evoked by brainstem stimulation.

Based on our results in section I, we went on to test the hypothesis that 5-HT7 receptors are necessary for 5-HT-induced locomotor-like activity in the spinal cord *in vitro*, by performing experiment using knockout mice lacking 5-HT7 receptors. To further elucidate whether 5-HT7 receptors are required for voluntary locomotor movements, we conducted experiments in the adult mouse in collaboration with Dr.

Pearson's lab at the University of Alberta using 5-HT7-/- mice. Overall our results clarify the underlying mechanisms of descending serotonergic pathways in the control of locomotion and 5-HT receptor subtypes involved in activation of locomotor networks. Identification of a population of 5-HT containing neurons in the PPR provides anatomical evidence for the possibility of specific tissue grafting to the spinal cord in order to improve locomotion after spinal cord injury.

9. Figure legends

Figure 1. A: Schematic drawing of midsagittal view of the brainstem with 5-hydroxytrypamine (5-HT) – immnuoreactive cells indicated by black dots. AQ, cerebral aqueduct; CLN, caudal linear nucleus; DRN, dorsal raphe nucleus; MRN, median raphe nucleus; NRM, nucleus raphe magnus; NRO, nucleus raphe obscurus; NRPa, nucleus raphe pdllidus; V4, 4^{th} ventricle (Modified from Jacobs 1992) (with permission from the American Physiological Society, copyright 1992).

Figure 2. Graphical representation of the current classification of 5-HT receptors. Receptor subtypes represented by coloured boxes and lower case designate receptors that have not been demonstrated to definitively function in native systems. Abbreviations: 3′-5′ cyclic adenosine monophosphate (cAMP); phospholipase C (PLC); negative (−ve); positive (+ve). (Reprinted from Hoyer et al, 2002) (with permission from Elsevier, copyright 2002).

10. Figures

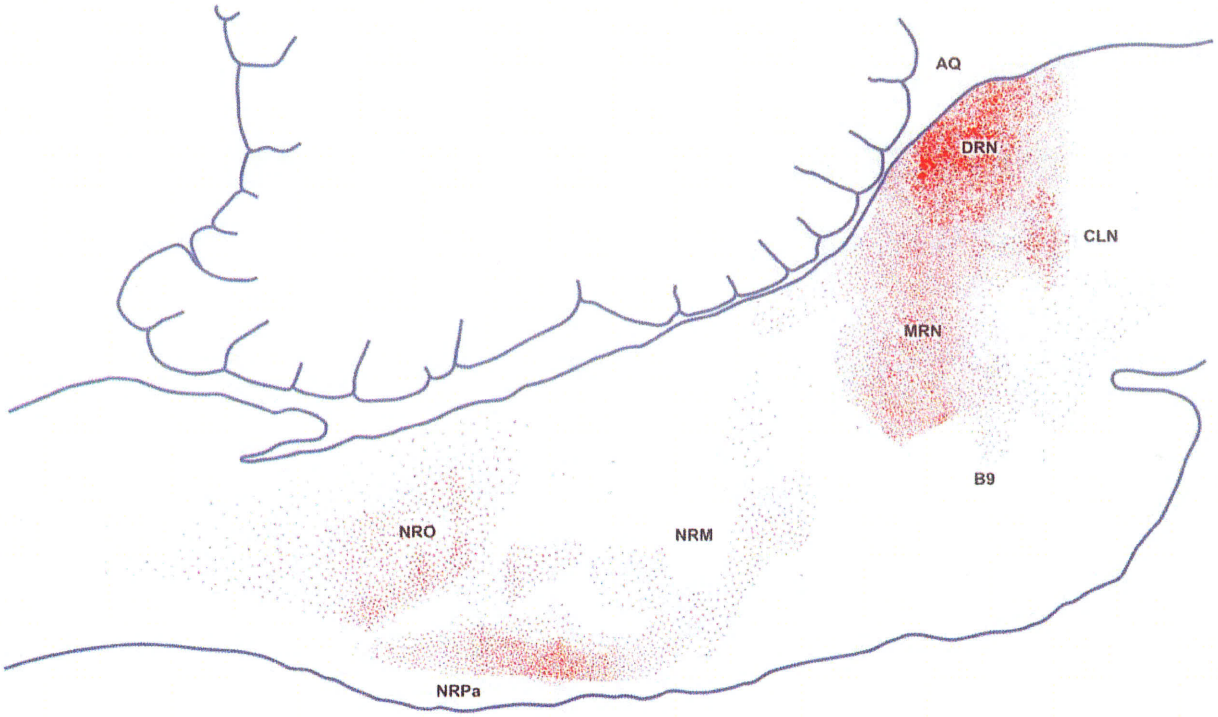


Figure 1

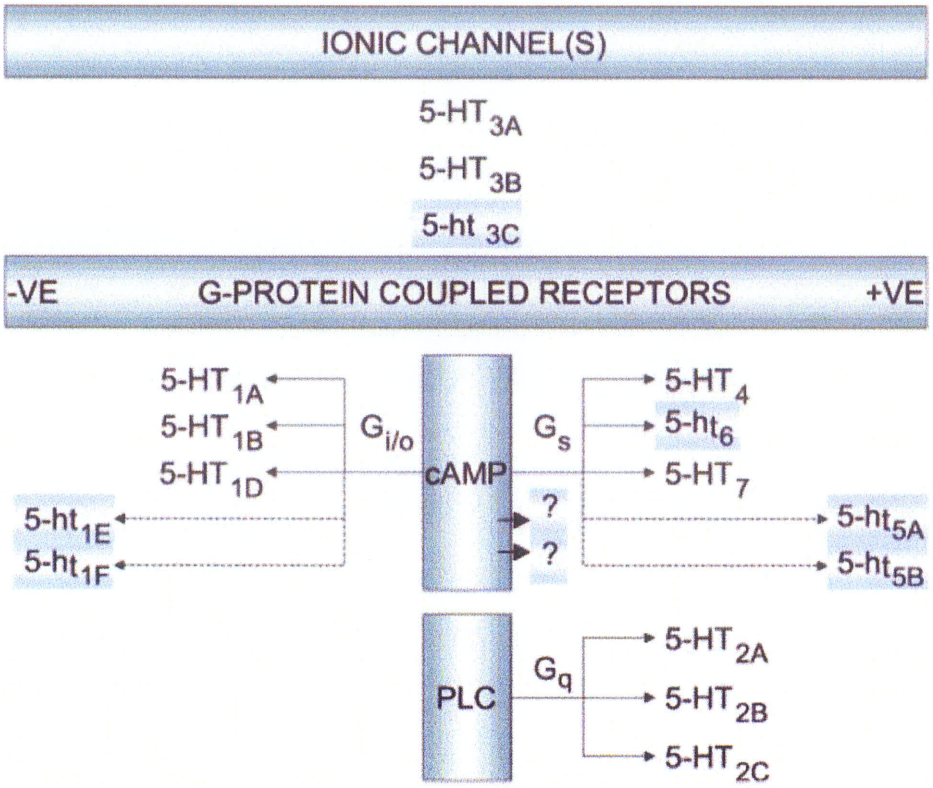

IONIC CHANNEL(S)
5-HT 3A
5-HT 3B
5-ht 3C
-VE
G-PROTEIN COUPLED RECEPTORS
+VE
5-HT 1A
5-HT 1B
5-HT 1D
G i/o
cAMP
G s
5-HT 4
5-ht 6
5-HT 7
?
?
5-ht 1E
5-ht 1F
5-ht 5A
5-ht 5B
PLC
G q
5-HT 2A
5-HT 2B
5-HT 2C

Figure 2

***Section 1*: 5-$HT_{2A/7}$ receptors subtypes are involved in locomotor-like activity induced by stimulation of the parapyramidal region.**

Section 1 is directly from the published paper: *J Neurophysiol. 2005 Aug;94(2):1392-404. Epub 2005 May 4.*

***Hypothesis I*:** electrical and chemical stimulation of the PPR containing 5-HT neurons in the brainstem can evoke locomotor-like activity in the neonatal rat brainstem-spinal cord preparation.

***Hypothesis II*:** activation of 5-HT_{2A} and 5-HT_7 in the spinal cord is required for brainstem evoked locomotor-like activity.

Abstract

Locomotion can be induced in rodents by direct application of 5-hydroxytryptamine (5-HT) onto the spinal cord. Previous studies suggest important roles for 5-HT_7 and 5-HT_{2A} receptors in the locomotor effects of 5-HT. Here we show for the first time that activation of a discrete population of 5-HT neurons in the rodent brainstem produces locomotion, and that the evoked locomotion requires 5-HT_7 and 5-HT_{2A} receptors. Cells localized in the parapyramidal region (PPR) of the mid-medulla produced locomotor-like activity as a result of either electrical or chemical stimulation, and PPR-evoked locomotor-like activity was blocked by antagonists to 5-HT_{2A} and 5-HT_7 receptors located on separate populations of neurons concentrated in different rostro-caudal regions. 5-HT_7 receptor antagonists blocked locomotor-like activity when applied rostral to the L3 segment; 5-HT_{2A} receptor antagonists blocked locomotor-like activity only when applied caudal to

the L2 segment. 5-HT_7 receptor antagonists decreased step cycle duration, consistent with an action on neurons involved in the rhythm-generating function of the central pattern generator (CPG) for locomotion. 5-HT_{2A} antagonists reduced the amplitude of ventral root activity without affecting step cycle duration, suggesting an action directly on cells involved in the output stage of the pattern generator for locomotion, including motoneurons and premotor cells. Thus, in the neonatal rat, the rhythm generating and the output functions of the CPG are performed by separate populations of neurons distinguishable on the basis of the receptors required for their response to 5-HT. Experiments with selective antagonists show that dopaminergic (D_1, D_2) and noradrenergic (α_1, α_2) receptors are not critical for PPR-evoked locomotor-like activity.

Introduction

Locomotion in mammals is controlled by networks of spinal neurons constituting a central pattern generator (CPG), and can be initiated by certain pathways that originate in the brainstem and descend to the spinal cord (Grillner 1981; Jordan 1991; Rossignol 1996; Grillner et al. 1997; Jordan 1998). In recent years, considerable progress on the neural mechanisms for the control of locomotion has been made using isolated spinal cord preparations from neonatal rats and mice (Bonnot et al 2002; Clarac et al 2004; Jiang et al 1999; Kiehn and Butt 2003; Kiehn and Kjaerulff 1998; Kudo and Yamada 1987; Nishimaru et al 2000; Schmidt and Jordan 2000; Smith and Feldman 1987; Whelan et al 2000). For this purpose, most studies have employed applications of neurotransmitters or other drugs into the bath

to elicit locomotor-like activity. These studies have shown that a variety of neurochemicals, including NMDA, noradrenaline, 5-hydroxytryptamine (5-HT), dopamine and cholinergic agonists can elicit rhythmic activity with a locomotor-like pattern (Alford et al 2003; Rossignol et al. 2002). One of the most reliable means for inducing locomotion, at least in the neonatal rat isolated spinal cord, is with bath-applied 5-HT (Cazalets et al. 1990; Cazalets et al 1992; Cowley and Schmidt 1994; Schmidt and Jordan 2000). Furthermore, the most effective site for 5-HT to induce locomotion has been localized to the lower thoracic and upper lumbar segments of the spinal cord (Cowley and Schmidt 1997), and transplantation of 5-HT neurons into the thoracic cord activates locomotion in adult chronic spinal rats (Gimenez y Ribotta et al 2000).

Several 5-HT receptor types have been identified in the spinal cord (Hochman et al 2001; Schmidt and Jordan 2000), and blockage of several of these receptors types can disrupt 5-HT induced locomotor activity. In both the neonatal rat and mouse spinal cord, selective antagonists of 5-HT_{2A} and 5-HT_{7} receptors have been shown to block 5-HT induced rhythmicity (Beato and Nistri 1998; Bracci et al 1998; Cazalets et al 1992; Cazalets et al. 1995; Hochman et al 2001; Jordan and Schmidt 2002; Madriaga et al. 2004; Schmidt and Jordan 2000). Nevertheless, the cells upon which these antagonists act to block locomotion are not known. It would be possible to block the locomotor output recorded in these experiments if the affected receptors were on interneurons of the CPG or on motoneurons. It has been shown that 5-HT induced locomotion requires 5-HT be applied at the lumbar level as well as in the supra-lumbar region, although 5-HT applied to the lumbo-sacral region

alone induced only tonic activity (Cowley and Schmidt 1997). There is ample evidence that 5-HT exerts an excitatory effect directly on motoneurons (reviewed in Schmidt and Jordan 2000). Both 5-HT_{2A} and 5-HT_7 receptors have been shown to be involved in mediating 5-HT effects on motoneurons (Jackson and White 1990; Takahashi and Berger 1990; Wang and Dun 1990; Gilmore and Fedirchuk 2004; Inoue et al. 2002).

Although virtually all of the 5-HT terminals in the rat spinal cord originate in brainstem nuclei (Lakke 1997)), the descending 5-HT cells responsible for inducing locomotion have not been identified. In fact, it has never been demonstrated that stimulation of any area in the brainstem containing 5-HT neurons is effective for the induction of locomotion. Brainstem evoked locomotion has been examined in isolated rodent brainstem-spinal cord preparations using chemical stimulation of the entire brainstem (Atsuta et al 1991; Smith et al 1988) or with electrical stimulation of sites within the brainstem, although without detailed anatomical identification of the effective sites (Atsuta et al 1988; Atsuta et al 1990; Atsuta et al. 1991; Gilmore and Fedirchuk 2004; Zaporozhets et al 2004).

Here, we attempt to identify a population of brainstem 5-HT containing neurons that can be stimulated to evoke locomotor-like activity in the isolated neonatal rat brainstem – spinal cord preparation. Further, we performed experiments designed to determine the receptors at the spinal level that are responsible for the brainstem-evoked locomotor-like activity, and to identify and localize the spinal cord cells that are influenced by the effective antagonists. Preliminary experiments that preceded this study have been published in abstract form and in a review (Fyda and Jordan

1999; Jordan and Schmidt 2002), but none of the data in the present study was included in those reports. A preliminary report of the data presented here was published in abstract form (Liu and Jordan 2004).

Methods

Preparation

All experiments were performed in accordance with Canadian Council on Animal Care guidelines and were approved by the University of Manitoba Animal Protocol Committee. The experiments were performed on brainstem-spinal cord preparations isolated from neonatal Sprague-Dawley rats (0-3 days old). Following anaesthesia with halothane, the animal was immediately decapitated just posterior to bregma, eviscerated and removed to a sylgard coated recording chamber. A laminectomy was performed, first removing the dorsal portions of the vertebrae, then the ventral side of the vertebrae. The dorsal and the ventral roots were cut from C1 to S3. The brainstem was transected at a site just rostral to the exit of the trigeminal nerve. The isolated brainstem - spinal cord was superfused with artificial cerebrospinal fluid (aCSF, concentration in mM: NaCI 128, KCI 3.0, NaH_2PO_4 0.5, $CaCI_2$ 1.5, $MgSO_4$ 1.0, $NaHCO_3$ 21, and glucose 30) and oxygenated with 95% and 5% CO_2 at room temperature. The preparation was pinned ventral side up to the sylgard surface, and Vaseline barriers were placed at the C5/6, T8/9 and L2/3 levels (unless otherwise specified in the text) to form separate pools. This was done because preliminary experiments showed that specific 5-HT antagonists had differential effects when applied to the lower thoracic/rostral lumbar or the lower lumbar regions of the

spinal cord (Schmidt and Jordan 2000; Jordan and Schmidt 2002). The barriers were tested for leakage before and after the experiments by their ability to maintain different depths of superfusate. Thus the spinal cord could be separated into rostral (T9-L2) and caudal (below L3) compartments.

Electrically evoked locomotion

Locomotor-like activity was evoked through electrical stimulation of the parapyramidal region (PPR) of the mid-medulla. A monopolar tungsten electrode (10 - 50KΩ, tip diameter 1-2 μm, 25 μm exposed tip, MICRO PROBE, INC.) was lowered into the PPR as follows: 1.0 – 1.2 mm caudal to the junction between the pons and medulla; 0.5 – 1.0 mm lateral to the midline, and 0.2 - 0.8 mm from the ventral surface. Small adjustments were made in the position of the electrode until locomotion could be reliably evoked. Square pulses of constant current, 5 ms duration, 2.5-3 Hz, were given at variable intensities (80-200uA). Glass or plastic suction electrodes were applied to record the L2 and L5 ventral discharges of both sides to monitor locomotor-like activity (Kjaerulff and Kiehn 1996; Cowley and Schmidt 1997). Ventral root recordings were amplified, filtered (30 Hz-2 KHz), digitized, stored and analyzed using software developed at the Winnipeg Spinal Cord Research Centre (see http://www.scrc.umanitoba.ca/doc/ for details). Locomotor-like activity consisted of alternating activity recorded between the segmental left and right L2 and L5 ventral root pairs and alternating activity between ipsilateral flexor (L2) and extensor (L5) related ventral root pairs (see Fig. 2).

Chemically evoked locomotion

To elicit locomotor-like activity by activation of neurons in the vicinity of the stimulating probe, while excluding activation of fibres of passage, chemical stimulation was employed. Glutamate (10-50 mM/1μl) or the GABA receptor antagonist bicuculine (10 mM/1μl) was injected through a glass micropipette (4-6 μm tip diameter) connected to a pneumatic PicoPump (World Precision Instruments, Inc.). Control saline injections (1 μl, n=4) did not produce locomotor-like activity. In each case, the injection site was the same as previously identified as effective for electrically-evoked locomotor-like activity in the same preparation.

Drugs

Various antagonists were tested to block the following receptors during electrically and chemically evoked locomotor-like activity: 5-HT_{2A} (ketanserin 5-20 μM, spiperone 5-20 μM), 5-HT_7 (clozapine 0.5-1.0 μM, SB269970 10-15 μM), noradrenergic α_1 (prazosin 1-10 μM), α_2 (yohimbine 5-25 μM, RX 821002 10-25 μM), dopominergic D_1 (SCH 23390 10-20 μM), D_2 (sulpiride, 10-20 μM). All antagonists were dissolved in distilled water. Drugs prepared for injection into the brainstem (glutamate or bicuculine) were dissolved in saline. All drugs were obtained from Sigma.

Immunohistochemistry

The neonatal rats were anethesized with halothane. The brainstems were dissected and placed in 4% paraformaldehyde for 24 hrs at 4°C, then postfixed in 15% sucrose solution for at least 72 hrs. 30 μm thick sections were cut on a cryostat. The sections were presoaked in 0.1 M phosphate buffered saline (PBS) for 1hr to remove the holding medium, then pretreated with 5% donkey serum in PBS-T (0.1%) for 30 min; After washing in PBS, sections were incubated with rabbit anti 5-HT antibody (1:200) in 0.1% PBS-T and 1% donkey serum for 48 hrs at 4°C, then incubated with donkey anti rabbit Cy3 antibody (1:250) in 0.1% PBS-T and 1% donkey serum for 2hrs at room temperature. Sections were washed in PBS, Tris-HCI (50mM), then coverslipped with vectashield. Section were then examined and photographed using a Nikon fluorescence microscope.

Measurements and Statistical analysis

The cycle duration was defined as the time interval between the onsets of successive bursts of ventral root activity and was measured for at least 10 consecutive cycles in each episode of locomotor-like activity. Amplitude was measured from these same consecutive cycles as the average peak-to-peak amplitude of the rectified and filtered waveforms. The effects of drugs on amplitude and duration were expressed quantitatively as a fraction of respective control values in the same experiment. The statistical values are indicated as the mean±SD; n refers to the number of experiments. One-way ANOVA or Student's t-test was used for statistical analysis, and the level of significance was set as $p < 0.05$. Data were pooled as required if the one-way ANOVA test did not reveal significant

differences between individual samples. The coupling strength between left/right or flexor/extensor discharges of the ventral roots was assessed using circular statistics (Kriellaars et al. 1994; Kjaerulff and Kiehn 1996) (see Fig. 2). The phase value (Φ) indicated by the direction of the vector was defined by dividing the latency between the onsets of paired root cycles by the step cycle period, so that a Φ value of 0.5 represents out-of-phase activity in the two roots, and values of 0 or 1 would occur when the roots are completely in phase. The mean value (r) indicating the concentration of phase values was expressed by the length of the vector, which ranged from 0 to 1. The Rayleigh test (Zar 1974) was used to determine the coupling strength.

Results

Stimulation within a cluster of 5-HT neurons in the parapyramidal region produces locomotor-like activity

The effective sites for evoking locomotor-like activity were located in a very restricted area of the mid-medulla just lateral to the pyramidal tract (PT) that others (Sasek and Helke 1989; Helke et al 1989) have termed the parapyramidal region (PPR). The effective area was 0.5 – 1.2 mm caudal to the junction between the pons and medulla (just rostral to the inferior olivary nucleus at the level of the nucleus of the VIIth cranial nerve), 0.5 – 1.0 mm lateral to the midline, and 0.2 – 0.5 mm beneath the ventral surface. Stimulation 0.5 mm or more lateral or medial to an effective site did not produce locomotor-like activity. It is well known that 5-HT neurons in the raphe nuclei (i.e, raphe magnus, raphe pallidus and raphe obscurus) project to the spinal cord, and stimulation of these midline regions was not effective

for producing locomotor-like activity. As shown in Fig.1A, a cluster of 5-HT neurons are labeled lateral to the PPR. Fig. 1B shows a typical lesion at the effective stimulus site, which is surrounded by many 5-HT-ir neurons. The sites of stimulation from 12 representative experiments are shown in Fig. 1C. The sites that were effective for evoking locomotor-like activity are just lateral to the PT, in an area rich in 5-HT neurons (Fig. 1A, B). In the adult rat, a high proportion of the 5-HT-containing cells in this area project to the spinal cord (Jones and Light 1992). Our effective stimulus sites likely overlap with the most effective sites for brainstem-evoked locomotion reported by Atsuta and collaborators (Atsuta et al 1988; Atsuta et al 1990). They did not specifically seek to stimulate within areas containing 5-HT neurons, however, and the relationship of their stimulus sites to 5-HT neurons is unknown.

Properties of locomotor-like activity evoked by electrical stimulation of the PPR

Locomotor-like activity with right/left and flexor/extensor alternation is shown in Fig 2. The raw recordings from the right (R) and left (L) L2 (flexor) and L5 (extensor) ventral roots over a 60 s period are shown in Fig. 2A, upper 4 traces, and rectified and filtered versions of these same recordings are repeated in the lower 4 traces. Alternation between the right (RL2 and RL5) and left (LL2 and LL5) flexor and extensor ventral roots is demonstrated in Fig. 2B by the rasterized overlay of 15 successive step cycles in these same rectified and filtered traces triggered at the onset of activity in RL2. In Fig. 2C the alternating activity is analyzed using polar plots derived from 11 preparations. The polar plots reveal that mean values (Φ)

were 0.44±0.03 (RL2/RL5), 0.43±0.05 (LL2/LL5), 0.51±0.04 (RL2/LL2), and 0.51±0.05 (RL5/LL5). r values reflecting the coupling strength for each of these root pairs were significant ($p<0.001$): 0.93±0.07 (RL2/RL5), 0.93±0.04 (LL2/LL5), 0.93±0.04 (RL2/LL2), and 0.91±0.05(RL5/LL5). Only preparations in which well-coordinated locomotor-like activity such as illustrated in Fig. 2 occurred are reported here.

The effective stimuli in these experiments were 80 – 200 μA square pulses of 5 ms duration at 2.5 – 3.0 Hz. The cycle duration of the PPR-evoked locomotion ranged from 2.75 to 5.20 s (mean = 3.88 ± 0.76 s, n = 86). During the locomotor-like activity, both the cycle duration and amplitude were remarkably stable. In most cases, the onset of the stimulus was followed immediately by locomotor-like activity, which ceased almost coincidentally with termination of the stimulus (Fig. 2A). Rarely, 1 to 3 cycles persisted after switching off the stimulus. Stimuli were typically applied for periods of 60 – 90 s, separated by 5 minute intervals during the control period, prior to drug application. Upon application of an antagonist, stimuli were applied after 1 min., 3 min. 5 min. 10 min., 15 min. and 20 min. (see Fig. 4).

Microinjection of drugs into the parapyramidal region produces locomotor-like activity

To verify that electrically-evoked locomotor-like activity was mainly due to the activation of cells in this area instead of descending fibers, the excitatory amino acid glutamate (1 μl /10 mM, n=4) and the $GABA_A$ receptor antagonist bicuculine (1 μl/10 mM, n=4) were microinjected into the sites that were effective for electrically-evoked locomotor-like activity. As would be predicted if the stimulus

were effective due to activation of neuronal somas in or around the stimulus site, locomotor-like activity was induced after 3-5 min of drug injection. The locomotion evoked in this manner was characterized by 5 - 10 episodes of alternating rhythmic activity lasting 30 - 40 min (Fig. 3A). In some cases episodes of well-coordinated locomotor-like activity were separated by one or two bursts of synchronous activity (see Fig. 3B in all four ventral roots).

Effects of 5-HT receptor antagonists on PPR-evoked locomotion

5-HT_{2A} receptor antagonists are only effective when applied to the caudal compartment: Although several authors have previously shown that the 5-HT_{2A} receptor antagonist ketanserin blocks locomotion induced by 5-HT (see Introduction), a striking feature of the effects of this drug in our experiments, shown in Fig. 4A, was that it has no effect on PPR-evoked locomotion when applied to the rostral compartment (20 μM, n = 5), but it consistently blocks locomotion within 3 min. when applied below L2 (20 μM, n = 6, Fig. 4B). A second antagonist with high affinity for the 5-HT_{2A} receptor, spiperone, also blocked PPR-evoked locomotion (Fig. 4C) when applied to the caudal compartment (15 μM, n = 6) but was without effect (data not shown) when the same concentration of the drug was applied to the rostral compartment (n = 5). The restriction of the locomotor blocking effects of the two 5-HT_{2A} antagonists to the caudal bath is consistent with their having an action on output cells of the locomotor system, including motoneurons, which are known to possess 5-HT_{2A} receptors (Cornea-Hébert et al. 1999; Doly et al 2004). It is noteworthy, however, that application of ketanserin or

spiperone in the compartment containing the L2 motoneurons (see Fig. 4A) did not reduce L2 motoneuron discharge. One possible explanation for this is that that there may be differential distribution of 5-HT_{2A} receptors on flexor motoneurons (predominating in L2) and extensor motoneurons (predominating in L5).

Prior to blocking locomotor-like activity, the 5-HT_{2A} antagonist ketanserin reduced the amplitude of the L5 ventral root discharge, but had no effect on cycle duration: Low concentrations of ketanserin (5 μM, n=6) reduced the amplitude of ventral root discharges within the first five minutes (to 0.73 ± 0.07, $P < 0.05$) when applied to the caudal compartment, but the amplitude recovered to almost normal after 20min (0.91±0.11, $P>0.05$). Increasing the concentration to 10 μM (n=6) gave rise to a more evident reduction in amplitude (to 0.45 ± 0.14, $P < 0.01$ at 10 min.), but no effect on cycle duration (1.07 ± 0.22, $P > 0.05$) (see Fig. 4D and Fig. 5A).

The effects of ketanserin on the amplitude of the L5 ventral root discharges and the duration of the step cycle for all experiments are summarized in Fig. 5A. A dose of 20 μM rapidly reduced the amplitude (by 42.5%, $p<0.01$) and increased the duration of the step cycle (by 31%, $p<0.05$) at 1 min. after addition of ketanserin, while lower doses (5 and 10 μM) reduced the amplitude over a longer period, and in the case of the 5 μM dose, recovery was observed after 10 minutes. The lower doses had very little effect on the step cycle duration, with the exception of a slight increase (by 25%) after 20 minutes with the 20 μM dose. These results suggest that blocking the 5-HT_{2A} receptor may serve to reduce motoneuron output, rather than to disturb the central pattern generating mechanisms controlling cycle duration. The fact that L2 activity is eventually blocked, even though ketanserin is applied only

below L3, suggests interneurons related to motoneuron output in L2 may be affected by the drug (see Discussion).

5-HT$_7$ receptor antagonists are effective only when applied to the rostral compartment: Clozapine (1 μM, n=5) a non-specific antagonist with a high affinity for the 5-HT$_7$ receptor (Hochman et al 2001) was without effect when applied to the caudal compartment (Fig. 6A). At the same dose (1 μM, n=7), clozapine completely blocked PPR-evoked locomotor-like activity when applied to the rostral compartment (Fig. 6B). The blockage achieved with this dose of clozapine was complete within 3 minutes of application. When clozapine was applied at a lower concentration (0.5 - 0.75 μM, n=6) to the rostral compartment it caused a substantial prolongation of cycle duration (Fig. 6C). As shown in the histograms in Fig. 5B, the cycle duration was approximately doubled at 10 min. after drug application (to 2.02 ± 0.19 sec, $p<0.001$). A decrease in amplitude also occurred after 10 minutes (to 0.70 ± 0.07 of the control, $p<0.05$). The 1 μM dose of clozapine rapidly increased cycle duration prior to complete block of locomotion (Fig. 5B). These results indicate that clozapine, rather than blocking locomotion through an action on motoneurons, does so by reducing an excitatory 5-HT mediated input to elements of the CPG for locomotion, thereby altering cycle duration. The amplitude of the ventral root discharges was also reduced, suggesting that the suppression of activity in interneurons of the CPG results in a reduced excitatory drive to the motoneurons. Clozapine also has a high affinity to 5-HT$_{2A}$ receptors, but it did not mimic the action of ketanserin in the caudal compartment, suggesting that its action was primarily on 5-HT$_7$ receptors.

The specific 5-HT_7 receptor antagonist SB269970 also produced a reversible dose-dependent blockage of locomotor-like activity, but only when the site of application included the rostral compartment (Fig. 7). SB269970 (15 μM, n = 7) completely blocked locomotion within 5 minutes of drug application (Fig. 7B). No changes in locomotor-like activity were observed when SB269970 (15 μM, n=4) was applied in the caudal compartment (Fig. 7A). SB269970 applied to the rostral compartment prolonged the step cycle duration at all concentrations used (Fig. 5C; Fig. 7C.). For example, at the lowest concentration used (5 μM, n=6) it caused a significant prolongation of cycle duration (to 1.80 ± 0.16, $p < 0.01$ at 20min) without a significant change in the amplitude of the ventral root discharges (0.86 ± 0.16, $p>0.05$ at 20 min). As the dose increased to 10 μM (n=6), for instance at 10 min after application of SB269970, ventral root amplitude steadily decreased (0.35 ± 0.14, $p < 0.001$) with the same time course as the increase in cycle duration (1.84 ± 0.21, $p < 0.001$). We propose that the decrease in amplitude results from decreased drive to motoneurons due to blockage by SB269970 of a descending 5-HT mediated excitation of CPG neurons. These actions are similar to those of clozapine.

SB269970 also blocked locomotor-like activity induced by microinjection of bicuculine or glutamate into the PPR (Fig. 8), first increasing the cycle duration (at 5 min.), and then later blocking locomotor-like activity (10 min.). This demonstrates that locomotor-like activity evoked by activation of only the neurons in the PPR and not the passing fibers can also be blocked by the 5-HT_7 receptor antagonist. However, episodes of synchronous activity persisted after SB269970 in

cases of locomotor-like activity produced by injections of bicuculine into the PPR (see Fig. 8, bottom 4 traces). Locomotor-like activity produced by injections of glutamate into the PPR was not accompanied by synchronous activity.

The restriction of the actions of the 5-HT_7 antagonists to the rostral compartment is consistent with the suggestion that 5-HT- mediated excitation of the locomotor CPG requires the supra-lumbar regions of the spinal cord (Cowley and Schmidt 1997; Schmidt and Jordan 2000; Jordan and Schmidt 2002). It is also consistent with the notion that the CPG for locomotion in the neonatal rat, although distributed throughout the thoraco-lumbar region, is most readily activated from the thoracic and rostral regions of the lumbar segments (Bertrand and Cazalets 2002; Cazalets et al 1995; Cowley and Schdmit 1997; Kiehn and Kjaerulff 1998; Kjaerulff and Kiehn 1996).

Effects of dopaminergic and noradrenercic antagonists

Dopaminergic (D_1 and D_2) antagonists do not alter PPR-evoked locomotor-like activity: To examine if dopamine is involved in the regulation of the locomotor-like activity evoked by electrical stimulation of the PPR, dopaminergic antagonists (the D_1 antagonist SCH23390 and the D_2 antagonist sulpiride) were applied to the whole spinal cord below the C5 segment. Sulpiride (20μM, n=3) altered neither the amplitude of the ventral root recordings (0.94±0.18, $p>0.05$) nor step cycle duration (0.98±0.13, $p>0.05$). Similarly, SCH23390 (20μM, n=3) had no significant effect on ventral root discharge amplitude (0.89±0.26) or cycle duration (1.05±0.21). Although clozapine has moderate to high affinity for dopamine receptors (van Tol

et al. 1991), the above results suggest that the action of clozapine is not due to blocking the D_1 or D_2 dopamine receptor. It also indicates that although 5-HT-evoked locomotion in the neonatal mouse can be disrupted by either D_1 or D_2 antagonists (Madriaga et al 2004), these receptors are not involved in the induction of locomotion from the PPR. According to Whelan and colleagues (Madriaga et al 2004), the 5-HT- evoked locomotion could be most effectively disrupted by a combination of the D_1 and D_2 antagonists, We therefore attempted to disrupt PPR-evoked locomotor-like activity with SCH23390 and sulpiride in combination (20μM each, n=3), but there was no significant effect (data not shown).

Noradrenergic antagonists: The α_2 antagonist yohimbine was applied to the whole bath. Low concentrations of the drug (1-10 μM, n=8) produced either no effect (amplitude: 0.94±0.17, $p>0.05$; duration: 1.06±0.21, $p>0.05$, n = 4) or an obvious decrease in cycle duration (amplitude : 0.88±0.19, $p>0.05$; duration 0.78±0.19, $p<0.05$, n = 4) after 5-10 min. of drug application, as shown in Fig. 9A. A high concentration of yomibine (20 or 25 μM, n=6) always blocked locomotor-like activity after 5-10 min of drug application (Fig. 9B). Addition of the specific noradrenergic α_2 antagonist RX 821002 (10-25 μM, n=6) caused no change either in amplitude (1.02 ± 0.23, $p > 0.05$) or duration (1.05 ± 0.20, $p > 0.05$). In addition, no changes in amplitude (0.92 ± 0.14, $p > 0.05$) or duration (1.10 ± 0.19, $p > 0.05$) were obtained when the α_1 antagonist prazosin (10 μM, n=4) was applied to the bath. In a recent study (Gabbay and Lev-Tov 2004a) on the locomotor-like activity induced by noradrenaline in the isolated neonatal rat spinal cord, the α_1 antagonist prazosin antagonized the drug-induced rhythmic activity, while high doses ($\geq$ 20

μM) of the α_2 antagonist yohimbine were required for blockage. Although our finding that similar doses of yohimbine block PPR-evoked locomotor-like activity is consistent with these results, the absence of any effect of the more specific α_2 antagonist RX 821002 or prazosin in our experiments suggest that neither α_1 nor α_2 noradrenergic receptors play a significant role in locomotor-like activity produced by PPR stimulation.

Discussion

5-HT neurons sufficient for evoking locomotor-like activity are located in the parapyramidal region (PPR)

We have demonstrated that electrical and chemical stimulation of a specific group of 5-HT neurons in the brainstem of the isolated neonatal rat brainstem and spinal cord preparation is sufficient for evoking locomotor-like activity. These cells are localized in the parapyramidal region (PPR) of the mid-medulla. 5-HT neurons that project to the ventral horn of the lumbar spinal cord via the ventral and ventrolateral funiculi have been localized in the raphé pallidus, raphé obscurus, and the ventral medulla (para-raphé area), including an area close to the ventral surface and lateral to the pyramidal tract, the PPR (Basbaum et al 1978; Bowker et al 1981b; Westlund et al. 1983; Dahlstrom and Fuxe 1965; Helke et al 1989; Hokfelt et al. 2000; Holstege 1987; Holstege and Kuypers 1987a; Holstege and Kuypers 1987b; Jones and Light 1992; Martin et al 1978; Schmidt and Jordan 2000; Skagerberg et al 1985; Steinbusch 1981). These and other studies show that fibers from the raphé and para-raphé nuclei are known to terminate at multiple levels of the spinal cord and on a variety of neuron types, including autonomic preganglionic neurons, neurons in

laminae VII, VIII and X, and α-motoneurons. It is noteworthy that a high proportion of the 5-HT neurons in the PPR (so-called para-raphé neurons) project to the lumbar spinal cord (Jones and Light 1992) in adult rats. Cells in the PPR region have been labeled trans-synaptically with pseudorabies virus injected into the gastrocnemius muscle of adult rats (Kerman et al 2003). Cells in this region were shown to be activated during treadmill exercise, because they were positive for the activity-dependent marker *c-fos* (Iwamoto et al 1996). We have evidence that PPR stimulation gives rise to release of 5-HT, dopamine, and noradrenaline in the thoraco-lumbar spinal cord (Fyda et al 1997).

We have shown that the effective stimulus site for evoking locomotor-like activity in the neonatal rat brainstem-spinal cord preparation is within the PPR, among a population of 5-HT neurons, and we conclude that this is the same area reported to send descending fibers to the lumbar cord in adults (Jones and Light 1992). Chemical activation with glutamate or bicuculine demonstrates that excitation of neurons in the PPR, and not fibers of passage, accounts for the evoked locomotion. The episodic nature of the chemically evoked locomotor-like activity cannot be readily explained on the basis of the current experiments, and we have not systematically pursued this issue. Neurons in the raphé pallidus and raphé obscurus did not give rise to locomotion when stimulated electrically, and are therefore not likely to be the source of the descending serotonergic pathway involved in locomotor-like activity evoked in our experiments. Although it is likely that our stimuli activate neurons other than those in the PPR that contain 5-HT, the fact that $5HT_7$ and $5\text{-}HT_{2A}$ receptor antagonists block the PPR evoked locomotor-like

activity supports the notion that PPR stimulation functions primarily through release of 5-HT in the spinal cord. These same antagonists have been shown in several studies to be effective in antagonizing the locomotor-like activity-producing action of bath-applied 5-HT in both rat and mouse *in vitro* preparations (Cazalets, Sqalli-Houssaini, and Clarac 1992;Cina and Hochman 1998;Hochman, Garraway, Machacek, and Shay 2001;MacLean, Cowley, and Schmidt 1998;Madriaga, McPhee, Chersa, Christie, and Whelan 2004a;Schmidt and Jordan 2000;Jordan and Schmidt 2002).

If non-5-HT containing cells in the PPR are involved in the production of locomotor-like activity, they are not sufficient for this purpose in the absence of concurrent actions of 5-HT. Our novel finding that 5-HT neurons in the PPR are sufficient for the production of locomotion has implications for future research on the function of specific 5-HT neurons in the control of movement, and for studies on the use of transplanted 5-HT neurons for the restoration of locomotion after spinal cord injury. Previous studies have demonstrated that 5-HT neurons of the raphé pallidus and raphé obscurus are active during locomotion (Heym et al. 1982; Fornal et al. 1985; Veasey et al. 1995), but there are no electrophysiological studies on the activity of PPR neurons during locomotion. Efforts to restore locomotion have involved the transplantation of 5-HT neurons into the spinal cord (Gimenez y Ribotta et al 2000; Orsal et al. 2002), and serotonergic reinnervation of the L1-L2 level of the spinal cord from these implants is correlated with locomotor recovery. It is possible that PPR serotonergic neurons were included in the transplants (Gimenez y Ribotta et al 2000). The effectiveness of re-innervating the upper

lumbar segments is consistent with our finding that the rhythm generating portions of the CPG activated by PPR stimulation is in the region above L3 (see below). Future attempts at reinnervation for restoring locomotion should focus on the use 5-HT neurons of the PPR.

5-HT$_7$ receptor activation is required for PPR-evoked locomotor-like activity

In a preliminary report of some of these results (Jordan and Schmidt 2002) we found that clozapine, a non-specific antagonist with a high affinity for the 5-HT$_7$ receptor, blocked brainstem-evoked locomotor-like activity. We also provided preliminary evidence that clozapine applied intrathecally could block brainstem-evoked locomotion in the decerebrate cat preparation (Schmidt and Jordan 2000). Here we show that both clozapine and the more specific 5-HT$_7$ antagonist SB-269970 block PPR-evoked locomotor-like activity, and that their action is limited to regions of the spinal cord rostral to the L3 segment. Application of either antagonist into the compartment caudal to the L2 segment was ineffective. This is consistent with the evidence that 5-HT$_7$ receptors play a critical role in the initiation of locomotion by direct application of 5-HT (Hochman et al 2001; Madriaga et al 2004). Various pharmacological and lesioning studies have localized the dominant components of the locomotor CPG to the lower thoracic-upper lumbar region (Bertrand and Cazalets 2002; Cazalets et al 1995; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997). Hochman and colleagues (Hochman et al 2001) have shown a similar distribution for cells stained with a 5-HT$_7$ receptor antibody. These same authors also demonstrated that a substantial

proportion of cells in this region of the cord labeled with the activity-dependent label sulforhodamine after 5-HT induced locomotor-like activity were positive for the 5-HT_7 receptor. We also have reported preliminary data (Jordan and Schmidt 2002) showing that many cells in the thoraco-lumbar area that are active during locomotion in the adult rat (as demonstrated with *c-fos* immunostaining after a treadmill locomotion task) are positive for the 5-HT_7 receptor. Thus, it is plausible that this distribution of 5-HT_7 receptors underlies our finding that 5-HT_7 receptor antagonists block PPR-evoked locomotor-like activity only if applied rostral to the L3 segment.

Neurons with 5-HT_7 receptors are involved in rhythmogenesis

The finding that 5-HT_7 receptor blockade results in a progressive reduction in step cycle duration before finally blocking PPR-evoked locomotor-like activity altogether (Figs. 5-8) strongly suggests that the affected 5-HT_7 receptors are located on cells that play a role in the rhythm-generating capacity of the locomotor network. These cells may either be involved in providing an excitatory drive to the CPG neurons producing the rhythm, or they may form part of the CPG. This is consistent with the apparent gradient of 5-HT responsive CPG neurons from thoracic to lumbar cord (Schmidt and Jordan 2000; Jordan and Schmidt 2002). Concomitant with blockage of this rhythm-generating function by the 5-HT_7 antagonists, the amplitude of the locomotor output recorded from motoneurons in the ventral roots is also reduced (Figs. 5-8). This does not appear to be due to a direct suppression of motoneuron excitability due to blockage of 5-HT_7 receptors on motoneurons,

because the antagonists did not alter the motoneuron activity recorded from the L5 ventral root when applied into the caudal compartment, which included the motor nuclei of the L5 segment. Rather, it is more likely due to a progressive reduction in the output of the more rostrally located elements of the rhythm generating network, resulting in a reduced excitatory drive to the motoneurons, either directly or through more caudally located elements of the CPG. Our data are not consistent with 5-HT_7 receptor blockage of locomotor-like activity solely due to an action directly on motoneurons (Inoue et al 2002).

It was previously hypothesized that 5-HT_7 receptor activation may be essential for 5-HT-induced locomotor rhythmogenesis (Hochman et al 20001; Schmidt and Jordan 2000; Madriaga 2004) and that an important component of the CPG for locomotion can be characterized by the presence of 5-HT_7 receptors (Jordan and Schmidt 2002). The results of the experiments reported here provide strong support for these hypotheses. Furthermore, the cells with 5-HT_7 receptors that are involved in the initiation of locomotion appear to be localized in the thoraco-lumbar cord in segments rostral to L3.

5-HT_{2A} receptor activation is also required for PPR-evoked locomotor-like activity

Consistent with numerous prior studies showing that antagonists with high affinity for the 5-HT_{2A} receptor block locomotor-like activity (Bracci et al 1998; Cazalets et al 1992; MacLean et al 1998; Madriaga et al 2004), we found that PPR evoked locomotor-like activity was blocked when ketanserin or spiperone were applied to the caudal compartment. The results with these two antagonists, taken

together, strongly suggest that their effects are exerted on 5-HT_{2A} receptors, because both have high affinity for this receptor, with non-overlapping affinities for other 5-HT receptors (Hochman et al 2001; Glennon et al. 2002). Our results further suggest that the cells possessing 5-HT_{2A} receptors involved in locomotor-like activity are part of the output stage of the locomotor pattern generator, not the rhythm-generating component. This is demonstrated by the fact that while the 5-HT_{2A} antagonists reduce the amplitude of the ventral root discharges when applied below L2, they have no effect on the step cycle duration. Furthermore, they are without any effect when applied above L3, where the 5-HT responsive CPG elements appear to be located (see above). It is likely that most if not all the effects of 5-HT_{2A} antagonists are on motoneurons, because immunolabeling for these receptors is particularly dense in lamina IX (Cornea-Hébert et al 1999; Doly et al 2004), and because the excitatory actions of 5-HT on motoneurons has been shown to be blocked by 5-HT_{2A} antagonists (Gilmore and Fedirchuk 2004; Jackson and White 1990; Takahashi and Berger 1990; Wang and Dun 1990).

It is striking that the 5-HT_{2A} antagonists, when applied above L3, in the compartment in which the L2 motoneurons are located, had no effect on the amplitude of the L2 ventral root discharge. A plausible explanation for this finding may be that flexor motoneurons do not possess 5-HT_{2A} receptors. Flexor motoneurons make up the majority of the cells recorded in the L2 root (Cazalets et al. 1996; Kiehn and Kjaerulff 1996) and the L2 ventral root is routinely used as a monitor of flexor motoneuron activity. There is currently no information on the distribution of 5-HT_{2A} receptors on motoneurons innervating specific muscles,

although it is clear that not all motoneurons possess 5-HT_{2A} receptors (Doly et al 2004). There is evidence that maturation of 5-HT effects is responsible for the development of repetitive firing properties in extensor but not flexor motoneurons (Pflieger et al. 2002). In the chick spinal cord, 5-HT fibers were differentially distributed among motor nuclei, and dense clusters of 5-HT terminals were found preferentially on extensor motoneurons (Okado et al. 1988). Hultborn and colleagues (Hounsgaard et al. 1988) found that bistable properties were induced in extensor but rarely in flexor motoneurons of spinal cats by intravenous 5-hydrpxytryptophan, a precursor of 5-HT. Further studies are needed to determine if there is a differential distribution of 5-HT_{2A} receptors on different functional groups of motoneurons.

An alternate explanation for the absence of effects of 5-HT_{2A} antagonists when applied in the bath containing L2 is based on the apparent gradient of excitability of locomotor networks from rostral to caudal regions within the thoraco-lumbar cord (Kjaerulff and Kiehn 1996; Kiehn and Kjaerulff 1998). If the rhythmic drive to L2 motoneurons is relatively greater than that to L5 motoneurons, then blocking specific 5-HT receptors may be effective in L5, but not in L2, because the increased activity in locomotor neurons in L2 may be sufficient to overcome any reduced excitability in motoneurons produced by 5-HT receptor blockage. Thus, L2 motoneurons may possess 5-HT_{2A} receptors, but their blockage does not suppress motoneuron output.

Another striking result was that although the 5-HT_{2A} antagonists did not alter cycle duration at low concentration (and hence can be considered to act solely on

the output components of the locomotor pattern generator), they blocked rhythmic activity in the L2 ventral root when applied below the L2 segment. The activity in L5 was always reduced prior to any effect on L2, however. This sequence of effects first on L5 and then on L2 was also observed by Whelan and colleagues (Madriaga et al 2004) for ketanserin antagonism of rhythmic activity in the neonatal mouse spinal cord induced by 5-HT. Thus, even with antagonist application over both motor nuclei simultaneously, the effect on L2 lags behind that on L5. One possible explanation for our observations is that motoneurons located in L2 receive an excitatory drive from more caudally situated interneurons that possess 5-HT_{2A} receptors, while the extensor motoneurons recorded in the L5 root are more directly affected by the antagonist due to their expression of 5-HT_{2A} receptors. There is evidence that neurons other than motoneurons are immunoreactive for the 5-HT_{2A} receptor (Cornea-Hébert et al 2004), but they have not been identified or localized in any detail. In the neonatal mouse spinal cord, α-methyl-5-HT, a 5-HT2 agonist, was shown to elicit locomotor activity (Madriaga et al 2004), suggesting that cells with 5-HT_{2A} receptors can contribute to the initiation of locomotion. It is possible that blockage of L2 rhythmic output by applying the 5-HT_{2A} antagonists in the compartment below the L2 segment in our experiments is due to suppression of PPR-evoked activity in such interneurons.

Simply blocking motoneuron output in the caudal compartment with the 5-HT_{2A} antagonists might also result in loss of L2 motoneuron firing. One possible route through which this might occur is by reduced drive to Renshaw cells from the motoneurons below L5, thereby disinhibiting Ia inhibitory interneurons and

increasing their inhibition of motoneurons in other segments. We have previously shown that blocking motoneuron excitation of Renshaw cells results in increased activity in Ia inhibitory interneurons during locomotion (Noga et al. 1987).

Dopaminergic (D_1, D_2) and noradrenergic (α_1, α_2) receptors are not necessary for PPR induced locomotor-like activity

Dopaminergic and noradrenergic agonists can induce or modulate rhythmic activity in rodent *in vitro* preparations(Gabbay and Lev-Tov 2004; Jiang et al 1999; Kiehn and Kjaerulff 1996; Kiehn et al 1999; Whelan et al 2000). In the functionally mature neonatal mouse preparation, dopamine was required for chemically evoked locomotion (Jiang et al 1999). Whelan and colleagues (Madriaga et al 2004) showed that dopaminergic D_1 and D_2 receptors potentiate 5-HT's ability to evoke locomotor activity in the neonatal mouse spinal cord. In the case of PPR evoked locomotor-like activity, however, neither D_1 nor D_2 antagonists had an effect. We conclude that these dopaminergic receptors do not contribute to PPR evoked locomotor-like activity.

The α_1 noradrenergic antagonist prazosin blocked noradrenaline-evoked rhythmic activity in the isolated neonatal rat spinal cord (Gabbay and Lev-Tov 2004), but was without effect in the case of PPR-evoked locomotor-like activity in our experiments. High doses of the α_2 antagonist yohimbine also blocked noradrenaline-induced locomotor-like activity (Gabbay and Lev-Tov 2004), and our results with yohimbine were similar for PPR-evoked locomotor-like activity. However, the absence in our experiments of any blocking effect of the more specific α_2 antagonist RX 82002 (Clarke and Harris 2002) does not confirm a role for α_2 receptors in PPR-

evoked locomotor-like activity. Yohimbine is known to have a marked affinity for the 5-HT1A receptor (Newman-Tancredi et al. 1998), where it acts as an agonist. There is evidence that 5-HT, acting via 5-HT1 receptors, can inhibit the locomotor rhythm in the isolated rat spinal cord (Beato and Nistri 1998). Perhaps this explains the locomotor blocking action of yohimbine in our experiments. We argue, therefore, that neither α_1 nor α_2 noradrenergic receptors are required for PPR-evoked locomotion. The ability of yohimbine at low concentrations to shorten the locomotor cycle is consistent with the finding that α_2 noradrenergic agonists decrease the frequency of NMDA-induce locomotor-like activity (Sqalli-Houssaini and Cazalets 2000), and suggests that these receptors can also modulate locomotion induced by PPR stimulation.

Summary

We have shown for the first time that the descending monoaminergic pathway responsible for locomotor-like activity in the neonatal rat originates primarily, if not exclusively, in the 5-HT-containing neurons located in the parapyramidal region of the medulla. The results also show that PPR - evoked locomotor-like activity can be blocked by antagonists to 5-HT_{2A} and 5-HT_7 receptors, and that the neurons possessing these two classes of receptors are concentrated in different rostro-caudal regions. 5-HT_7 receptor antagonists were effective when applied to the rostral compartment (above L3); 5-HT_{2A} receptor antagonists blocked locomotor-like activity only when applied to the caudal compartment (below L2). Low doses of 5-HT_7 receptor antagonists decreased step cycle duration, suggestive of an action on

neurons involved in the rhythm-generating function of the CPG for locomotion. 5-HT_{2A} antagonists acted in a different way, reducing the amplitude of ventral root discharges without affecting step cycle duration, consistent with an action directly on motoneurons, or on premotor cells involved in controlling the recruitment of motoneurons during locomotor-like activity. Dopaminergic (D_1, D_2) and noradrenergic (α_1, α_2) antagonists are not required for PPR-evoked locomotor-like activity.

Figure legends

Figure 1. A: Fluorescence photomicrographs of 5-HT containing cells labeled in the PPR of the mid-medulla. It can be seen that a cluster of 5-HT labeling positive cells are located just lateral to the pyramidal tract. B: A representative coronal brainstem section 0.8 mm caudal to the ponto-medullary junction showing the effective site of electrically-evoked locomotor-like activity and 5-HT immuno-positive neurons. The arrow indicates the site of the lesion produced by anodal DC current applied through the stimulating electrode (500 μA, 10 sec). B: Schematic drawing of a coronal section of the brainstem 0.5-1.0 mm caudal to the ponto-medullary junction from 12 typical experiments demonstrating the effects at various stimulus sites. Sites producing locomotor-like activity (▲) are concentrated in the para-pyramidal region, among the para-raphe 5-HT neurons. Other effects observed were alternating discharge of one or two ventral roots of 3-5 sec duration (Δ), increased tonic activity in one or more ventral roots (■) or no response (○). Sites of the pyramidal tract (PT), the nucleus of the VIIth cranial nerve (VII) the

gigantocellular reticular nucleus, pars alpha (GiA), the nucleus raphé obscurus (ROb) and the nucleus raphé pallidus (RPa) are indicated (modified from Paxinos et al 1991).

Figure 2. A: Locomotor-like activity evoked by electrical stimulation of PPR in the neonatal rat preparation at 160 uA, 5 ms duration, 3 Hz and 60 s stimulation. Rhythmic activity was recorded from the left and right L2 and L5 ventral roots. *Upper panel*: raw waveforms. *Lower panel*: rectified and filtered waveforms. **B**: Rasters of 15 step cycles during the rhythmic activity. The start of RL2 discharge at a 400ms delay was used to define the onset of each step cycle, and the window of the frame was 4.6 s. **C**: Polar plots showing the phase value and coupling strength of alternating activities between ipsilateral ventral roots (RL2/RL5 and LL2/LL5) and the contralateral roots (RL2/LL2 and RL5/LL5) from 11 preparations (number of cycles from each preparation was 10-20). The gray vectors represent the phase values (Φ) averaged from 10-20 step cycles of single brainstem-spinal preparations; and the black ones represent the mean results from the 11 individual phase values. The length (r) of the vector indicates the coupling strength of the step cycles of the paired ventral roots.

Figure 3. A: Microinjection of glutamate (1 μM/10 mM) to the site effective to electrically evoked locomotion induced locomotor activity. B: An example of Microinjection of bicuculline (1 μM/10 mM) to the PPR to evoke well-coordinated

locomotor-like activity. The episodic locomotor-like activity occurred 3-5 min after the injection and lasted 20-30 min.

Figure 4. The 5-HT_{2A} receptor antagonists ketanserin and spiperone block PPR-evoked locomotor-like activity when applied to the caudal bath. *Upper panels* in **A** and **B** show the control rhythmic activity before ketanserin application. Stimulus intensity: 160 μA, 5 ms duration at 2.6 Hz (A); 120 μA, 5 ms duration at 2.6 Hz (B). **A**: Ketanserin (20 μM) produced no change in rhythmic activity when applied to the rostral bath (*lower panel*). Stimulation was delivered at 5 min after drug application. **B**: Rhythmic activity was blocked when ketanserin (20 μM) was applied to the caudal bath (*lower panel*). Stimulation was applied at 3 min after drug application. **C**: Spiperone (15 μM) abolished PPR-evoked locomotor-like activity 3 min after drug application when applied to the caudal bath. *Upper panel*: Control PPR-evoked locomotor-like activity before drug application. *Lower panel*: Stimulation was given at 3 min after drug application. Stimulus intensity: 100 μA, 5 ms duration at 2.8 Hz. **D**: The application of a low concentration of ketanserin (10 μM) to the caudal bath caused a substantial decrease in the amplitude of the L5 ventral root discharges, whereas the frequency remained unchanged. *Upper panel*: control PPR-evoked locomotor-like activity. *Lower panel*: stimulation was delivered at 10min after ketanserin application. Stimulus intensity: 100 μA, 5 ms duration at 2.8 Hz. Note that the L2 ventral root discharges are unaffected, while the output via the L5 ventral root was steadily diminished.

Figure 5. A: Histograms of ventral root discharge amplitude of (*left panel*) and cycle duration (*right panel*) produced by administration of ketanserin to the caudal bath at the various concentrations as a function of time course (~20 min). Note that ketanserin predominantly reduced ventral root discharge amplitude rather than cycle duration. The ordinate represents proportion of normalized amplitude or cycle duration. Error bars represent standard deviation. Ø indicates the cessation of locomotor-like activity. Similar histograms for the effects of clozapine (**B**) and SB269970 (**C**) to the rostral bath at the various concentrations as a function of time course (~20min). Both clozapine and SB269970 caused a substantial prolongation of cycle duration; and a moderate decrease in ventral root discharge amplitude. *: <0.05; **: <0.01; ***:<0.001 The ordinate represents amplitude or cycle duration normalized to the control value.

Figure 6. The 5-HT_7 receptor antagonist clozapine blocks PPR-evoked rhythmic activity when applied to the rostral lumbar region. *Upper panels* in **A** and **B** showed control rhythmic activity evoked by electrical stimulation of the PPR before drug application. Stimulus intensity: 140 μA, 5 ms duration at 2. 5 Hz (A); 160 μA, 5 ms duration at 2.6 Hz (**B**). **A:** The rhythmic activity was not disrupted when clozapine (1μM) was applied to the caudal bath (*lower panel*). Stimulation was given at 5min after drug application. **B:** The rhythmic activity was abolished when clozapine (1 μM) was applied to the rostral bath at 3min after drug application (*lower panel*).**C:** A low concentration of clozapine when applied to the rostral bath gave rise to a considerable prolongation in the cycle duration. Upper panel: control PPR-evoked

locomotor-like activity. Stimulus intensity: 140 μA, 5 ms duration at 3.0 Hz. Lower panel: the application of clozapine (0.5 μM) in the rostral bath increased the cycle duration. Stimulation was applied at 10 min after drug application.

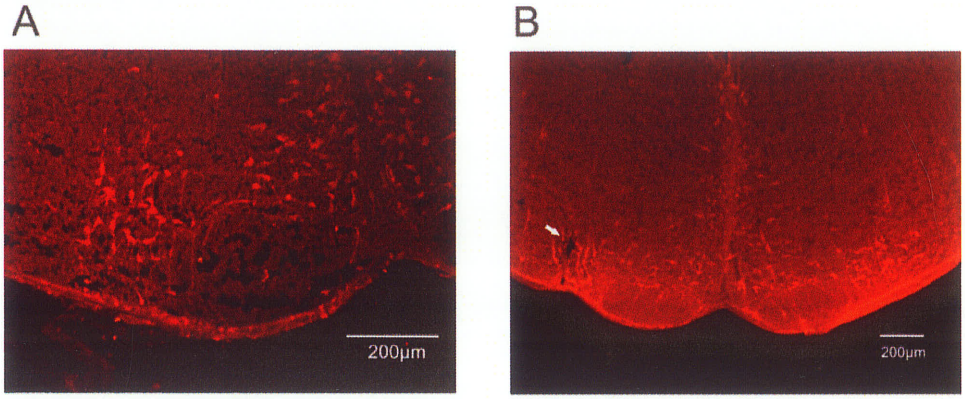
Figure 7. The specific 5-HT_7 receptor antagonist SB269970 blocked PPR-evoked locomotor-like activity when applied to the rostral bath. *Upper panel* in **A** and **B** show control rhythmic activity evoked by electrical stimulation of the PPR before drug application. Stimulus intensity: 160 μA, 5 ms duration at 2.6 Hz (A); 180 μA, 5 ms duration at 2.6 Hz (**B**). **A**: Rhythmic activity was not affected when SB269970 (15 μM) was applied to the caudal bath as shown in *lower panel.* Stimulation was delivered at 5min after drug application. **B**: As shown in *lower panel*, rhythmic activity was abolished when applied to the rostral bath at the same concentration at 5 min after drug application. **C**: A low concentration of SB269970, when applied to the rostral bath, caused a significant prolongation of step cycle duration. *Upper panel*: control PPR-evoked locomotor-like activity before drug application. Stimulus intensity: 80 μA, 5 ms duration at 2.8 Hz. *Lower panel*: a low concentration of SB 269970 (10 μM) when applied to the rostral bath caused a substantial prolongation of the cycle duration as well as a decrease in the amplitude of ventral root discharges. Stimulation was delivered at 10 min after drug application.

Figure 8. The 5-HT_7 receptor antagonist SB269970 (10μM) gave rise to a significant inhibitory effects on the locomotor-like activity evoked by the

microinjection of bicuculine (1 μl/10 mM) in a PPR previously effective for locomotor-like activity evoked by electrical stimulation. The control locomotor-like activity before drug application is shown in the *upper panel.* The locomotor-like activity was suppressed approximately 5 min after application of SB269970 (*middle panel*), and the rhythmic activity was abolished after 10 min (*bottom panel*).

Figure 9. The α_2 noradrenergic receptor antagonist yohimbine brought about variable effects on PPR-evoked locomotor-like activity at different concentrations. Stimulus intensity: 160 μA, 5ms duration at 2.6 Hz (**A**); 180 μA, 5ms at 2.6 Hz (**B**). *Upper panel* in **A** and **B** showed control rhythmic activity before the addition of yohimbine. **A**: Yohimbine (5 μM) caused a significant shortening of cycle duration (*lower panel*) when applied to the whole bath after ~10 min. **B:** A high concentration of yohimbine (25 μM) eliminated locomotor-like activity after 5 min. of drug application (*lower panel*).

Figures



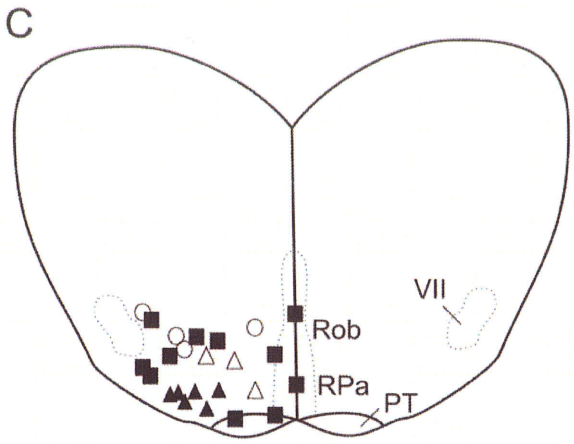


Figure 1

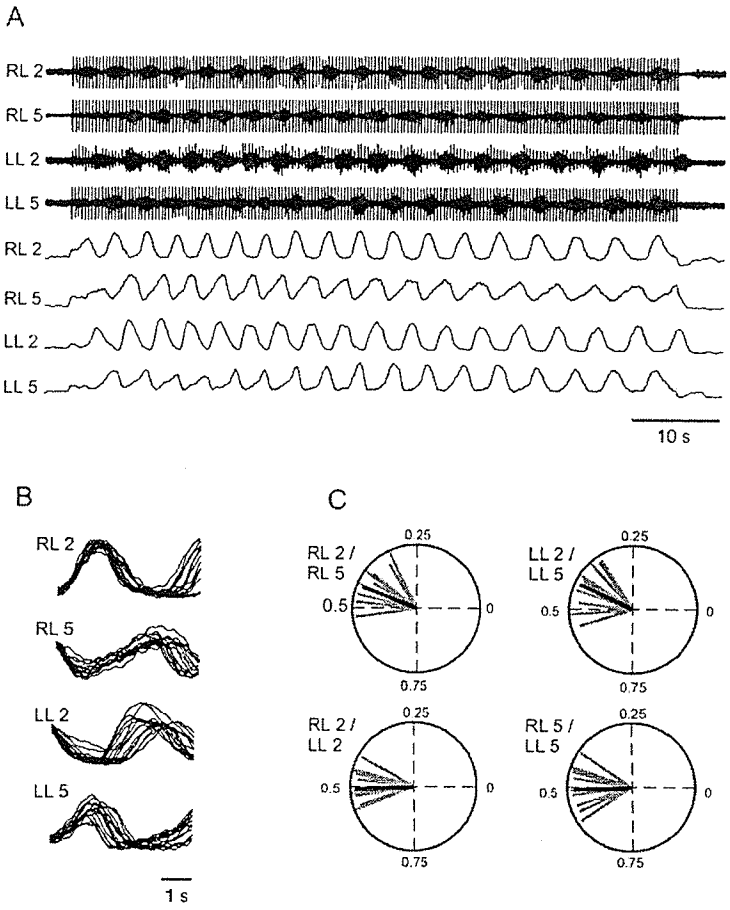

A
RL 2
RL 5
LL 2
LL 5
RL 2
RL 5
LL 2
LL 5
10 s
B
RL 2
RL 5
LL 2
LL 5
1 s
C
RL 2 / RL 5
LL 2 / LL 5
RL 2 / LL 2
RL 5 / LL 5
0.25
0.5
0
0.75

Figure 2

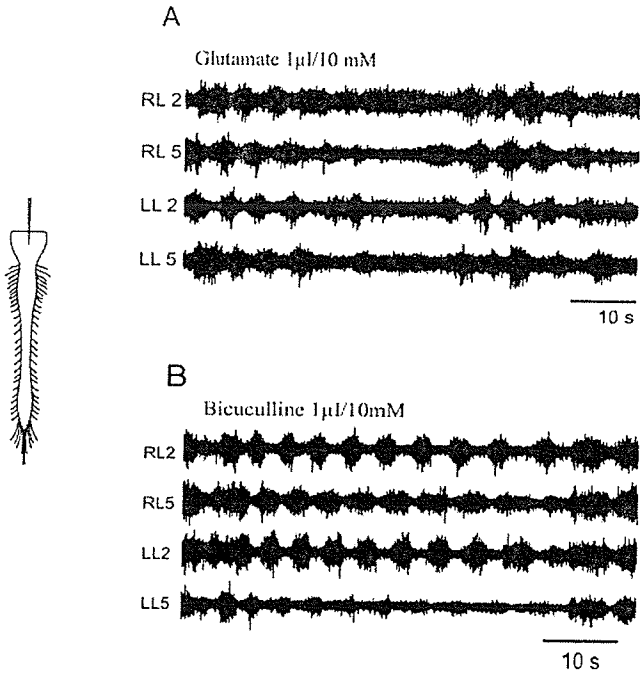

A
Glutamate 1µl/10 mM
RL 2
RL 5
LL 2
LL 5
10 s
B
Bicuculline 1µl/10mM
RL2
RL5
LL2
LL5
10 s

Figure 3

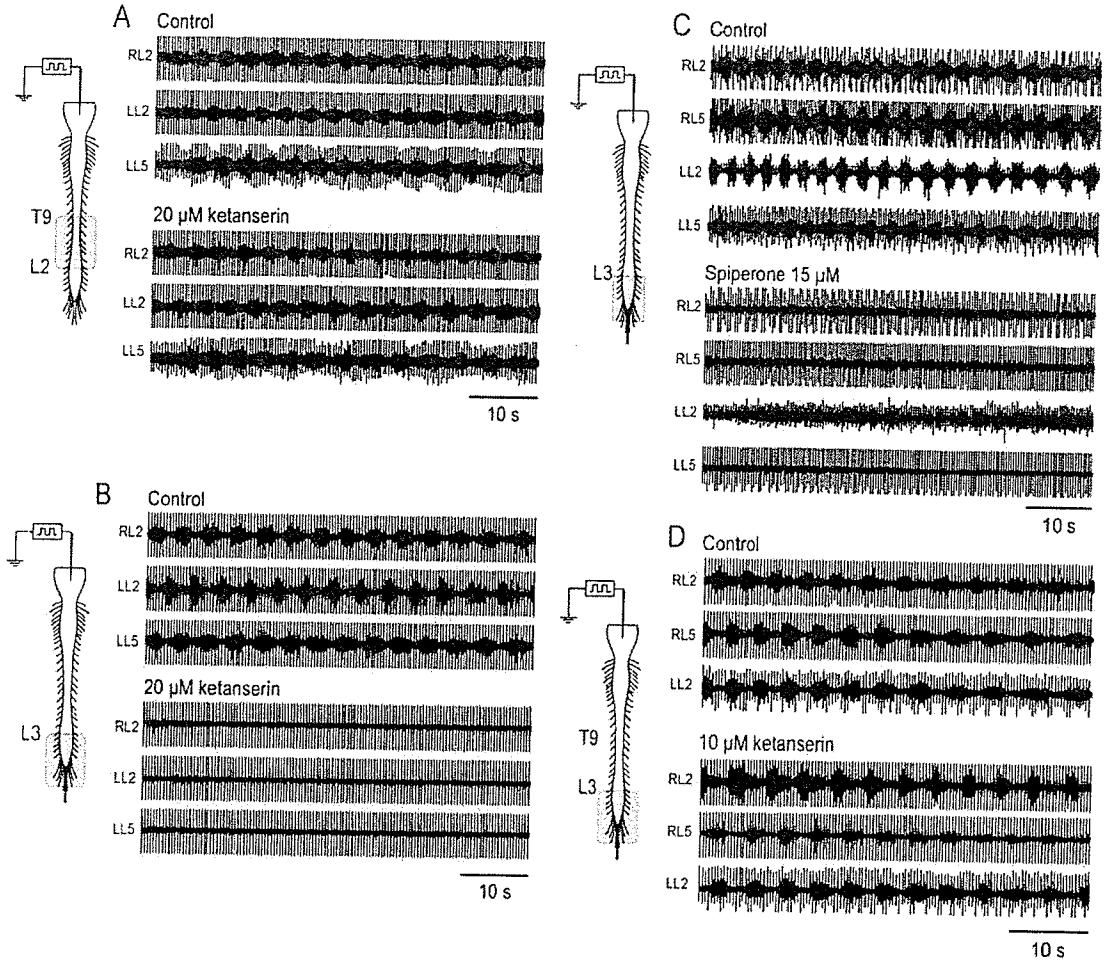

A
Control
RL2
LL2
LL5
T9
L2
20 µM ketanserin
RL2
LL2
LL5
10 s
B
Control
RL2
LL2
LL5
L3
20 µM ketanserin
RL2
LL2
LL5
10 s
C
Control
RL2
RL5
LL2
LL5
L3
Spiperone 15 µM
RL2
RL5
LL2
LL5
10 s
D
Control
RL2
RL5
LL2
T9
L3
10 µM ketanserin
RL2
RL5
LL2
10 s

Figure 4

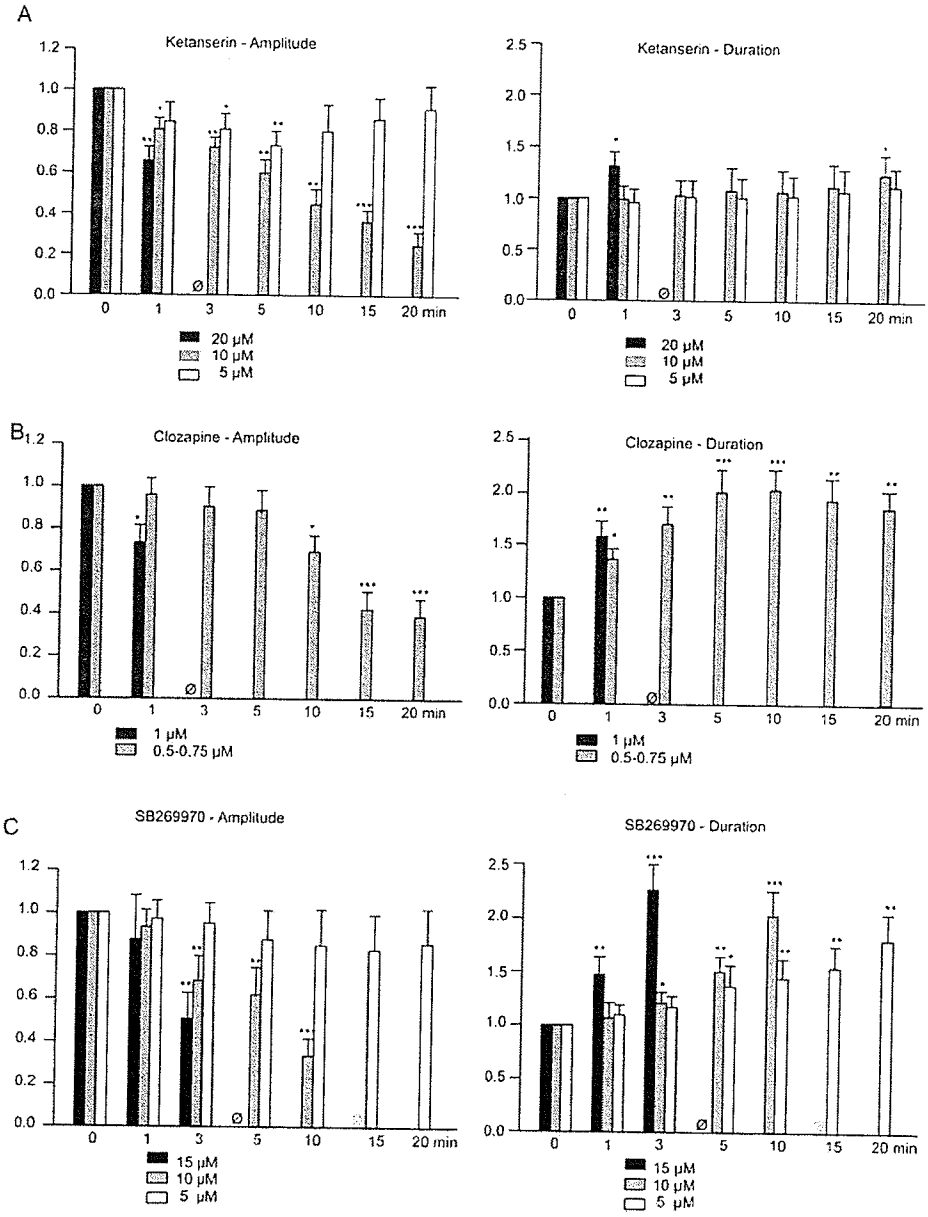

A
Ketanserin - Amplitude
Ketanserin - Duration
20 µM
10 µM
5 µM
B
Clozapine - Amplitude
Clozapine - Duration
1 µM
0.5-0.75 µM
C
SB269970 - Amplitude
SB269970 - Duration
15 µM
10 µM
5 µM
0
1
3
5
10
15
20 min

Figure 5

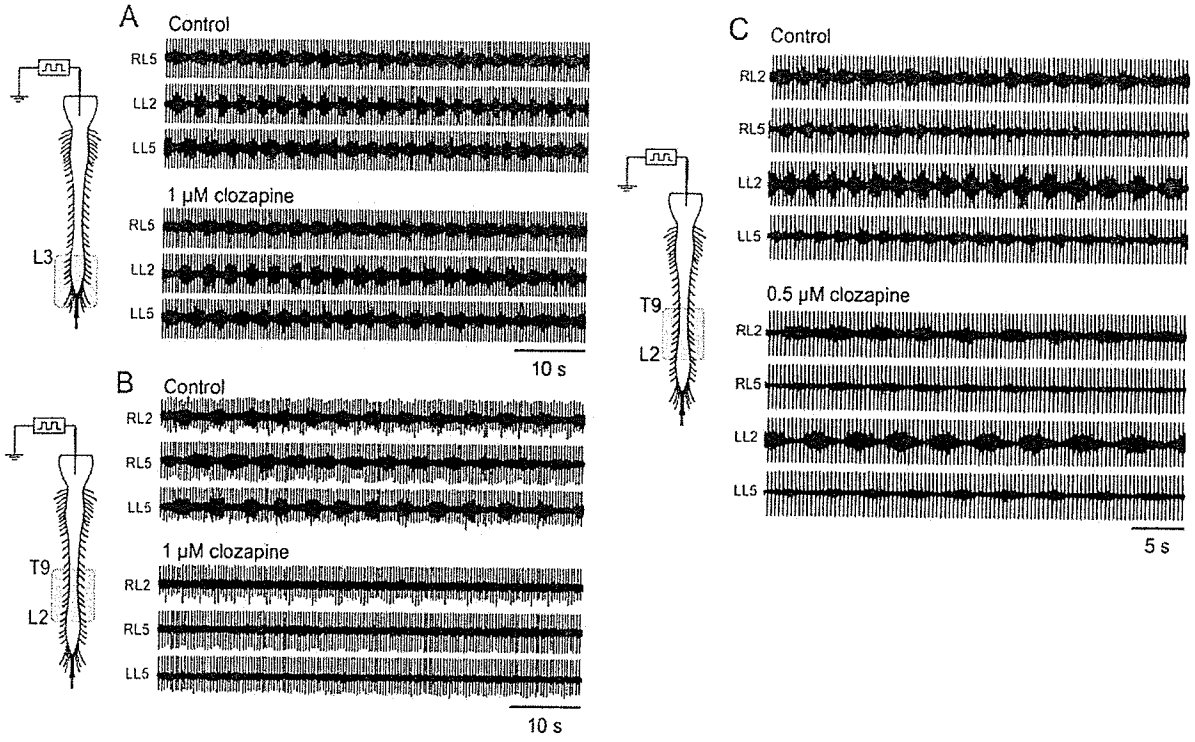

A
Control
RL5
LL2
LL5
1 μM clozapine
RL5
LL2
LL5
10 s
L3
B
Control
RL2
RL5
LL5
1 μM clozapine
RL2
RL5
LL5
10 s
T9
L2
C
Control
RL2
RL5
LL2
LL5
0.5 μM clozapine
RL2
RL5
LL2
LL5
5 s
T9
L2

Figure 6

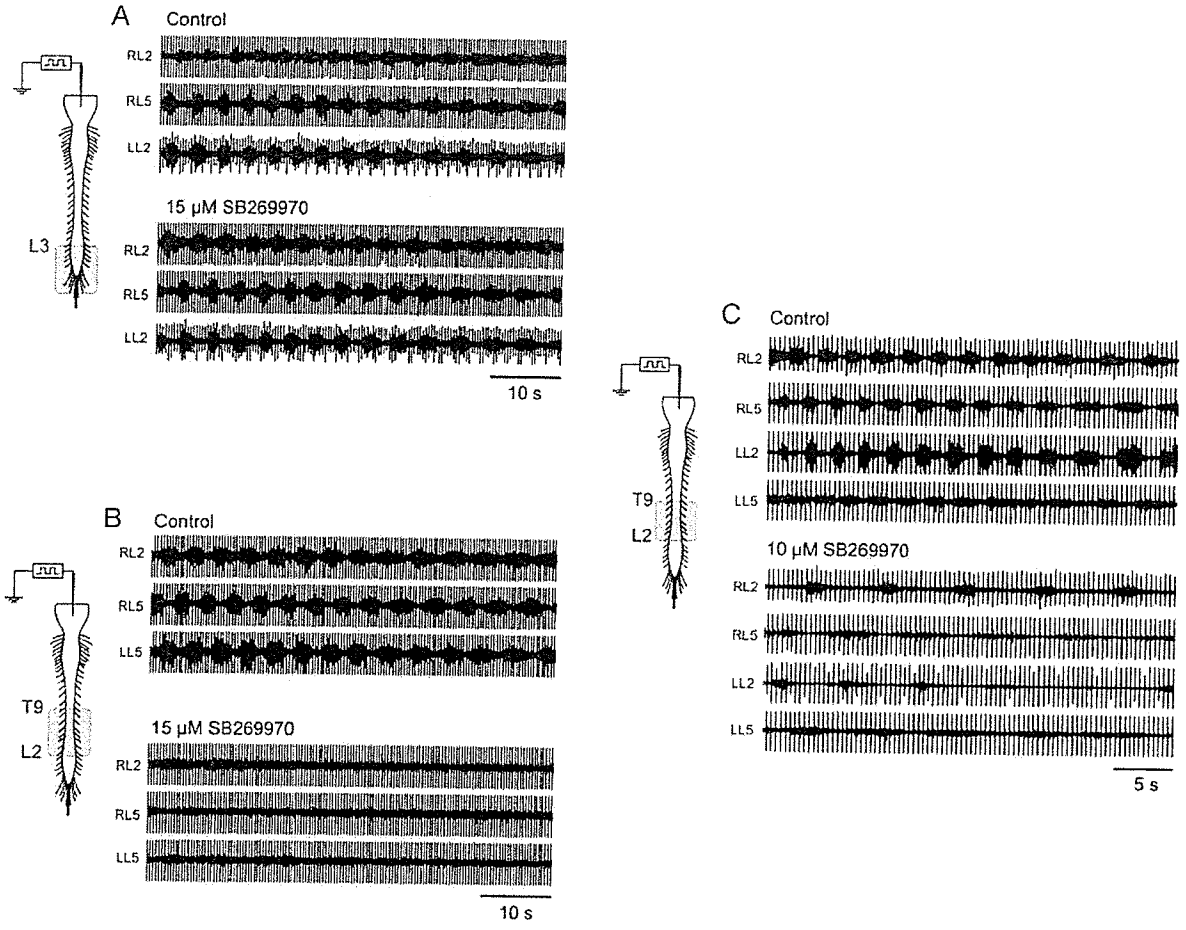

A
Control
RL2
RL5
LL2
15 μM SB269970
RL2
RL5
LL2
L3
10 s
B
Control
RL2
RL5
LL5
15 μM SB269970
RL2
RL5
LL5
T9
L2
10 s
C
Control
RL2
RL5
LL2
LL5
10 μM SB269970
RL2
RL5
LL2
LL5
T9
L2
5 s

Figure 7

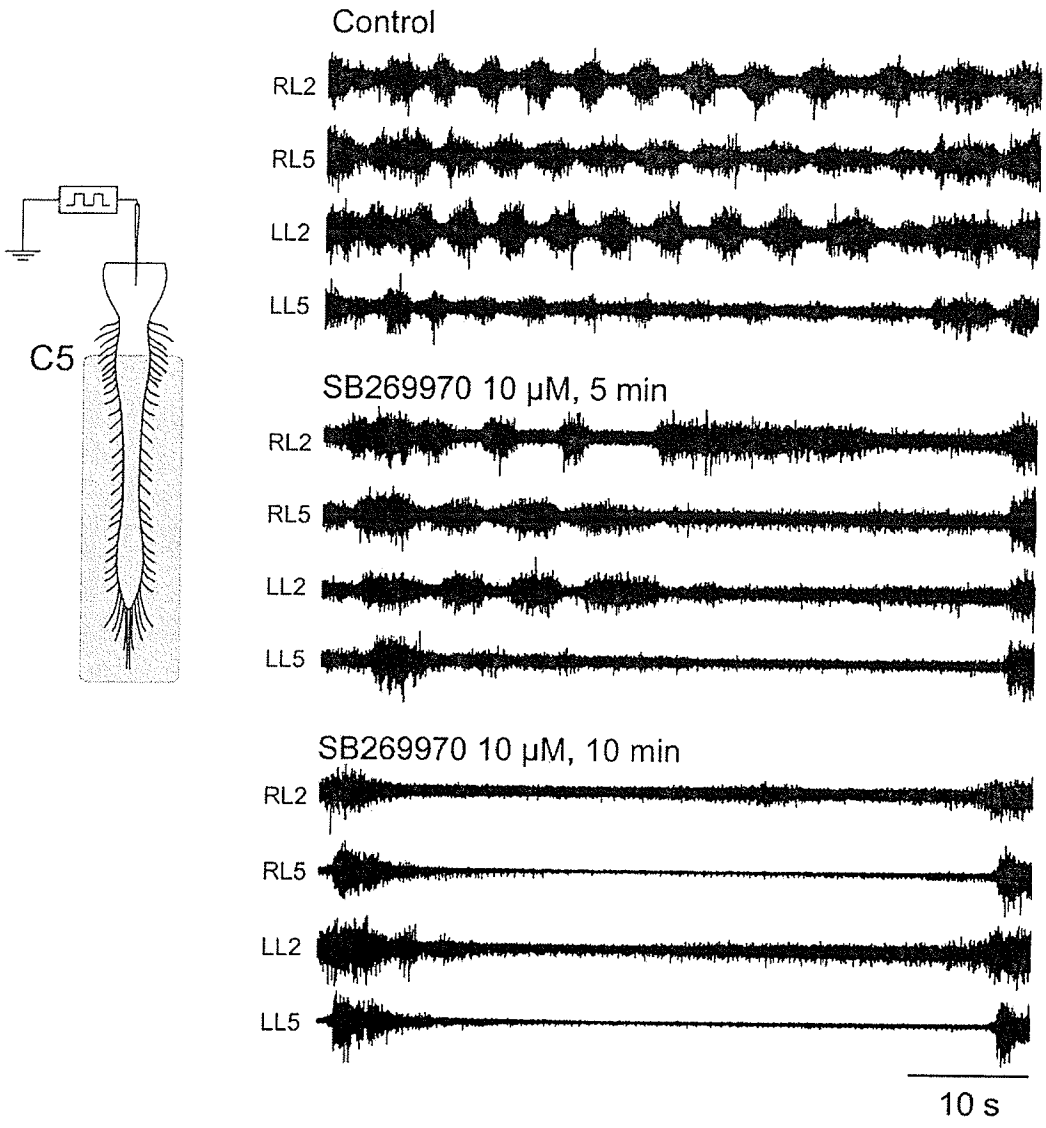

Control
RL2
RL5
LL2
LL5
C5
SB269970 10 µM, 5 min
RL2
RL5
LL2
LL5
SB269970 10 µM, 10 min
RL2
RL5
LL2
LL5
10 s

Figure 8

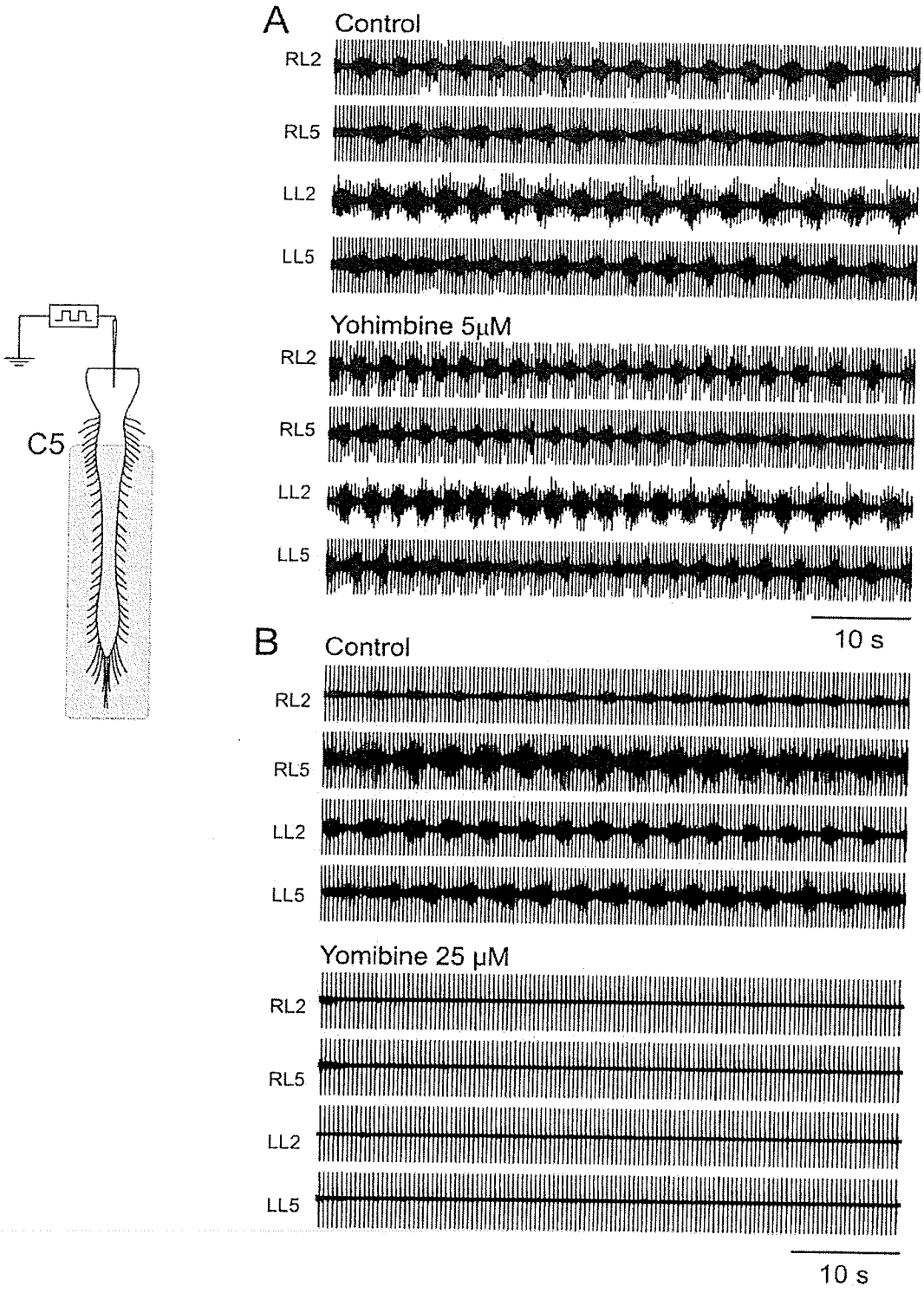

A
Control
RL2
RL5
LL2
LL5
Yohimbine 5μM
RL2
RL5
LL2
LL5
10 s
B
Control
RL2
RL5
LL2
LL5
Yomibine 25 μM
RL2
RL5
LL2
LL5
10 s
C5

Figure 9

Section 2: **5-HT$_7$ receptors play an important role in locomotor activity: the in vitro neonatal and adult studies on 5-HT$_7$ receptor knockout mice.**

Hypothesis I: 5-HT induced locomotor-like activity is defective in neonatal mice lacking 5-HT$_7$ receptors.

Hypothesis II: 5-HT$_7$ receptors are activated during normal voluntary locomotion in adult mice.

Abstract

Numerous reports have demonstrated that 5-HT elicits locomotor-like activity in rodent *in vitro* spinal cord preparations. Several lines of evidence have shown that activation of 5-HT$_7$ receptors in the spinal cord contributes to the control of locomotor-like activity. In this study, we have used a strain of mice with a 5-HT$_7$ receptor gene disruption to test the following hypotheses that: 1) 5-HT induces locomotor-like activity in the isolated neonatal mouse spinal cord by acting at the 5-HT$_7$ receptor; 2) Blocking 5-HT$_7$ receptors disrupts coordinated voluntary locomotion in adult mice. *In vitro* experiments were performed on the isolated spinal cord of postnatal day 0-5 animals, and recordings were made from the L2 (flexor) and L5 (extensor) ventral roots bilaterally. 5-HT was bath applied into the recording chamber to activate spinal motor circuits. We found that 53.8% (n=7/13) of 5-HT$_7^{+/+}$ preparations displayed the typical locomotor-like response to 5-HT.

This locomotor-like activity was disrupted and subsequently blocked by SB269970, a specific 5-HT7 receptor antagonist. However, 5-HT application resulted in (1) uncoordinated rhythmic activity in 70% (n=7/10) of 5-$HT_7^{-/-}$ mice, or (2) ipsilateral synchronous discharges of the ventral roots (20%, n=2/10). Locomotor-like activity was only observed in one preparation of 5-$HT_7^{-/-}$ mice, whereby the rhythm was not affected by SB269970, but blocked by the ketanserin. This uncoordinated rhythmic activity activity in the 5-$HT_7^{-/-}$ animals was not affected by the 5-HT_7 antagonist SB269970, but could be abolished by the 5-HT2A antagonists ketanserin and spiperone.

In *in vivo* experiments, adult mice were placed in a walkway and a laminectomy was performed to expose the L2 and L3 spinal segments for direct drug application to the spinal cord. Quantitative methods, kinematic measurements and EMG recording were utilized to monitor and analyze voluntary locomotion prior to and after drug administration. Intrathecal administration of SB269970 consistently disrupted locomotion in 5-$HT_7^{+/+}$ mice, producing "hyper-extension" of both hindlimbs such that the stance phase often persisted beyond the point where weight support could be maintained and resulting in slowed locomotion and "dragging" of the hindlimbs. In most (4 out of 6) 5-$HT_7^{-/-}$ mice, SB269970 produced no overt effects on locomotion were observed compared to those in wild type animals. Although 2 5-$HT_7^{-/-}$ animals exhibited hindlimb "hyper-extension" after SB269970, the severity of changes in hindlimb joint angles and the frequency of occurrence of hyperextension were much less than in 5-$HT_7^{+/+}$ mice. These results suggest that 5-HT_7 receptors are required for the production of 5-HT-induced locomotor-like

activity in the spinal cord *in vitro* and activation of 5-HT_7 receptors plays an important role in the control of voluntary locomotion in adult mice.

Introduction

Strong evidence suggests that 5-hydroxytryptamne (5-HT) is a transmitter involved in a descending command system sufficient for production of locomotion. The earliest indication of this was the demonstration by Viala and Buser (1971) that administration of 5-HTP, a precursor of 5-HT, enhanced or induced locomotor activity in spinal rabbits. Subsequent progress on the development of the concept that a descending 5-HT locomotor command pathway is sufficient to produce locomotion has been reviewed (Schmidt and Jordan 2000; Jordan and Schmidt 2002).

There is increasing interest in the possibility that recovery of locomotion after injury can be facilitated by use of 5-HT agonists or transplantation of 5-HT neurons into the spinal cord. Intrathecal application of 5-HT or systemic administration of quipazine, a 5-HT agonist, produced locomotion in adult spinal rats (Feraboli-Lohnherr et al. 1999), and recovery of locomotor activity in spinal rats was improved by 5-HT agonists (Kim et al. 2001; Antri et al 2002; Antri et al. 2005; Landry et al 2006). Transplantation of 5-HT neurons into the spinal cord below the lesion (Gimenez y Ribotta et al 2000; Slawinska et al 2000; Majczynski et al 2005) also improves locomotor recovery. The specific 5-HT neurons that are involved in the 5-HT locomotor command system have not been identified.

Reticulospinal cells located in the medial reticular formation, including the area containing descending 5-HT neurons, are required for locomotion evoked from the MLR (Shefchyk et al 1984; Noga et al 1991; Noga et al 2003). We have now localized a discrete population of 5-HT neurons in the parapyramidal region (PPR) of the medulla that, when stimulated, elicits locomotor-like activity in the neonatal rat isolated brainstem-spinal cord preparation (Liu and Jordan 2005). This 5-HT pathway constitutes the first identified descending command system for the initiation of locomotion. Locomotion evoked by stimulation of PPR neurons can be disrupted or blocked by 5-HT_7receptor antagonists in the isolated brainstem-spinal cord preparation of the neonatal rat (Liu and Jordan 2005), and MLR-evoked locomotion in the decerebrate cat can also be blocked by a 5-HT_7 antagonist (Schmidt and Jordan 2000).

The first suggestion of involvement of 5-HT_7 receptors in the control of locomotion induced by 5-HT was proffered by Cina and Hochman (1998). This notion has been confirmed by others (Madriaga et al 2004; Pearlstein et al 2005; Landry et al 2006). The *in vitro* locomotor rhythm is slowed and coordination among antagonist flexor and extensor nerve pairs and left and right sides is disrupted by a selective 5-HT_7 receptor antagonist (Madriaga et al 2004; Liu and Jordan 2005; Pearlstein et al 2005), suggesting that neurons of the central pattern generator for locomotion (CPG) possess 5-HT7 receptors.

Animals with a targeted deletion of 5-HT_7 receptors (5-HT_7 $^{-/-}$ mice) have been produced (Hedlund et al. 2003; Roberts et al. 2004), and we have employed these animals in experiments designed to test the hypotheses that 5-HT_7 receptors are

involved in modulation of 5-HT-induced locomotor-like activity in the mouse pinal cord in vitro. It is presumed that 5-HT decending system paly an important role for locomotion in adult animals. We reasoned that if 5-HT_7 receptors normally participate in the production of locomotor activity, then the ability of 5-HT to induce locomotor-like activity *in vitro* would be disrupted in 5-HT7 $^{-/-}$ mice. Moreover, if 5-HT_7 receptors normally play a role in the production of locomotion in adult animals, then it would be expected that voluntary locomotion would be disrupted by a 5-HT_7 receptor antagonist in adult wild-type mice (5-HT7 $^{+/+}$) but not in 5-HT7 $^{-/-}$ mice.

Methods

Mice lacking 5-HT_7 receptors were created by targeting the disruption of the 5-HT_7 receptor gene, previously described in Hedlund et al (2003). Crossings of heterozygous 5-$HT_7^{+/-}$ mice produced homozygous wild-type (5-$HT_7^{+/+}$), homozygous knockout mice (5-$HT_7^{-/-}$) and heterozygous 5-$HT_7^{+/-}$ mice at the expected Mendelian frequency. Genotyping consisted of detecting the neomycin resistance gene and a part of exon II of the receptor gene using PCR (Fig. 1). Exon

II primers: F: GAC AAA GTG TGC TTG ATC AGC CAG G

R: ATG CAG CTA CAG GAG GTG CCA CAG

400 base pairs

Neo primers: F: CTT GGG TGG AGA GGC TAT TC

R: AGG TGA GAT GAC AGG AGA TC

280 base pairs

Control primers: Mouse beta casein (MBC)

F: GAT GTG CTC CAG GCT AAA GTT CAC

R: AGA AAC GGA ATG TTG TGG AGT GGC

500 base pairs

Animals and procedures, <u>in vitro</u> experiments: The experiments were performed on neonatal C57B/6J 5-HT_7 receptor $^{-/-}$ and wild-type mice (0-5 days old). All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and were approved by the University of Manitoba Animal Protocol Committee. Following anesthesia with halothane, the animal was immediately decapitated, eviscerated and removed to a Sylgard coated recording chamber. A laminectomy was performed and the spinal cord was isolated. The spinal cord was transected below T1 and was superfused with artificial cerebrospinal fluid (aCSF, concentration in mM: NaCI 128, KCI 3.0, NaH2PO4 0.5, CaCI2 1.5, MgSO4 1.0, NaHCO3 21, and glucose 30) and oxygenated with 95% and 5% CO_2 at room temperature. The preparation was pinned ventral side up to the sylgard surface and allowed to stabilize for 30 min before recording. During experiments the temperature of the bath solution was maintinaed at 25°C

Animals and procedures, <u>in vivo</u> experiments: CD1/ICR (4) and C57B/6J (9) mice were used in these experiments. Adult wild-type and 6 littermates (C57B/6J background) with a targeted disruption of the 5-HT_7 receptor gene (Hedlund et al 2003) were used to determine if 5-HT_7 receptors are essential for the action of SB-

269970. Prior to surgery, the opioid receptor agonist buprenorphine (0.15 mg/kg) was injected subcutaneously. Anesthesia was induced with Forane (Isoflurane, Baxter Corporation, Toronto, Ontario) as previously described (Akay et al. 2006). A laminectomy was performed at the T12 and T13 vertebrae to expose the L1 and L2 segments of the spinal cord. The dura matter was removed with a fine spring scissor. Additionally little openings were also made on the pia to ease the drug diffusion into the spinal cord. The 5-HT_7 antagonist SB-269970 (1-10 mM) or vehicle (saline) was then applied for 30 minutes in a bath created with Vaseline around the opening in the dura. At the end of the 30 minute period the drug or saline solution and the Vaseline was quickly removed, the wound was closed and the anesthesia was discontinued (See Fig. 2). The mouse was placed on a transparent walkway, as previously described (Fig. 3) (Pearson et al. 2005; Akay et al 2006). The mice normally commenced voluntary locomotion approximately one or two minutes after recovery from the anesthesia. Due to the application of the opioid receptor agonist buprenorphine the mice walked undisturbed without any signs of pain from the surgery. "Pre-control", "drug application", and "post-control" recordings were captured. The effect of the applied drug lasted up to 2 hours, dependent on the dose. After the recordings were made, the animals were anesthetized again and Sodium Pentobarbital (0.96 mg/ml) 0.2 ml was injected intraperitoneally for euthanization.

Electrophyisiological recordings, <u>in vitro</u> experiments: Glass suction electrodes were applied to record the L2 and L5 ventral discharges of both sides as monitors of

locomotor-like activity (Kjaerulff and Kiehn 1996; Cowley and Schmidt 1997). Ventral root recordings were amplified, filtered (30 Hz-2 KHz), digitized, stored and analyzed using software developed at the Winnipeg Spinal Cord Research Centre (see http://www.scrc.umanitoba.ca/doc/ for details). Locomotor-like activity consisted of alternating activity recorded between both the left and right L2 and L5 ventral root pairs, as previously described for in vitro isolated mouse spinal cord (Nishimaru et al 2000; Whelan et al 2000; Madriaga et al 2004). Locomotor-like activity was evoked by bath application of 5-HT (20-50 μM). In some experiments, SB-269970 (5-20 μM) was added to block 5-HT_7 receptors, respectively. All drugs were obtained from Sigma-Aldrich.

Electrophyisiological recordings, in vivo experiments: The fabrication and implantation of EMG electrodes was as previously described (Pearson et al 2005; Akay et al 2006). For implantation, the mice were anesthetized with isoflurane and the hind legs and the dorsum of the neck were shaved. Small incisions were made in the skin over the dorsal neck area and tibialis (TB, ankle flexor) and gastrocnemius (GS, ankle extensor) muscles, and bipolar electrodes were drawn under the skin from the neck incision to the leg incisions. The electrodes were inserted into the muscles as previously described (Pearson et al 2005). The incisions on the legs were closed, and a headpiece with the electrodes sutured to it was stitched to the skin near the neck incision. Buprenex (buprenorphine hydrochloride, Reckitt Benckiser Healthcare Ltd., Hull, UK, 0.15 mg/kg) was injected subcutaneously for

analgesia, and the mice were left in their cages for recovery from surgery at least two days before performing the experiments.

Kinematic recordings during overground locomotion in adult mice: Custom-made three-dimensional reflective markers (2 mm diameters) were glued onto the shaved skin at the level of the iliac crest, hip, knee, ankle, paw, and tip of the fourth digit (toe) of the left hind leg, and one on the wrist of the left foreleg (Fig. 3A). Knee position was calculated by triangulation from the position of hip and ankle joint markers, using the measured lengths of the femur and tibia. For the video recordings during free walking, the mice where placed into a custom made Plexiglas walkway (90cm long, 5cm wide and 13cm high). After recovery from anesthesia, the mice walked freely back and forth in the walkway. The center of the walkway was viewed with a high speed camera (Photron Fastcam) with the capture rate at 250 frames/s (Fig. 3B). Video data were stored in computer memory and were analyzed using Peak Motus 8.2 motion analysis software (ViconPeak, Denver, Co). Kinematic parameters of the stepping movements, such as swing and stance durations, were measured from data files created by the Peak Motus System. The coordination between the legs was determined from video images captured by a mirror placed underneath the walkway set at approximately 45degrees from vertical. These images allowed measurement of the time of swing onset of each leg, defined as time of the onset of forward movement of the paw.

Measurements and Statistical analysis:

Step cycle frequency and duration were determined by measuring right or left L2 ventral root ENG activity (in vitro experiments) or TA and GS EMG after filtering and rectification (in vivo experiments). A five-minute episode of data with stable bursting was used for analysis after drug administration. For the EMG recordings, the measurements were taken at immediately after removal of the anesthetic and up to 90 minutes after removal from anesthesia. The statistical values are indicated as the mean ± SD; n refers to the number of experiments. Student's t-test was used to compare the differences between the mean values and P values less than 0.05 were considered significant. Circular statistics was used to determine the coupling strength (Φ; also called phase values) between flexor/extensor and right/left ventral roots or left and right TA and GS EMG recordings (Kriellaars et al 1994; Kjaerulff and Kiehn 1996; Liu and Jordan 2005). A phase value of 0.5 indicates strict alternation between the onsets of bursts of activity in the chosen roots or muscles. The mean value (r) reflecting the concentration of phase values was plotted as the length of the vector, which ranged from 0 to 1. In our experiments, cross-correlation analysis was also used to determine the coupling between the flexor/extensor and right/left ventral roots ENG recordings or ipsilateral TA/GS and right/left TA or GS EMG recordings, measured as the minimum negative value at time 0 subtracted from the maximum first positive value of the cross-correlogram, or peak-to-trough correlation coefficient (PTCC) (Madriaga et al 2004). The mean PTCC was averaged of all individual values observed in a given experimental condition. The maximum PTCC is defined as -2. In video recordings of the *in vivo* experiments,

the cycle periods were measured from the onset of swing (defined as the start of forward movement of the toe) to the onset of the following swing phase.

Results

Properties of locomotor-like activity produced <u>in vitro</u> by 5-HT in wild-type mice:

Bath application of 5-HT (20-50 μM) induced locomotor like activity in 53.8% (7 of 13) 5-HT_7 $^{+/+}$ spinal cord preparations. The mean stepping frequency was 0.12 ± 0.09 Hz, and the burst duration was 5.07±2.82s. The initial exposure to 5-HT always resulted in an increase in tonic discharges in all four neurograms, then followed by rhythmic alternating activity (n=7, Fig. 4A). In 2 preparations uncoordinated rhythmic activity was observed in the first 15 min after 5-HT application followed by alternate locomotor activity. A representative example of locomotor-like activity recorded in the ventral roots and evoked by 30 μM 5-HT is presented in Fig. 4A. Fig. 4B shows a raster display of the four ventral roots from 15 cycles during which the alternation between flexor (L2) and extensor (L5) and right and left ventral roots was clearly observed. Locomotor-like activities from seven preparations were pooled in polar plots (Fig. 4D). The mean value of the phase (ϕ) between right and left side (RL2-LL2) was 0.47 ± 0.08; coupling strength was highly significant, r=0.87 ($p<0.001$). The mean value of the phase (ϕ) between flexor and extensor ventral roots (RL2-RL5) was 0.46 ± 0.07, and the coupling strength was also significant, r=0.89 ($p<0.001$). An example of cross-correlation was shown in Fig. 4C, from the same preparation as in Fig 4A. The cross-correlation coefficients between flexor/extensor and right/left ventral roots were

1.46 and 1.50, respectively. The mean (n = 7) cross-correlation coefficients between flexor/extensor and right/left ventral roots were 1.25 ± 0.26 and 1.44 ± 0.31, respectively. Data from both qualitative and quantitative analysis suggest that 5-HT induces well coordinated locomotor-like activity in 5-HT_7 receptor wild type animals. An increase in tonic activities of ventral roots was observed in remaining 6 preparations. In mice with initial failure of induction of locomotion, increasing 5-HT concentration to 60-100 μm (n=3) was ineffective, indicating that the concentration of 5-HT (20-50 μM) in our experiments is appropriate for inducing locomotor-like activity and increasing the dose does not improve the occurrence of locomotor-like activity.

5-HT induced locomotor-like activity was disrupted by 5-HT7 receptor blockade:

To test the effectiveness of 5-HT7 receptors in the production of 5-HT induced locomotion, SB-269970, a specific 5-HT7 receptor antagonist, was applied to the bath after locomotor-like activity was induced by 5-HT (n=5). Fig. 5A illustrates an example of locomotor-like activity evoked by 30 μM 5-HT. ϕ and r value between flexor/extensor and right/left side ventral roots were 0.48, r=0.99 ($p<0.001$) and 0.49, r=0.98 ($p<0.001$), respectively. After application of SB-269970 (15 μM), rhythmic activity was first disrupted at 5 min (Fig. 5B). ϕ and r value between flexor/extensor and right/left side ventral roots were changed to 0.36, r=0.44 ($p>0.05$) and 0.28, r=0.30 ($p>0.05$). Locomotor-like activity was blocked after 10min (Fig. 5C). The blockade of locomotor-like activity by SB269970 was consitently observed in 5 preparations. The results indicate that 5-HT_7 receptors

play a critical role in 5-HT-induced locomotor-like activity, consistent with previous experiments in rats (Hochman et al 2001; Liu and Jordan 2005; Pearlstein et al 2005) and mice (Madriaga et al 2004), and provide the background for examining the 5-HT-induced effects in the spinal cords of mice lacking 5-HT_7 receptors.

5-HT-induced locomotor-like activity <u>in vitro</u> was disrupted in 5-$HT_7^{-/-}$ mice:

5-HT (20-50 μM) was applied to 5-HT7 $^{-/-}$ mouse spinal cord preparations (n=10). Uncoordinated rhythmic bursting was observed in 7 of these preparations. A representative example of uncoordinated activity is shown in Fig. 6A. Fig. 6B is a raster display of the four ventral roots from the same preparation, in which uncoordinated discharges in the 4 roots is evident. An example of the histogram of correlation-coefficient is shown in Fig. 6C and correlation-coefficients between flexor/extensor and right/left ventral roots were 0.55 and 0.50, respectively. Pooled data from 7 preparations are presented in polar plots, showing poor coupling and strength of discharges between flexor/extensor and right/left ventral roots (Fig. 6D). The mean correlation-coefficients between flexor/extensor and right/left ventral roots in the 7 preparations were 0.49 ± 0.23 and 0.56 ± 0.13, respectively. Ipsilateral synchronous discharges were evoked in 2 preparations (example in Fig. 6F). In 3 preparations with uncoordinated rhythmic activity, increasing the 5-HT concentration to 60-100 μM did not significantly change the discharge pattern. Well-coordinated locomotor-like activity was evoked in only 1 preparation, in which Φ and r value between flexor/extensor and right/left ventral roots were 0.52,

0.90 ($p<0.001$) and 0.47, 0.83 ($p<0.001$). 5-HT was rarely able to evoke well-coordinated fictive locomotion in the mice lacking 5-HT_7 receptors (1/10 cases). In this one case, the frequency of locomotor-like activity was markedly slower (0.06 - 0.07 Hz) than that seen in wild-type animals (mean = 0.12 Hz). SB269970 had not effect on this rhythmic activity; but it was blocked by ketanserin. Together, the results clearly demonstrate that 5-HT produced uncoordinated rhythmic activity in most cases.

To test whether SB269970 has effects on 5-HT induced rhythmic activity in 5-HT_7 $^{-/-}$ mice, SB-269970 (15-30 μM) was applied after uncoordinated rhythmic activity was evoked by 5-HT in 4 preparations. Fig. 7A is an example of 5-HT induced uncoordinated rhythmic activity. As shown in the polar plots, ϕ and r between flexor/extensor and right/left ventral roots were 0.38, r=0.54 ($p>0.05$) and 0.15, r=0.60. Ten minutes after adding SB-269970 (15μM), ϕ and r between flexor/extensor and right/left ventral roots were 0.31, r = 0.41 ($p>0.05$) and 0.18, r=0.19 ($p>0.05$). There were no significant changes in ventral root discharges before and after SB-269970 administration (Fig. 7B). Ketanserin, a 5-HT_{2A} antagonist, effectively blocked uncoordinated discharges in all tested preparations (n=4). Fig. 7C shows an example of 5-HT-evoked uncoordinated rhythmic activity that was blocked by 15 μM ketanserin. These results demonstrate that the actions of SB-269970 on 5-HT-induced locomotor-like activity require 5-HT_7 functional receptors. Our results further suggest that the largely uncoordinated rhythmic activity induced by 5-HT in the 5-HT7 $^{-/-}$ mice is due to activation of 5-HT_{2A} receptors. As a test of whether 5-HT_{2A} receptors are involved in this uncoordinated

rhythmic activity, a second 5-HT_{2A} receptor antagonist, spiperone, was used to apply to the bath. Spiperone (10 μM, n=4) consistently abolished this bursting (not shown). Blockage by both ketanserin and spiperone is taken as an indication that the action of 5-HT is due to 5-HT_{2A} receptors (Glennon et al 2002; Hochman et al 2001; Liu and Jordan 2005). Previous experiments demonstrated that 5-HT_2 receptors play a role in 5-HT-induced locomotor-like activity in mouse preparations (Madriaga et al 2004).

5-HT_7 receptor blockade disrupts voluntary locomotion in adult wild-type mice:

To test the hypothesis that 5-HT_7 receptors are involved in the control of voluntary locomotion in adult mice, we used adult 5-HT_7 $^{+/+}$ mice and directly applied the 5-HT7 receptor antagonist, SB-269970 (1 mM or 10 mM), to the exposed spinal cord at the level of the L1-L2 segments. The most pronounced effect of SB-269970 was prolongation of extension in the hindlimbs that severely impaired locomotion. This occurred in both CD1/ICR (4/4) and C57B/6J (8/9) strains of mice (Fig. 8 and 9). This could be associated with prolonged extension of the entire limb beyond the point of successful weight support, as illustrated in Fig. 8, or sustained extension at the ankle joint with failure of plantar placement of the hindpaw, as shown in Fig. 9. Recovery from the effects of SB-269970 occurred on average after 51.75 ± 25.33 minutes. All of the above results were obtained with doses of 10 mM SB-269970. In 3 cases, 1mM SB-269970 was used, but only very transient effects on locomotion were observed in these cases.

The prolonged extension induced by SB-269970 for 8 out of 9 animals examined in these experiments is illustrated in Fig. 10. The drug had little effect on the timing of the onset of hindlimb movements after removal of the anesthetic in either 5-HT_7 $^{+/+}$ or 5-HT_7 $^{-/-}$ mice (Fig. 10A). However, the prolonged hindlimb extension was far more dramatic in the 5-HT_7 $^{+/+}$ mice compared to 5-HT_7 $^{-/-}$ mice (Fig. 10B). The mean time to recovery from the SB-269970-induced prolonged extension in wild-type animals was 51.75 ± 25.33 min, significantly ($p = 0.0012$) longer than the time to normal extension in the control condition (5.78 ± 2.49). The mean time to recovery from the prolonged extension was 18 ± 17.22 min in knockout animals, statistically indistinguishable from the control condition (6.67 ± 2.07). The difference in time to recovery from prolonged extension between wild-type and knockout animals was highly significant ($p = 0.012$).

SB-269970 administration appeared to slow locomotion, increase the duration of the locomotor cycle and result in slowing of locomotion in some cases (Fig. 8), but there was considerable variability in the cycle duration due to the fact that the locomotion was voluntary and overground. There was no statistically significant difference in cycle duration for wild-type animals before and after SB-269970 administration.

Kinematic and EMG analysis was performed to determine the changes in joint angles and muscle activation induced by SB-269970 in wild-type animals. Increased hip extension and persistent extension of the ankle joint occurred in the example shown in Fig. 11. The increased hip extension was observed in 8/9 cases, and the changes in ankle joint angle were also observed in these same 8 animals..

The joint angle changes were accompanied by disturbed coordination between ipsilateral ankle flexor (TA) and extensor (GS) muscles (Fig. 11A) and left and right flexor muscles (Fig. 11B). Polar plots and correlation analysis were used to more clearly demonstrate the altered coordination. Fig. 12 shows an example of EMG recording. Prior to SB269970 application, alternation between flexor-extensor and right-left side are illustrated in polar plots and histograms of correlation-coefficient (Fig. 12A). Following the drug application, flexor/extensor and left/right coupling were disturbed, with frequent co-contractions of ipsilateral flexors and extensors (Fig. 12B). Recovery occurred within 60 minutes after drug application as shown in Fig. 12C). Fig. 13 summarizes EMG polar plots in four wild-type animals in the control and SB-269970 treated conditions; disturbed or decreased flexor-extensor and left-right coupling was observed in all cases. In one of these animals, the ipsilateral flexor and extensor coupling became synchronous (see also Fig. 13). These disturbances in EMG coordination are similar to the effects of SB-269970 on ventral root recordings in vitro (Fig. 6). Forelimb walking was not affected by SB-269970 during these experiments, indicating that the effects of the drug were confined to the lumbar area of the spinal cord. No indications of systemic actions of the drug were observed. All animals recovered fully from the drug treatment.

Voluntary locomotion in adult mice lacking 5-HT$_7$ receptors:

In 5-HT$_7$ $^{-/-}$ mice, voluntary locomotion was not distinguishable from that of wild type animals (See Fig. 14). The persistence of locomotor capability in these mice

was previously reported (Hedlund et al 2003), consistent with the presence of multiple activating pathways for the control of locomotion. Another explanation is that it might be due to developmental compensation mechanisms (see Discussion for detail). Analysis of cycle periods over the entire period of recording (up to 30 minutes) showed that the step cycle periods of 5-HT_7 $^{-/-}$ mice (n=6) were not significantly longer that those of 5-HT_7 $^{+/+}$ mice (n=9). In 4 out of 6 5-HT_7 $^{-/-}$ mice, the 5-HT_7 antagonist SB-269970 did not produce prolonged extension in 5-HT_7 $^{-/-}$ mice (see Figs. 10 and 14). An example of a 5-HT_7 $^{-/-}$ mouse prior to (control) and after SB-269970 administration is illustrated in Fig. 14. The striking impairment of locomotion induced by this drug in wild-type animals (Figs. 8 and 9) was not observed in this 5-HT7 $^{-/-}$ animal. Kinematic analysis shows that the step cycle and joint angle excursions are not significantly altered by the drug in 5-HT_7 $^{-/-}$ mice. In 2 out of 6 5-HT_7 $^{-/-}$ animals, SB269970 resulted in appreciable prolongation of hinblimbs, but the severity of changes in joint angles and frequency of loss of weight support were much less than in wild type mice. This is in contrast with the dramatic disruption of voluntary locomotion induced by SB-269970 in wild-type mice. The mechanisms of SB269970 on locomotion in 5-HT_7 $^{-/-}$ mice are not clear. It is likely that SB269970 at this concentration binds to other 5-HT receptor subtypes, for instance, 5-HT_5 receptors (Thomas and Hagan 2004). Thus, it is most likely that the action of SB-269970 on locomotion in adult animals requires functional 5-HT_7 receptors. We conclude that 5-HT_7 receptors play an important role in the control of voluntary locomotion in normal adult mice.

Discussion

5-HT_7 receptors are required for 5-HT induced locomotor-like activity:

We have shown that locomotor-like activity could be evoked by bath application of 5-HT in 58.3% (7 /13) of 5-HT_7 $^{+/+}$ mice. Such locomotor activity evoked by 5-HT was disrupted and blocked by a specific 5-HT_7 receptor antagonist, SB269970. We also showed uncoordinated rhythmic activity was induced by 5-HT in the majority (90%, 9/10) of 5-HT_7 $^{-/-}$ mice, an event which was not affected by SB269970, but was blocked by ketanserin, a 5-HT_{2A} receptor antagonist. These results clearly demonstrate that functional activation of 5-HT_7 receptors is required for locomotor-like activity evoked by 5-HT in the neonatal mouse spinal cord. This finding extends and strengthens our previous report (Liu and Jordan 2005) and the observation by Madriaga et al (2004). There is evidence that an increase or decrease in 5-HT levels during the developmental stage interferes with maturation of CPG networks for locomotion in the rodent. Thus, the possibility that spinal locomotor networks are poorly developed in the neonatal mice lacking 5-HT_7 receptors can not be ruled out.

5-HT induced uncoordinated rhythmic activity or synchronous discharges of the ventral roots in 5-$HT_7^{-/-}$ mice, which were blocked by the 5-HT_{2A} receptor antagonist ketanserin, indicating that activation of 5-HT_{2A} receptors are likely involved in rhythmic activity in 5-$HT_7^{-/-}$ mice. Ketanserin has a much higher affinity for 5-HT_{2A} receptors (pKi=8.9) than 5-HT_{2C} receptors (pKi=7.0) and 5-HT_{2B} receptors (pKi=5.4) (Barnes and Sharp 1999). However, we cannot entirely exclude the possibility of 5-$HT_{2B/2C}$ receptor subtypes involved in the rhythmic

activity induced by 5-HT in 5-HT_7 $^{-/-}$ mice. A nonspecific 5-HT_2 agonist, α-methyl-5HT, could elicit coordinated locomotor-like activity in the isolated spinal cord of neonatal mice (Madriaga et al 2004; Pearson et al 2003), an effect which was interrupted by ketanserin, providing evidence that locomotor network may be activated by 5-HT2 receptor agonists.

5-HT also modulates NMDA-induced fictive locomotion in the neonatal rat. For instance, 5-HT antagonists blocked NMDA induced rhythmic locomotor activity and abolished TTX-resistant NMDA receptor mediated oscillations of motoneurons (MacLean et al 1998), but it remains to be determined whether a 5-HT_7 receptor mechanism is involved. Pearlstein et al (2005) reported that 5-HT_7 receptors play a role in refining NMDA-induced locomotor activity in the neonatal rat. Thus, it is highly possible that 5-HT_7 receptors are also involved in NMDA induced rhythmic activity *in vitro*.

Previous work in our lab showed that c-fos expression after treadmill walking was substantially increased in the caudal thoracic and lumbar cord, and intrathecal administration of clozapine, a non-specific 5-HT_7 receptor antagonist abolished MLR induced locomotion in the cat (Schmidt and Jordan 2000). Thus, our results provide further evidence for the hypothesis that 5-HT_7 receptors play an important role in 5-HT induced locomotor-like activity in neonatal mammalians (Liu and Jordan 2005; Madriaga et al 2004; Schmidt and Jordan 2000).

5-HT induced locomotor-like activity is impaired in neonatal 5-HT_7 $^{-/-}$ mice

In the present study, blockade of 5-HT_7 receptors disrupted and abolished 5-HT induced locomotor-like activity. Furthermore, uncoordinated rhythmic activity or synchronous discharges of the ipsilateral ventral roots in the majority (90%) of animals lacking 5-HT_7 receptors was observed following 5-HT application, suggesting 5-HT_7 receptors play an important role in the production of well coordinated locomotor activity. However, adult 5-HT_7 $^{-/-}$ mice do not express any overt behavioral phenotype and perform normal locomotion (Hudlend et al 2003). We assume that other descending systems are active or compensation mechanisms take over with development in the 5-$HT_7^{-/-}$ knockout during development. Certainly, it is recognized that there are redundant mechanisms for activtion of spinal locomotor circuits and that these circuits are capable of being reconfigured (Rossignol 2006). The uncoordinated rhythmic activity or synchronous discharges of the ventral roots may be attributed to the direct activation of motoneuron pools or premotor neurons. In one case, well coordinated locomotion with very slow step frequency induced in 5-HT_7 $^{-/-}$ mice was observed, providing clues that compensation might occur in the postnatal stage and other 5-HT receptor subtypes (e.g., 5-HT_2) may take on the role of rhythmgenesis for 5-HT induced locomotor-like activity. Studies have shown that 5-HT_{2A} receptors are found in lumbar dorsal horn (laminae I-II) and ventral horn (lamina IX) (Doly et al 2004), whereas 5-HT_{2C} receptors are diffusely expressed throughout the gray matter of the spinal cord, especially in Lamina IV-IV (Fonseca et al 2001), providing an anatomical basis for 5-HT_2 receptor involvement in the control of locomotion. α-methyl-5HT has a higher affinity for 5-HT_{2C} (pKi=8.57) and 5-HT_{2B} (pKi=8.16) receptors than 5-

HT_{2A} (pKi=7.8) receptors (Knight et al. 2004), thus the role of 5-$HT_{2B/2C}$ receptors involved in α-methyl-5HT induced locomotor rhythm remains to be further examined.

5-HT_7 receptor activation is required for normal voluntary locomotion in adult wild-type mice:

Our data show that application of SB269970 to the exposed spinal cord (L1 and L2) resulted in a pronounced effect on locomotion in 8 out of 9 5-$HT_7^{+/+}$ mice, such as prolongation of extension or synchronous activation of antagonist muscles. Such disrupted locomotion was observed in both CD1/ICR and C57B/6J strains of mice. In one case, SB269970 had no any effect on locomotion. The feasible explantation for this is that the drug may never access into the spinal cord at all due to bleeding or limited exposure of the spinal cord.

Several lines of evidence demonstrate that descending 5-HT projections play an important role in the control of motor activity (Jacobs et al 2002). For example, *in vivo* studies have shown that caudal raphe serotonergic neurons are activated during treadmill-induced locomotion (Veasey et al 1995) and both electrical and chemical stimulation of caudal raphe nuclei result in a tonic excitation of lumbar motoneurons (Roberts et al. 1988; Fung and Barnes 1989). But 5-HT receptor subtypes involved in this action have not been determined. Our results provide for the first time essential evidence that a 5-HT command system is active and 5-HT_7 receptors are involved in the production of coordinated locomotion in adult wild-type mice. It needs to be pointed out that the final concentration of SB269970 in

the spinal cord was unknown in our experiments. Given the fact that appreciable changes of prolongation of extension were observed in 2 out of 4 in 5-HT_7 $^{-/-}$ mice at the same dose (10 mM) of SB269970 as in wild-type mice, the binding specificy for 5-HT_7 receptors at such concentration needs to be taken into account, for instance, 5-HT_5 receptors (Thomas and Hagan 2004). That the severity of prolongation of extension and frequency of loss of weight support were much less compared to 5-HT_7 $^{+/+}$ mice provides evidence that the results obtained with SB269970 are more specific to the 5-HT_7 receptor.

There was a striking similarity between the disrupted ventral root coordination seen in vitro and the uncoordinated EMG activity observed in the adult mice. For example, disrupted rhythmic activity and ipsilateral synchronous discharges were both seen in the in vitro and adult mice. Although the mechanisms underlying these similarities can not be addressed in the present study, it is possible that neurons responsible for the onset of the swing phase may possess 5-HT_7 receptors, and failure to activate them adequately due to blockage by the antagonist would lead to the prolonged extension observed in these experiments. It is well-known that one of the functions of sensory feedback during walking is to control the timing of the transition from stance to swing. This has been shown in cats (Grillner and Rossignol 1978; Pearson 1995; Whelan et al. 1995) and in human infants (Pang and Yang 2000). The sensory receptors involved in mediating the stance-to-swing transition include Golgi tendon organs in ankle extensor muscles (Pearson et al.

1998; Whelan et al. 1995) and muscles spindles from hip flexor muscles (Hiebert et al. 1996).

An explanation for the uncoordinated locomotion produced by 5-HT in 5-HT_7 receptor knockout mice might be that 5-HT_7 receptors normally regulate the excitability of coordinating interneurons. In the absence of activation of sufficient numbers of these cells in knockout mice or in the presence of SB-269970, rhythmic activity ensues, but coordination among the neurons producing excitation of motoneurons is lacking. Certainly one of the probable mechanisms causing co-contraction of flexors and extensors following SB269970 application is that inhibitory interneurons responsible for reciprocal inhibition during walking possess 5-HT_7 receptors, and their excitability might be suppressed after the drug application. During locomotion alternate contraction of antagonist muscles at the same joint is realized partly by reciprocal inhibition through excitation of inhibitory interneurons (Jordan 1983; Shefchyk and Jordan 1985; Orsal et al. 1986). Blocking the action of inhibitory interneurons produces synchronous discharge of ventral roots in the isolated neonatal rat spinal cord (Cowley and Schmidt 1995). Ia inhibitory interneurons may be involved in this inhibition (Pratt and Jordan 1987), but they appear to not be required for reciprocal inhibition during walking (Gosgnach et al. 2006).

The possibility that 5-HT modulation of afferent input underlies the observed results must be considered (Jankowska et al 1997; Millan 2002). Of course, this cannot be a factor in the case of *in vitro* fictive locomotion, but it could be

important in the interpretation of results from intact adult animals. 5-HT descending pathways modulate synaptic actions of muscle spindle and tendon organ afferents on spinal interneurons and excitability of group II premotor interneurons (Jankowska et al 1997; Maxwell et al 2000). Feedback from muscle receptors is known to contribute to the timing of the onset of the swing phase of locomotion, as discussed above, and alterations in the transmission of related afferent input could account for the prolonged extension in intact animals.

Multiple descending command systems in the control of locomotion

Another interesting observation derived from this study was that 5-HT_7 $^{-/-}$ mice do not show any deficiency in locomotion as previously reported (Hedlund et al 2003). This supports the notion that there are parallel descending pathways that can activate and modulate spinal locomotor circuits (Rossignol 2006).

The importance of 5-HT for the initiation of locomotion using pharmacological depletion methods was examined in Dr. Jordan lab and it was found that substantial reductions in spinal 5-HT did not disrupt voluntary locomotion in intact cats or locomotion produced by stimulation of the Mesencephalic Locomotor Region (MLR) in decerebrate cats (Steeves et al. 1980). Similar depletion of noradrenaline also did not alter MLR-evoked locomotion. These results led authors to the conclusion that other descending command systems for initiation of locomotion must exist. Depletion of 5-HT with the 5-HT synthesis inhibitor ρ-chlorophenylalanine in neonatal rats resulted in decreased velocity of locomotion

and prolonged extension of the hindlimbs (Myoga et al. 1995; Nakajma et al 1998). In mice lacking *Pet-1*, a gene that plays a critical role in 5-HT neuron development, the majority of brainstem 5-HT neurons fail to differentiate, giving rise to a transient decrease in exploratory locomotion (Hendricks et al. 2003). Recent experiments using *Lmx1b* conditional knockout mice resulted in elimination of all 5-HT neurons, yet no overt locomotor deficits were observed in these mice (Zhao et al. 2006). However, their measures of locomotion were limited to the accelerating rotorod test and total activity in an open-field test. A more detailed analysis of the locomotor capability of these animals might resolve some of the conflicting results. It is particularly important to note that the rotorod test, considered a skilled locomotor task, also recruits neural systems for control of voluntary movement and balance. Nevertheless, the authors concluded, "The central serotonergic system is not required for normal locomotor activity". This conclusion is consistent with the widely accepted notion that there are multiple descending locomotor command systems capable of eliciting locomotion (Orlovsky et al 1999), such that "...none of the descending pathways plays an indispensable role in the basic generation of locomotion...." (Rossignol 2006). This is especially true in cases where developmental adaptation to decreased or absent function of one of the pathways is possible.

This redundancy in locomotor command systems is encouraging from the point of view of functional recovery. The alternate pathways that may be sufficient for serving as locomotor command systems include descending systems containing

norepinephrine, dopamine, excitatory amino acids, and certain neuropeptides (Rossignol et al 2002; Alford et al 2003; Fouad and Pearson 2004), and an intraspinal cholinergic system of propriospinal neurons (Jordan and Schmidt 2002). It is plausible that remaining descending systems coordinating locomotion could compensate in whole or in part after depletion of 5-HT neurons (Zhao et al 2006).

Another potential form of redundancy is the fact that many of the descending 5-HT neurons contain co-transmitters such as thyrotropin releasing hormone (TRH), substance P or glutamate (see General Introduction). It is possible that the normal locomotion seen in 5-HT_7 $^{-/-}$ mice can be accounted for by involvement of one or more of these co-transmitters when 5-HT is no longer effective due to absence of the 5-HT_7 receptor.

Summary:

In the current study, first we have used 5-HT_7 $^{-/-}$ mice to test the hypothesis that 5-HT induces locomotor-like activity in the isolated neonatal mouse spinal cord by acting at the5-HT_7 $^{-/-}$ receptor. Experiments were performed on the isolated spinal cord (below T1) of post-natal day 0-5 animals and recordings were made from the L2 (flexor) and L5 (extensor) ventral roots bilaterally. 5-HT (20-50uM) was applied into the recording chamber for evoking ventral root discharges. We found that spinal cords from 53.8% of 5-HT_7 $^{-/-}$ animals (n=7/13) displayed the typical locomotor-like response to 5-HT. This locomotor-like activity was disrupted and then blocked by SB269970, a specific 5-HT_7 receptor antagonist. However, 5-HT

application resulted in uncoordinated rhythmic activity in a majority of 5-HT_7 $^{-/-}$ mice (70%, n=7/10) or synchronous discharges of the ventral roots (20%, n=2/10). Coordinated locomotor-like activity was observed in only one case. Such rhythmic activity in the 5-HT_7 $^{-/-}$ animals was not affected by the 5-HT_7 antagonist SB269970, but could be abolished by 5-HT_{2A} antagonists ketanserin and spiperone. The results suggest that locomotor-like activity induced by 5-HT applied to the isolated neonatal mouse spinal cord is mediated by 5-HT7 receptors.

In the second project, we used the same strain of 5-HT_7 $^{-/-}$ mice to test if 5-HT_7 receptors are involved in the control of voluntary locomotion in adult mice. Quantitative methods i.e., kinematic measurements and EMG recording were utilized to monitor spontaneous locomotion before and after drug administration to the spinal cord. SB269970 consistently altered locomotion in wild type animals, producing over-extension of both hindlimbs such that the stance phase often persisted beyond the point where weight support could be maintained, resulting in slowed locomotion and "dragging" of the hindlimbs. Mice lacking 5-HT_7 receptors, when treated with SB-269970, showed no overt effects on locomotion compared to those in wild type animals. These results demonstrate that activation of 5-HT_7 receptors plays an important role in the control of voluntary locomotion in adult mice.

Figure legends

Figure 1. Examples of PCR results of 5-HT_7 receptor genotyping. Only the exon II product was detected in –HT7 $^{+/+}$ mice. Only the neo product was detected in 5-HT_7 $^{-/-}$ mice. Both the exon II and the neo product was detected in 5-HT_7 $^{+/-}$ mice. Mouse beta casein (MBC) was used as the housekeeping gene.

Figure 2. Schematic drawing of drug application in the adult mouse. Anesthesia was induced with isoflurane. Prior to surgery the opioid receptor agonist buprenorphine (0.15mg/kg) was injected subcutaneously. A laminectomy was performed at the T12 and T13 vertebrae to expose the L1 and L2 segments of the spinal cord. Isoflurane was shut off after 30 min. The control recording was performed in a walkway for 30 min. Following the control recording, the animal was anethsitized again and the drug (SB-269970) or vehicle (saline) was applied to the exposed cord. After 30min, drug or saline was removed and the animal was placed back to the walkway for recording.

Figure 3. **A**: schematic drawing showing the location of three-dimensional reflective markers (2 mm diameters) glued on the surface of shaved skin, and one on the wrist of the foreleg. The markers represent the site of iliac crest, hip, knee, ankle, paw, tip of the fourth digit (toe) of the left hind leg. **B**: The schematic showing the Plexiglas walkway used to allow mice to walk freely during recording (90 x 5 x 13 cm^3). A high speed camera was placed 1.5-2.0 m away in front of the walkway for capturing movements of the mouse. The captured area is shown in a

square of the dashed line. The mirror placed underneath the walkway was set at approximately 45 degrees from vertical, which was used to determine the coordination between legs.

Figure 4: 5-HT elicits locomotor-like activity in the in vitro neonatal 5-HT_7 $^{+/+}$ mouse. **A**: a representative example of the alternate locomotor activity induced by bath application of 30 μM 5-HT in a 5-HT_7 $^{+/+}$ mice. **B**: Raster display of 15 step cycles triggered by the onset of RL2 bursts showing the coordinated locomotor pattern between ipsilateral flexor/extensor and right/left ventral roots. **C**: The histogram of cross-correlation between flexor and extensor and right/left ventral roots from the same mouse showing strong correlation. The cross-correlation coefficient between flexor/extensor and right/left ventral roots are 1.46 and 1.50. 1 lag = 100ms. **D**: polar plots of paired RL2/LL2 and RL2/RL5 from seven 5-HT_7 $^{+/+}$ receptor mice showing the well-coordinated locomotor pattern. The mean value of the phase (ϕ) and coupling strength (r) between flexor/extensor and right /left side are 0.46 ± 0.07; 0.89 ($p<0.001$) and 0.47 ± 0.08; 0.87 ($p<0.001$).

Figure 5: Bath application of a 5-HT_7 receptor antagonist disrupted and abolished 5-HT induced locomotor-like activity in 5-HT_7 $^{+/+}$ preparation. **A**: locomotor-like activity induced by 30μM 5-HT (*left*) and polar plots (*right*). ϕ and r value between flexor/extensor and right/left side ventral roots were 0.48, r=0.99 ($p<0.001$) and 0.49, r=0.98 ($p<0.001$) respectively. **B**: disrupted locomotor activity following application of 15μM SB269970. Polar plots showing the disorganized rhythmic

activity. ϕ and r value between flexor/extensor and right/left side ventral roots were changed to 0.36, r=0.44(p>0.05) and 0.28, r=0.30 (p>0.05). **C:** the locomotor rhythm was abolished completely 10 min after the application of SB269970.

Figure 6: 5-HT produced uncoordinated or disorganized rhythmic bursting in the spinal cord of 5-HT_7 $^{-/-}$ mice. The uncoordinated rhythmic bursting was observed in 9 out of 10 preparations. **A**: An example of uncoordinated activity by 5-HT (40μM). **B**: Raster of 17 bursts triggered by the onset of RL2 showing disorganized bursting in the ventral roots. **C**: The histogram of cross-correlation between flexor/extensor and right/left ventral roots from the same mouse showing weak correlation. The cross correlation coefficient between flexor/extensor and right/left ventral roots are 1.46 and 1.50.0.55 and 0.50. 1 lag = 50 ms D: Polar plot of 10 preparations showing the phase relationship between RL2/LL2 and RL2/RL5. E: An example of the synchronous discharges in the ventral roots induced by 5-HT (30 μM).

Figure 7: The uncoordinated rhythmic bursting produced by 5-HT in the 5-HT_7 $^{-/-}$ mice was blocked by 5-HT_2 receptor antagonists. **A**: Uncoordinated ventral roots bursting induced by 30μM 5-HT. ϕ and r between flexor/extensor and right/left ventral roots were 0.38, r=0.54 (p>0.05) and 0.15, r=0.60 (p>0.05). **B**: Application of SB269970 had no effect on the pattern of the discharges. ϕ and r between flexor/extensor and right/left ventral roots were 0.31, r = 0.41 (p>0.05) and 0.18,

r=0.19 ($p>0.05$). C: Ketanserin (15μM), a 5-HT receptor antagonist, blocked the uncoordinated rhythmic discharges

Figure 8. Prolongation of locomotion after application of SB269970 in a CD1/ICR mouse. Selected frames from a video recording showing the step cycle of the left hindlimb during a control recording (*left*), 18 minutes after the addition of 10mM SB 269970 (*middle*), and during recovery (*right*) approximately 1 hour after the drug was administered. Frames were chosen to represent the entire step cycle for the left hind limb for control and recovery trials, beginning with mid-stance and proceeding at intervals as indicated.

Figure 9. Prolongation of locomotion after application of SB269970 in a C57B/6J mouse. Selected frames from a video recording showing the step cycle of the left hindlimb during a control recording (*left*) and after the addition of 10 mM SB 269970 (*right*).

Figure 10: **A**: The delay to the first hindlimb movements in 5-HT_7 $^{+/+}$ (n=9) and 5-HT7 $^{-/-}$ (n=6) mice measured from the time point of removal of isoflurane anaesthetic was similar in wild-type and knockout mice. Open circles represent the delays measured after the application of 0.9% NaCl (mean delay = 2 ± 0.53 min for WT and 1.5 ± 0.55 min for KO), and the closed circles represent the delays after application of 10mM SB-269970 diluted in 0.9% NaCl (mean delay = 4.38 ± 1.6 min for WT and 3 ± 2.53 min for KO). **B**: The recovery from the prolonged

extension induced by SB-269970 (filled circles) in wild-type mice (mean and SD shown by the horizontal line and shaded area) differed significantly ($p=0.012$) from the saline controls (open circles). In the 5-$HT_7^{-/-}$ mice, recovery from the prolonged extension required a mean of only 18 ± 17.22 min, not significantly different from the saline controls (6.67 ± 2.07 min).

Figure 11: Kinematic and EMG analysis demonstrating the effect of SB-269970 on joint angles and patterns of EMG activity during voluntary locomotion in 5-$HT_7^{+/+}$ mice. **A**: Stick figure representations of a single step cycle in the control condition 23 min after removal of the anesthetic (*left panel*) and in the SB-269970 treated condition (*right panel*), 27 min after termination of the anesthetic. The step cycle is clearly altered by SB-269970, with dramatically increased hip extension and prolonged extension of the ankle joint such that ankle flexion does not occur and the foot in never placed for weight support, the mouse is walking on its knees. The changes in joint angle are quantified in the traces below for several step cycles. **B**: An example of rectified and filtered EMG recordings from the ipsilateral TA and GS muscles showing regular alternations between flexor and extensor muscles around the ankle in the control condition, this coordination is disturbed during SB-269970 treatment. There are often co-contractions of TA and GS along with irregular bursts in TA. This animal is the same one illustrated in Fig. 9. EMG records taken from a second 5-$HT_7^{+/+}$ mouse show the appearance of co-contractions and irregular bursts in the ipsilateral TA after SB-269970 treatment, with recovery of the normal pattern after 65 minutes. The contralateral TA (RTA)

also displayed altered activity, with only very weak bursts, occasionally occurring atypically in synchrony with the LTA.

Figure 12: Hindlimb voluntary locomotion was disrupted in adult 5-HT_7 $^{+/+}$ mice after administration of the 5-HT_7 receptor antagonist to the spinal cord. **A**: An example of EMG recording from a 5-HT_7 $^{+/+}$ mouse. *left panel*: control raw EMG recording from left extensor (LGS), left flexor (LTA) and right flexor (RTA) showing alternating EMG activity before drug administration; *middle panel*: polar plots showing the coordinated locomotion between flexor/extensor nerves ($\phi = 0.72$, $r = 0.84$, $p < 0.001$), and right/left side ($\phi = 0.40$, $r = 0.76$, $p < 0.001$); *right panel*: cross-correlation showing the strong coupling between flexor/extensor EMGs (PTCC = -1.39) and right/left sides (PTCC = -1.32). **B**. Administration of 5-HT_7 receptor antagonist, 10 mM SB-269970, caused a deficit in voluntary locomotion. *left panel*: raw EMG recording of disrupted locomotion after 30 min removal of the drug; *middle panel*: polar plot showing synchronous discharge between flexor/extensor EMGs ($\phi = 0.99$, $r = 0.75$, $p < 0.01$), and disorganized right/left side alternation ($\phi = 0.66$, $r = 0.38$, $p > 0.05$); *right panel*: the histogram of cross-correlation showing the weak coupling between flexor/extensor (PTCC = 0.43) and right/left side (PTCC = -0.30). **C**, recovery of normal voluntary locomotion 60min after removal of SB-269970; *left panel*: raw EMG recording showing the recovery of voluntary locomotion; *middle panel*: polar plots showing well the coordinated locomotion between flexor/extensor ($\phi = 0.70$, $r = 0.76$, $p < 0.001$), and right/left side ($\phi = 0.49$, $r = 0.80$, $p < 0.001$); *right panel*: the histogram of

cross-correlation showing the strong coupling between flexor/extensor (PTCC = -1.36) and right/left side (PTCC = -1.22).

Figure 13: Pooled polar plots of EMG recoding from four 5-HT_7 $^{+/+}$ mice before and after SB269970 administration *Control* (Top panel) showing well coordinated voluntary locomotion. The mean value Φ and r between the flexor/extensor and right/left side are 0.57 ± 0.16, $r = 0.82 \pm 0.10$ ($p < 0.001$) and $\Phi = 0.47 \pm 0.07$, $r = 0.85 \pm 0.06$ ($p<0.001$). *SB-269970* administration (bottom panel) resulted in disruption of EMG coupling, with co-contractions and weaker coupling of ipsilateral flexor and extensor muscles, and much weaker coupling of left and right TA.

Figure 14: Voluntary locomotion is unperturbed by SB-269970 in 5-HT_7 $^{-/-}$ mice. The left panel shows sequential frames from videos taken before (Control) and after (SB-269970) application of the 5-HT_7 receptor antagonist. A full step cycle is illustrated in each case, beginning with the onset of the stance phase. In the right panels, two examples of stick figures and joint angles before and after SB-269970 are presented. These examples are typical of the lack of step cycle alteration produced by SB-269970 in 5-HT_7 $^{-/-}$ mice.

Figures

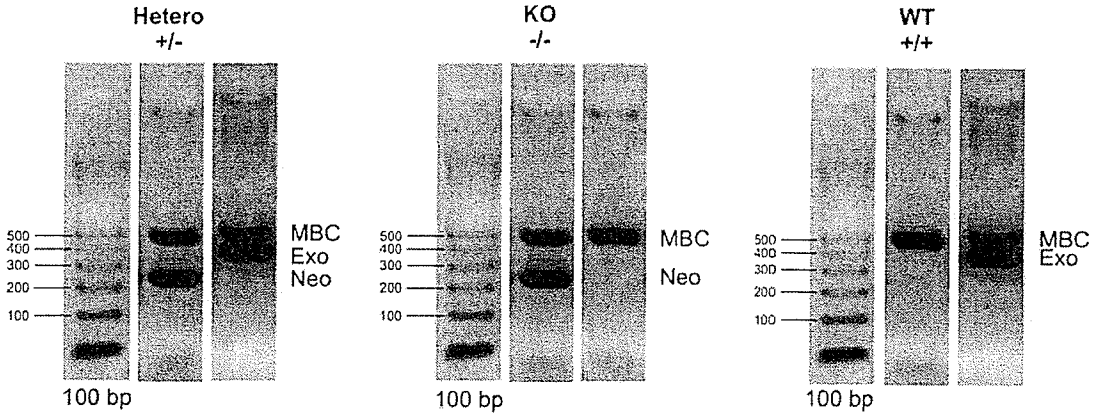


Figure 1

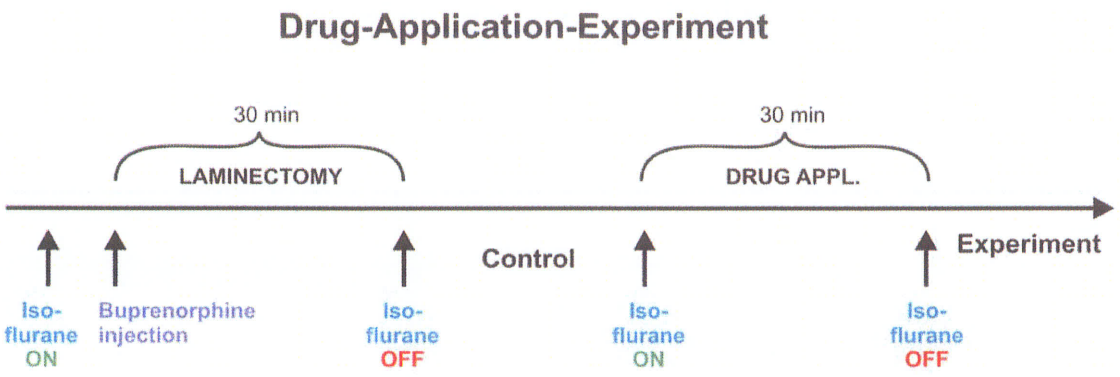

Drug-Application-Experiment
30 min
LAMINECTOMY
30 min
DRUG APPL.
Control
Experiment
Iso-
flurane
ON
Buprenorphine
injection
Iso-
flurane
OFF
Iso-
flurane
ON
Iso-
flurane
OFF

Figure 2

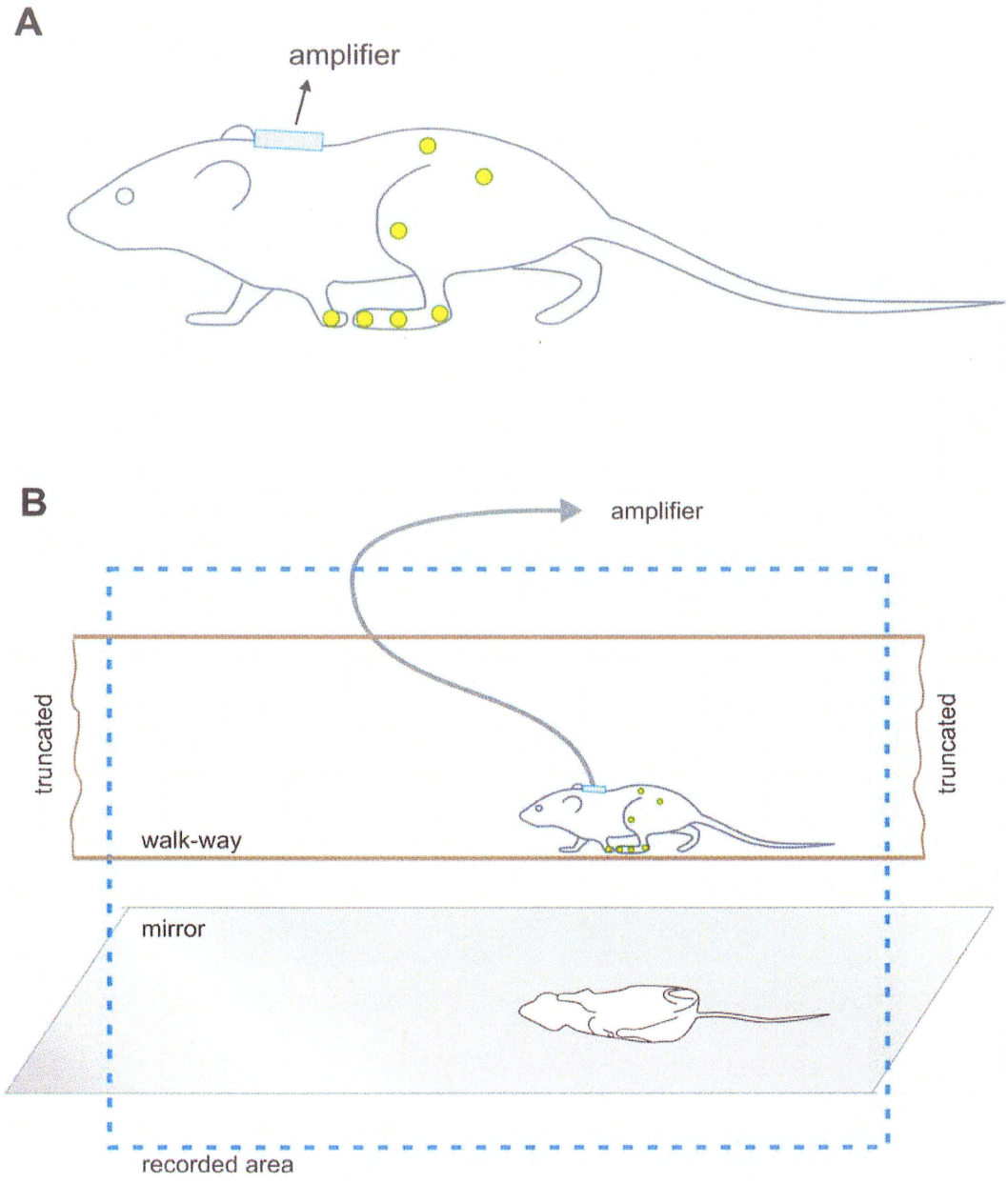

A
amplifier
B
amplifier
truncated
truncated
walk-way
mirror
recorded area

Figure 3

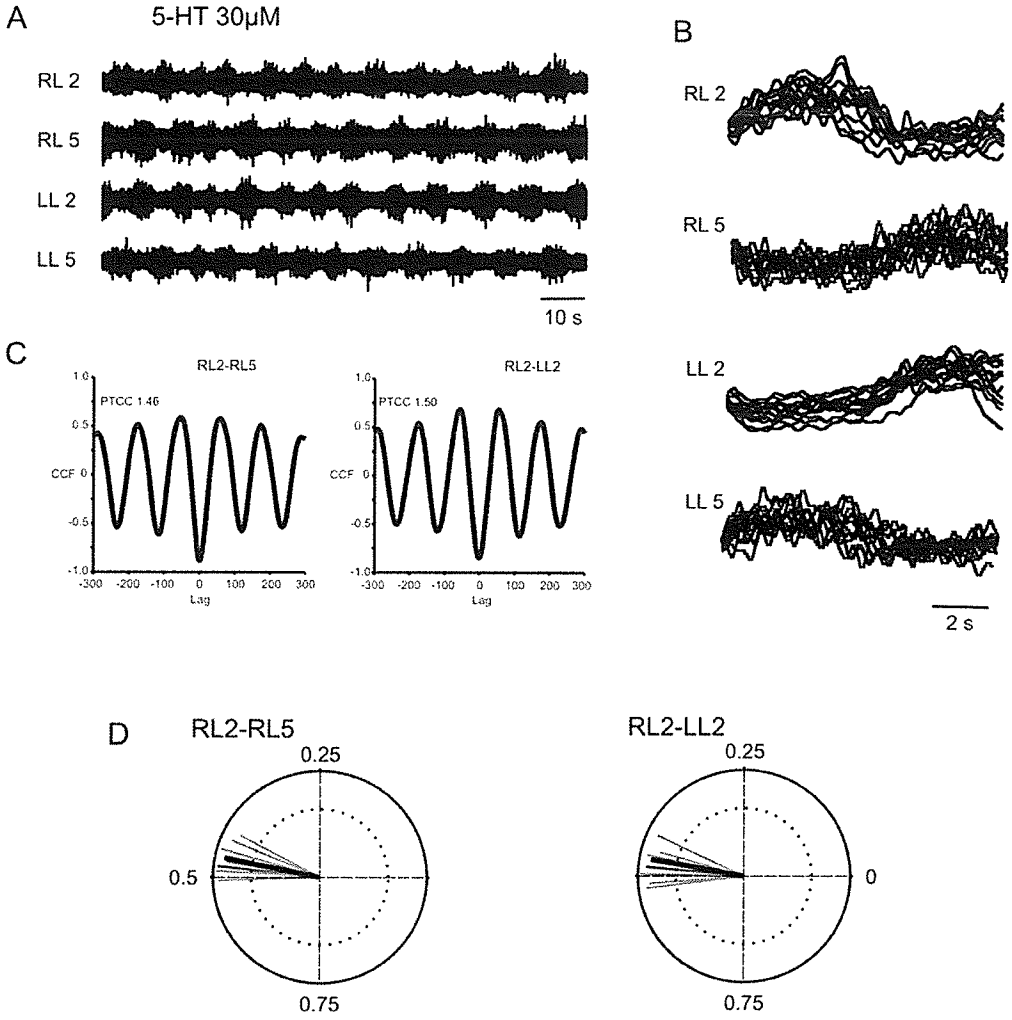

A
5-HT 30μM
RL 2
RL 5
LL 2
LL 5
10 s
B
RL 2
RL 5
LL 2
LL 5
2 s
C
RL2-RL5
RL2-LL2
PTCC 1.46
PTCC 1.50
CCF
Lag
D
RL2-RL5
RL2-LL2
0.25
0.5
0
0.75

Figure 4

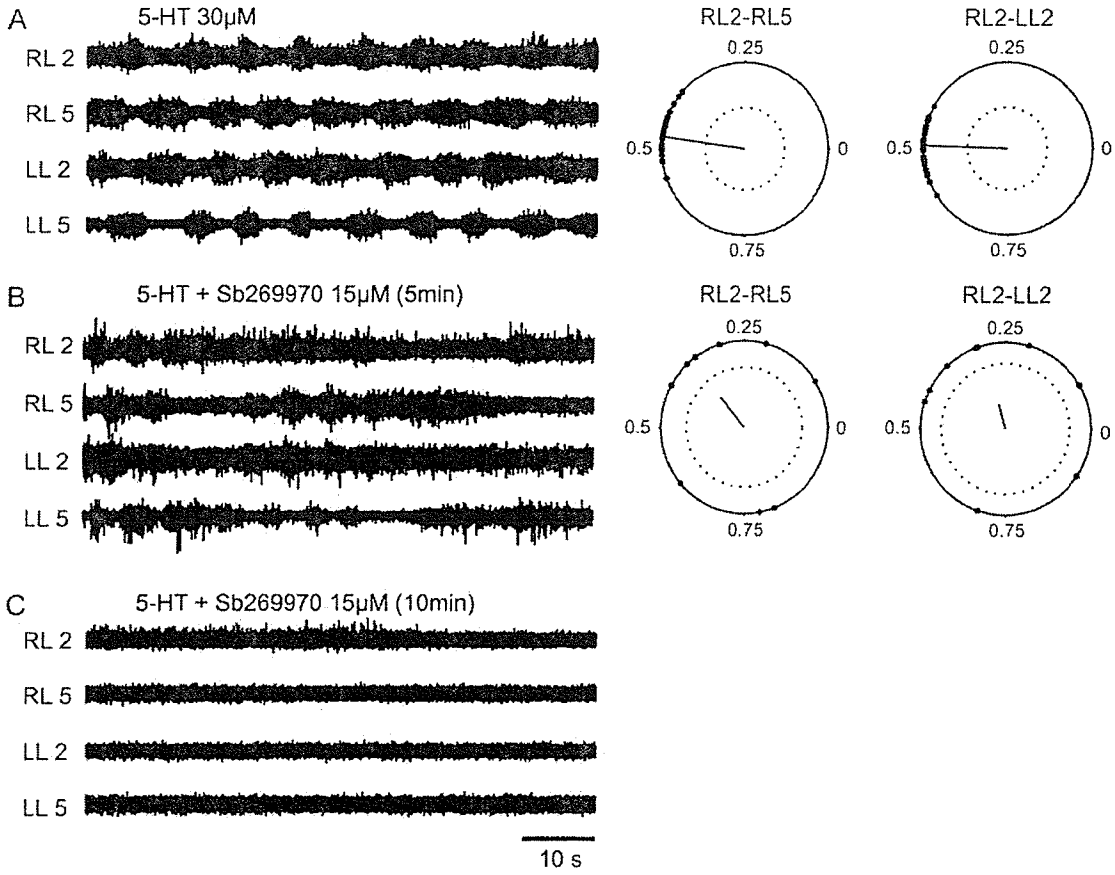

A
5-HT 30µM
RL 2
RL 5
LL 2
LL 5
RL2-RL5
RL2-LL2
0.25
0.5
0
0.75
B
5-HT + Sb269970 15µM (5min)
C
5-HT + Sb269970 15µM (10min)
10 s

Figure 5

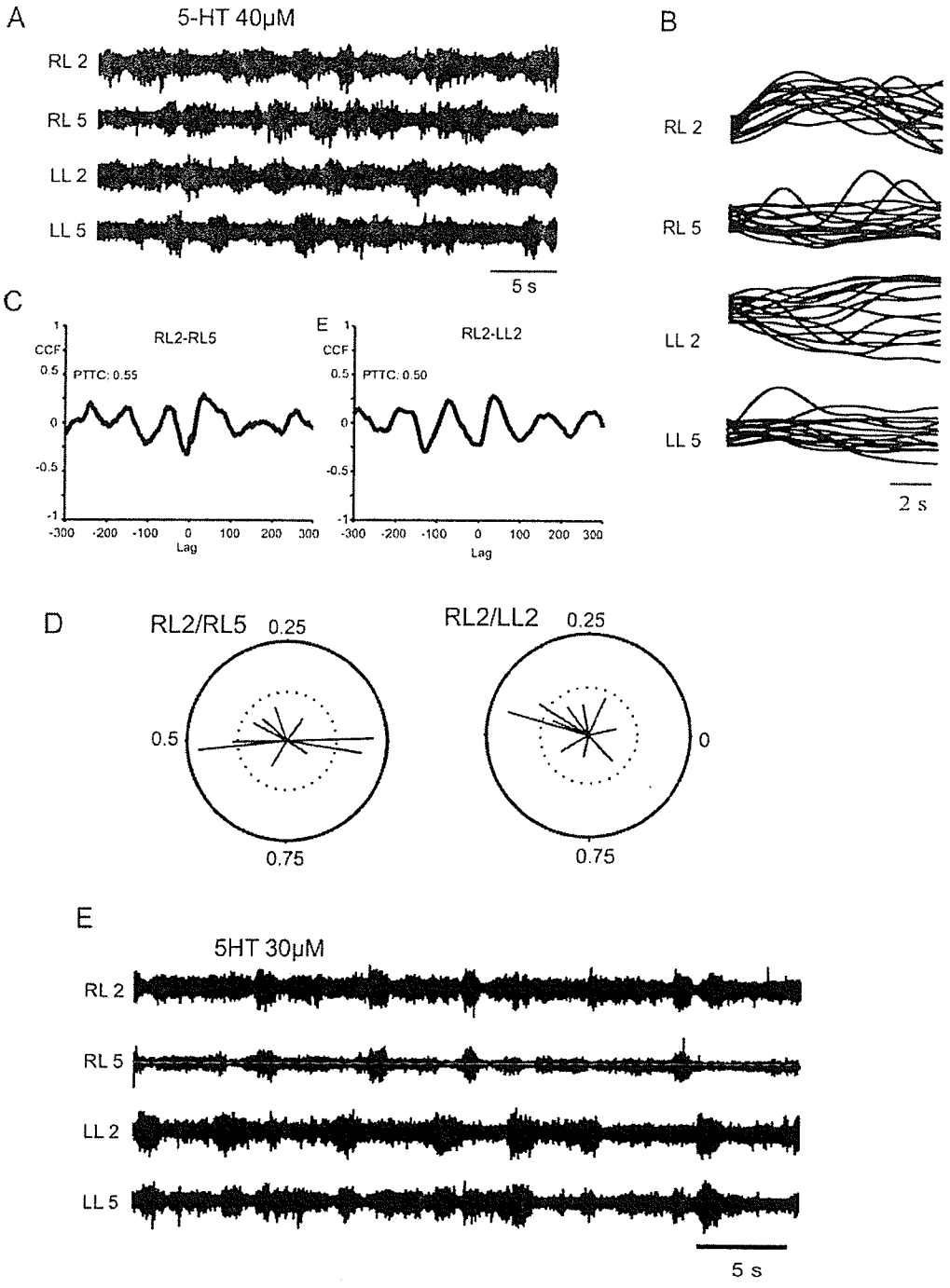

A
5-HT 40μM
RL 2
RL 5
LL 2
LL 5
5 s
B
RL 2
RL 5
LL 2
LL 5
2 s
C
RL2-RL5
CCF
PTTC: 0.55
Lag
E
RL2-LL2
CCF
PTTC: 0.50
Lag
D
RL2/RL5
RL2/LL2
0.25
0.5
0
0.75
E
5HT 30μM
RL 2
RL 5
LL 2
LL 5
5 s

Figure 6

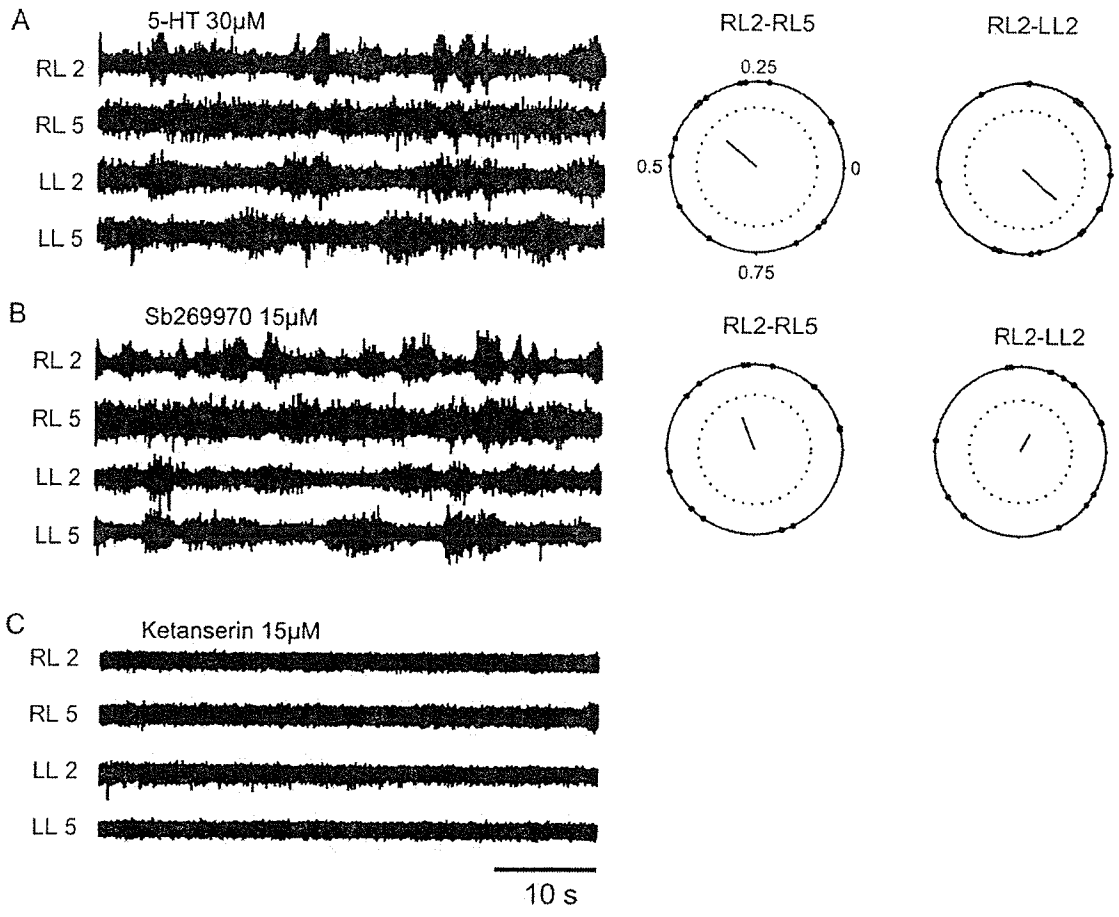

A
5-HT 30µM
RL 2
RL 5
LL 2
LL 5
RL2-RL5
RL2-LL2
0.25
0.5
0
0.75
B
Sb269970 15µM
RL 2
RL 5
LL 2
LL 5
RL2-RL5
RL2-LL2
C
Ketanserin 15µM
RL 2
RL 5
LL 2
LL 5
10 s

Figure 7

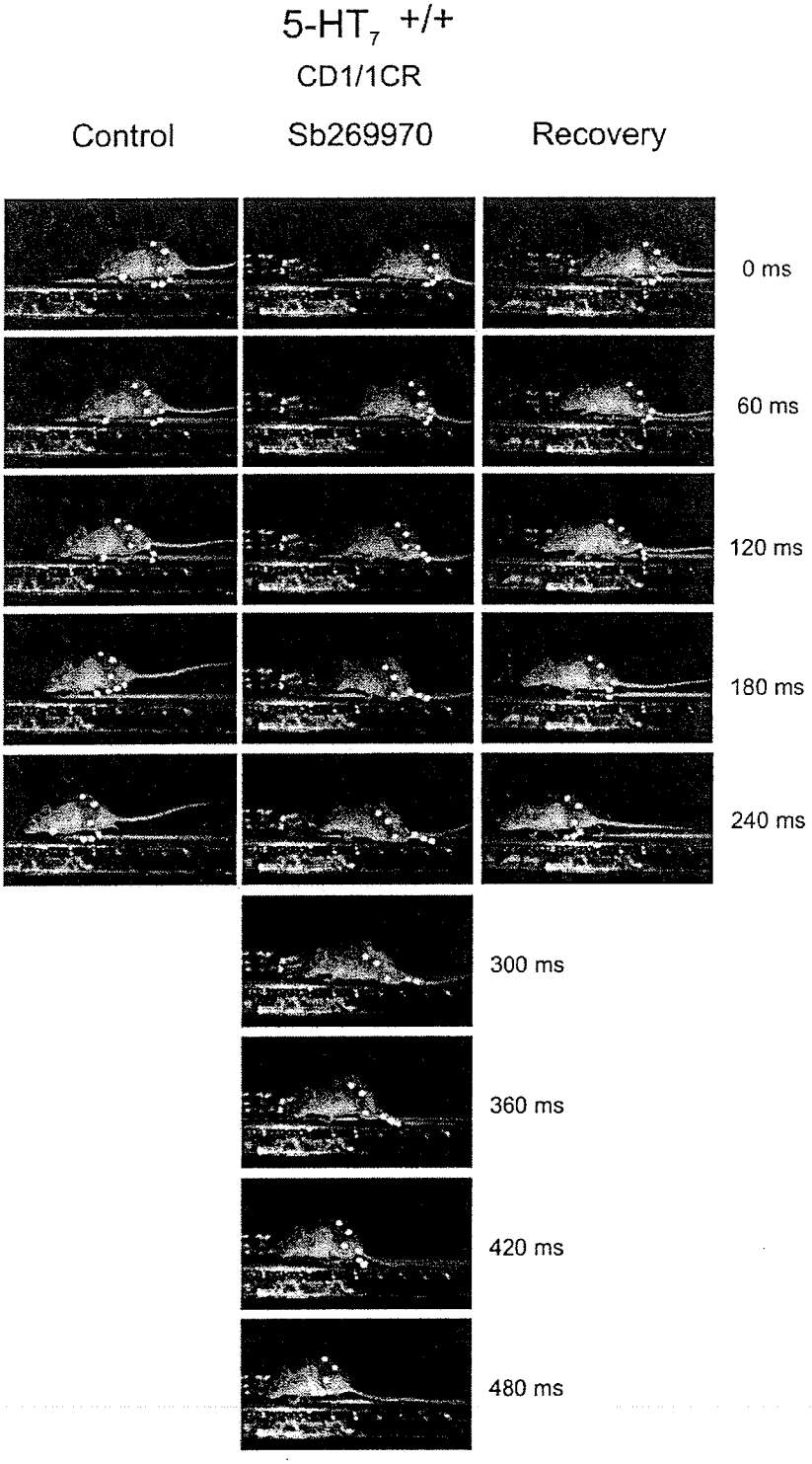

5-HT7 +/+
CD1/1CR
Control
Sb269970
Recovery
0 ms
60 ms
120 ms
180 ms
240 ms
300 ms
360 ms
420 ms
480 ms

Figure 8

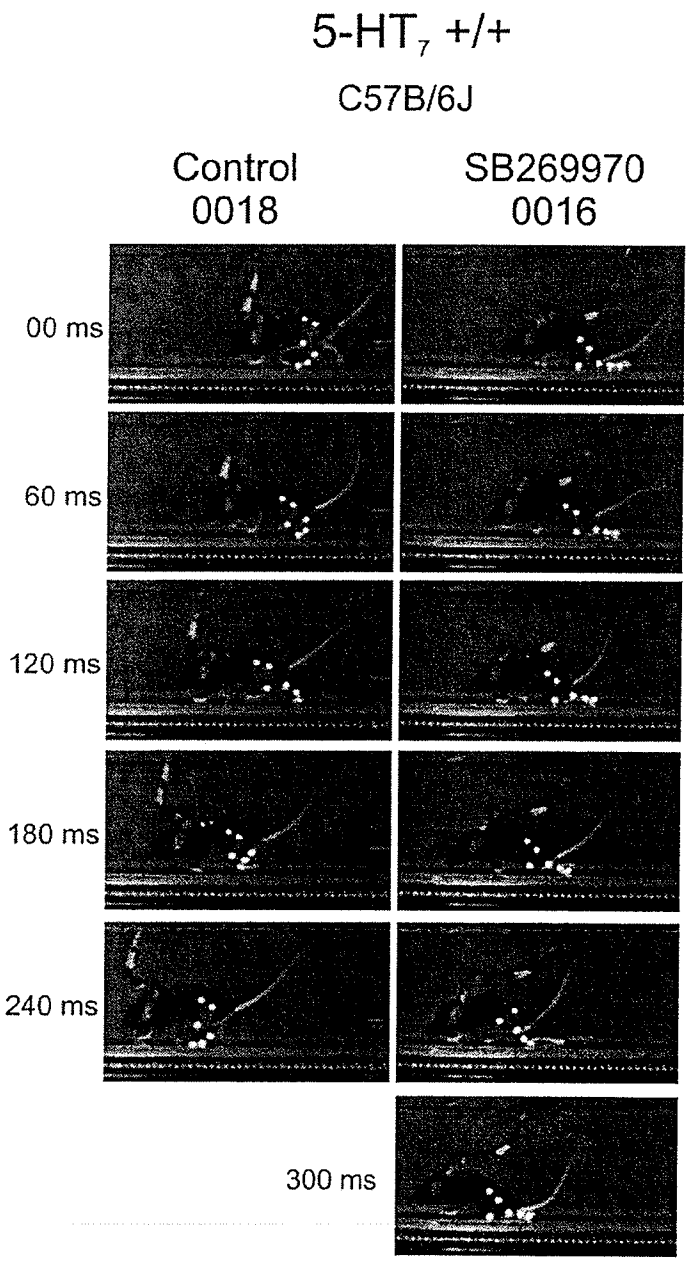

5-HT7 +/+
C57B/6J
Control
0018
SB269970
0016
00 ms
60 ms
120 ms
180 ms
240 ms
300 ms

Figure 9

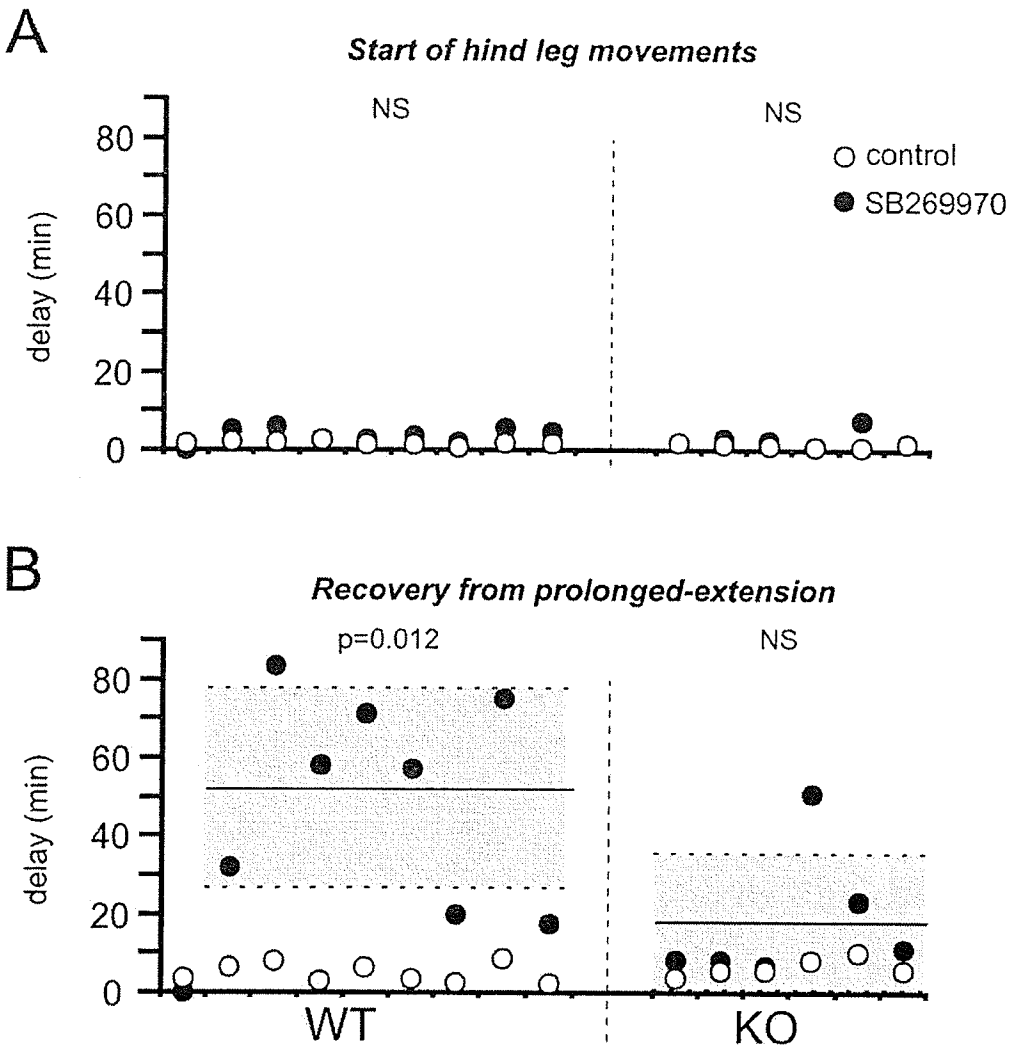

A
Start of hind leg movements
NS
NS
control
SB269970
delay (min)
80
60
40
20
0
B
Recovery from prolonged-extension
p=0.012
NS
delay (min)
80
60
40
20
0
WT
KO

Figure 10

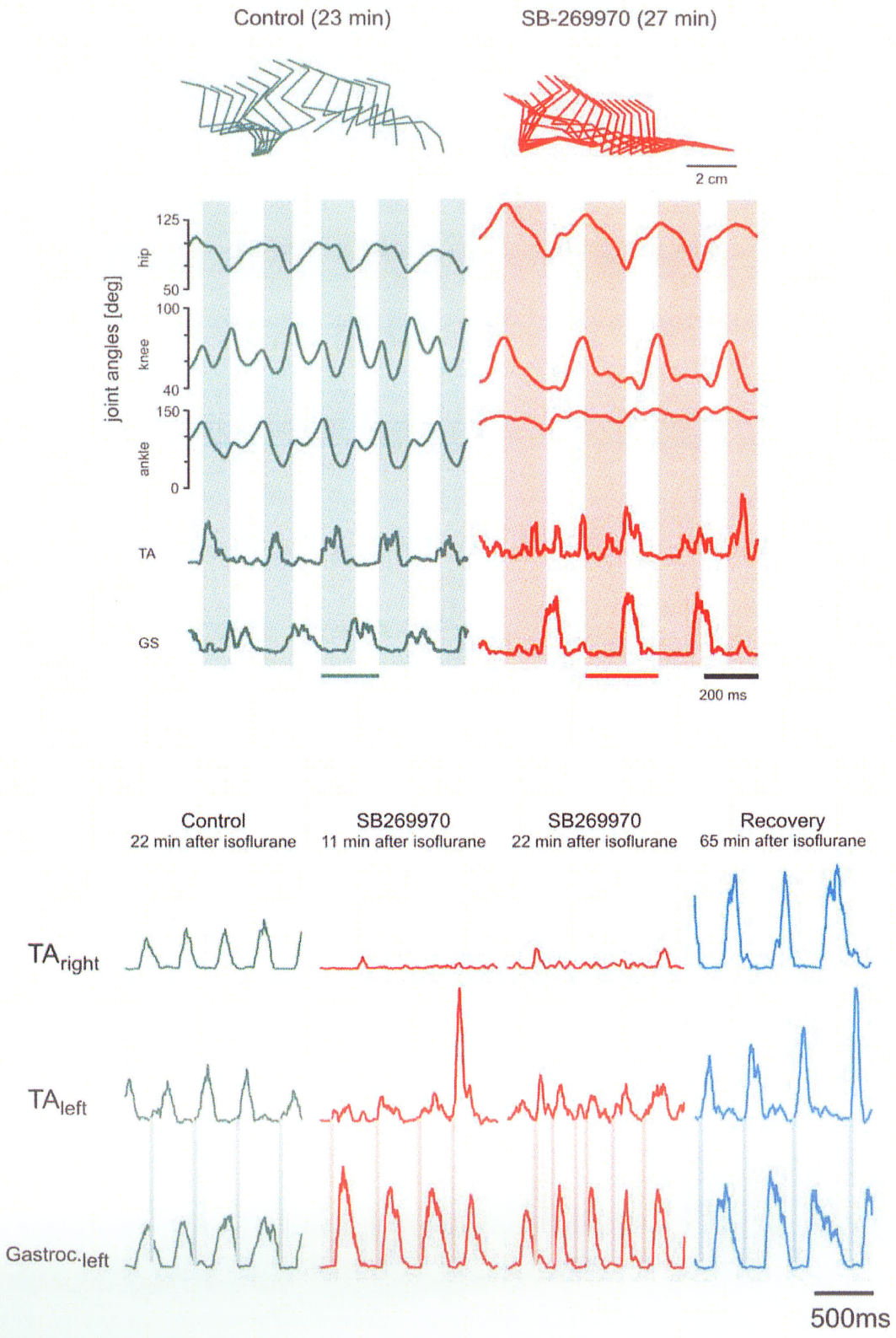

Control (23 min)
SB-269970 (27 min)
2 cm
joint angles [deg]
125
hip
50
100
knee
40
150
ankle
0
TA
GS
200 ms
Control
22 min after isoflurane
SB269970
11 min after isoflurane
SB269970
22 min after isoflurane
Recovery
65 min after isoflurane
TA right
TA left
Gastroc. left
500ms

Figure 11

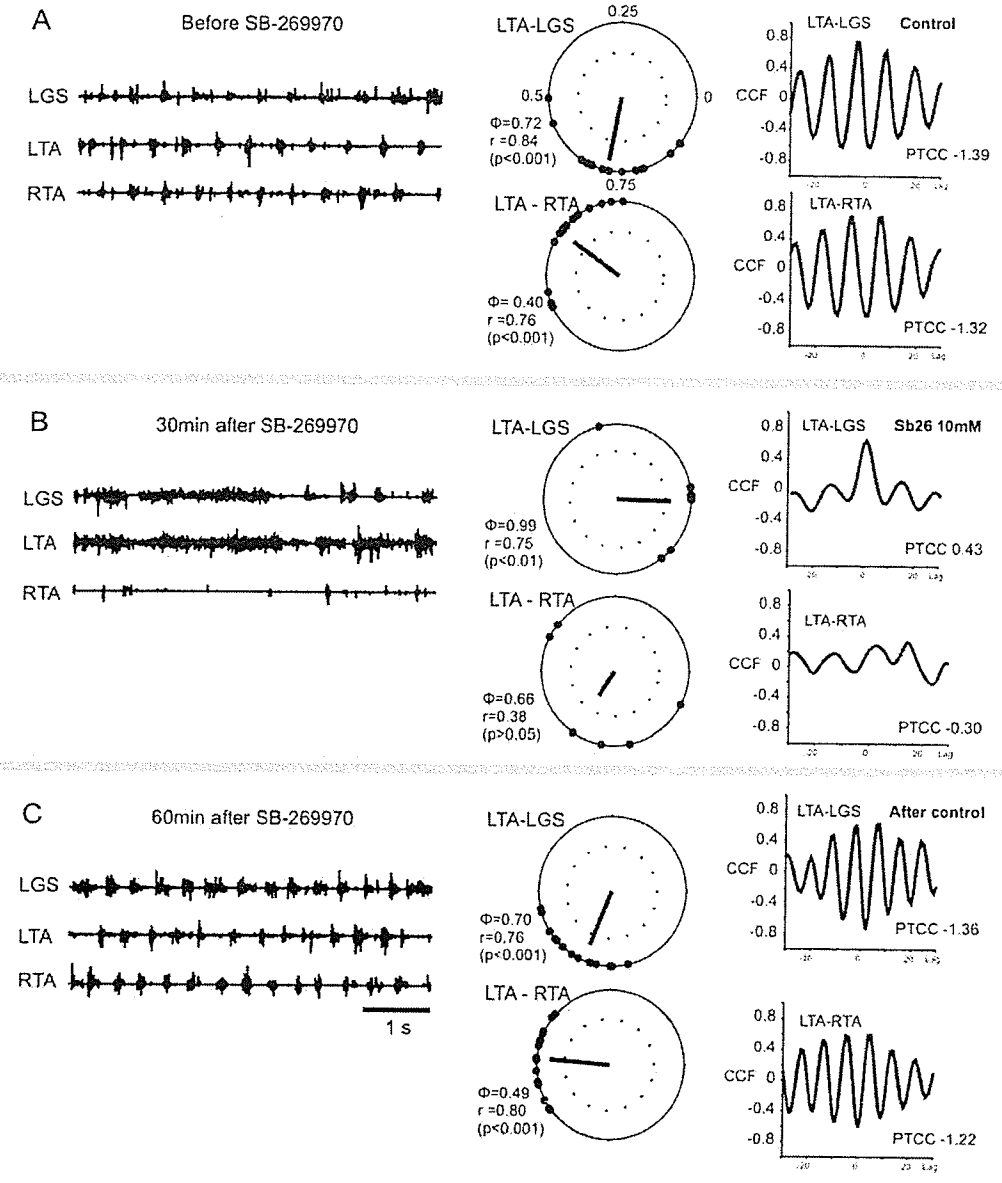

A
Before SB-269970
LGS
LTA
RTA
LTA-LGS
0.25
0.5
0
0.75
Φ=0.72
r =0.84
(p<0.001)
LTA - RTA
Φ= 0.40
r =0.76
(p<0.001)
LTA-LGS Control
CCF
PTCC -1.39
LTA-RTA
PTCC -1.32
B
30min after SB-269970
LGS
LTA
RTA
LTA-LGS
Φ=0.99
r =0.75
(p<0.01)
LTA - RTA
Φ=0.66
r=0.38
(p>0.05)
LTA-LGS Sb26 10mM
PTCC 0.43
LTA-RTA
PTCC -0.30
C
60min after SB-269970
LGS
LTA
RTA
1 s
LTA-LGS
Φ=0.70
r=0.76
(p<0.001)
LTA - RTA
Φ=0.49
r =0.80
(p<0.001)
LTA-LGS After control
PTCC -1.36
LTA-RTA
PTCC -1.22

Figure 12

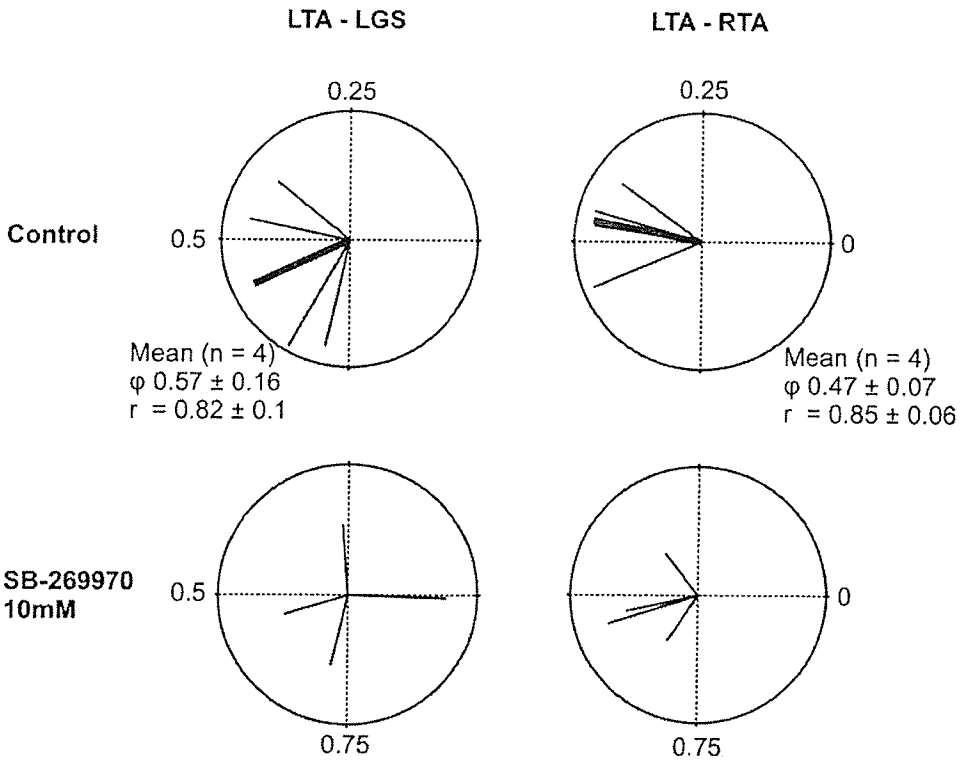
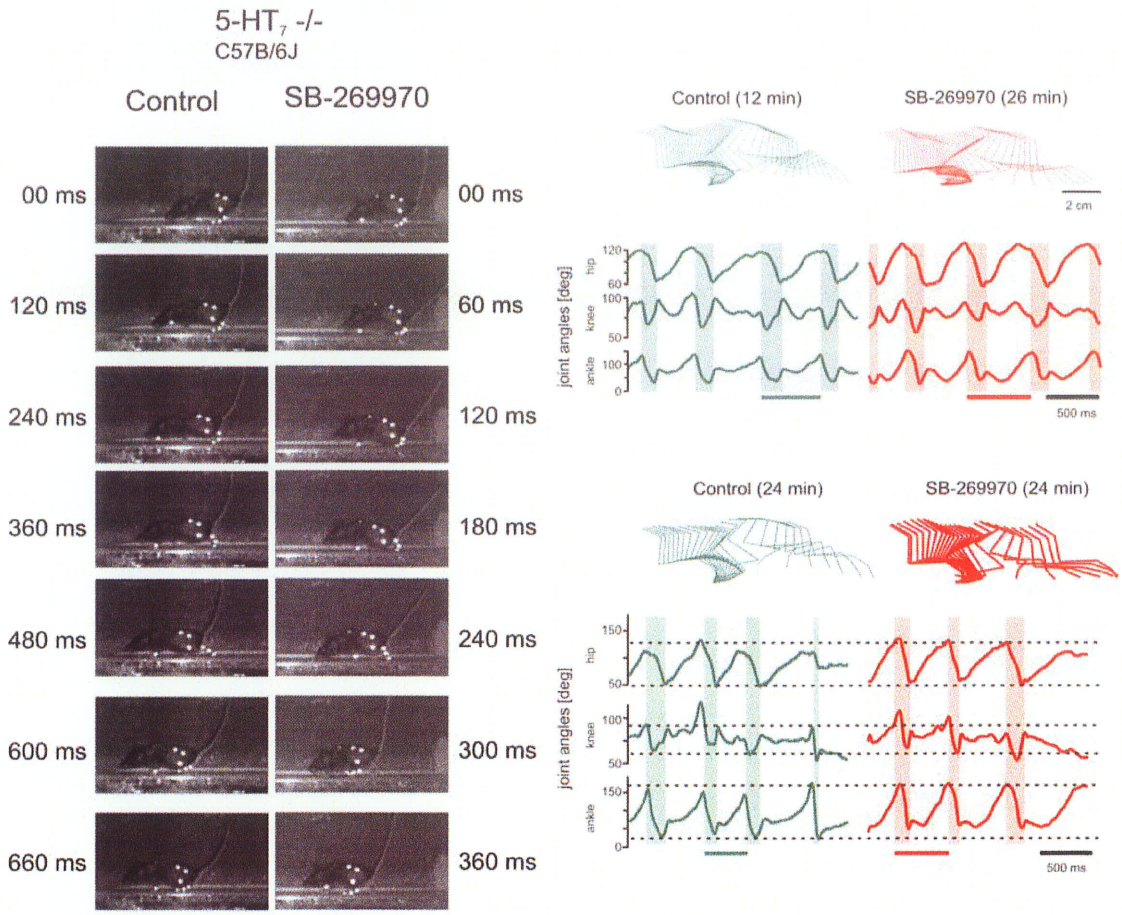

LTA - LGS
LTA - RTA
Control
SB-269970
10mM
0.25
0.5
0
0.75
Mean (n = 4)
φ 0.57 ± 0.16
r = 0.82 ± 0.1
Mean (n = 4)
φ 0.47 ± 0.07
r = 0.85 ± 0.06

Figure 13


5-HT7 -/-
C57B/6J
Control
SB-269970
00 ms
120 ms
240 ms
360 ms
480 ms
600 ms
660 ms
00 ms
60 ms
120 ms
180 ms
240 ms
300 ms
360 ms
Control (12 min)
SB-269970 (26 min)
2 cm
joint angles [deg]
hip
knee
ankle
500 ms
Control (24 min)
SB-269970 (24 min)
joint angles [deg]
hip
knee
ankle
500 ms

Fgiure 14

GENERAL DISCUSSION

The main findings in this thesis are: 1) activation of a discrete population of 5-HT neurons in the brainstem can evoke locomotor-like activity in the *in vitro* brainstem-spinal cord of the neonatal rat; 2) activation of both 5-HT_7 and 5-HT_{2A} receptors is required for PPR-evoked locomotion, but the neurons with these receptors function at different spinal segments. Neurons with 5-HT_7 receptors located in the low thoracic and upper lumbar cord may comprise CPG elements and play an important role in the production of alternating locomotor rhythm, whereas 5-HT_{2A} receptors are functionally involved in the control of CPG elements and output elements (including motoneurons) in the lower lumbar cord; 3) 5-HT_7 $^{-/-}$ neonatal mice exhibit a deficit in 5-HT-induced locomotor-like activity in vitro; 4) 5-HT_7 receptors are activated during normal voluntary locomotion in adult mice; 5) 5-HT_7 $^{-/-}$ mice appear to walk normally. Because a detailed discussion of the results has been dealt with in each section, here we mainly incorporate interrelated findings, extrapolate their significance, specify limits of the experiments and put forward probable approaches to further verify and extend our work.

There is evidence that the electrical stimulation of the medulla is able to elicit locomotor-like activity in the neonatal rat (Atsuta et al 1988; Zaporozhets et al 2004; Gilmore and Fedirchuk 2004), but the detailed location has not been specified in their studies. In this study, we have shown that stimulation of the PPR, which contains a discrete population of 5-HT cells, is sufficient for evoking locomotor-like activity in the *in vitro* brainstem-spinal cord preparation of the neonatal rat, suggesting that a serotonergic descending command system in the PPR, when stimulated, is capable of

activating the CPG networks for locomotion. The concept of a command system is derived from the term, "command neurons" referring to a group of neurons of which their activation is both necessary and sufficient to initiate a behavioral output (Kupfermann and Weiss 1978). Morphological studies have shown that the PPR is composed of heterogeneous neurons, for example, glutamatergic, serotonergic and some peptide-releasing cells (Helke et al 1989; Holstege and Kuypers 1987b; Skagerberg and Bjorklund 1985). Given that PPR-induced locomotor-like activity could be blocked by 5-HT_{2A} or 5-HT_7 receptor antagonists at the different segments of the spinal cord, it is strongly implicated that 5-HT neurons in the PPR play a critical role in the PPR-evoked initiation of locomotion.

As discussed in the General Introduction, the roles of the PPR in central chemoreception and modulation of autonomic functions have been extensively explored. Both morphological and physiological studies indicate that 5-HT neurons in this area are involved in these functions (Helke et al 1989; Toth et al 2006; Richerson 2004; Jones and Light 1992). Here, we provide, for the first time, evidence that 5-HT neurons in the PPR also play an important role in control of locomotion, which is consistent with the concept that a "central command" might exert more than one function in response when activated (Secher 2007). The role of PPR 5-HT cells involved in control of locomotion should be examined further by further experiments. For example, serotonergic cells in the PPR can be detected by microinjecting dyes into lumbar laminae VII, VIII and the central canal where the CPG elements are proposed to be located. Using intracellular recording combined with electrical stimulation of the PPR could allow to establish whether the neurons in the PPR have contact with lumbar interneurons or motoneurons. An

complementary approach would be to examine *c-fos* expression of the PPR following locomotion on the treadmill. The PPR 5-HT cells might be expected to show much more *c-fos* expression after a locomotor task compared to the control.

Immunohistochemical studies have demonstrated that individual 5-HT neurons in the PPR may contain one, two or even three neurotransmitters, such as some peptides, glutamate and GABA (see General Introduction). Furthermore, there is evidence that peptides e.g., SP, TRH, arginine vasopressin (AVP), and GABA are involved in the modulation of locomotor-like activity (Barriere et al 2005; Cowley and Schmidt 1994; Cowley and Schmidt 1995; Pearson et al 2003). It is likely that activation of 5-HT neurons in the PPR results in co-release of other neurotransmitters which excite and/or modulate the spinal locomotor network. These neurotransmitters may act in harmony on CPG interneurons to generate alternating rhythmic activity at an appropriate step frequency. It can be speculated that blockage of receptors of one of these transmitters at the spinal levels would disturb PPR-induced locomotion.

The physiological properties of locomotor command cells in the brainstem are well understood in the lamprey (Brocard and Dubuc 2003; Deliagina et al. 2002). Reticulospinal neurons (RN) in the brainstem of the lamprey, of which many are glutamatergic neurons, and exhibit NMDA mediated plateau properties (Di Prisco et al. 1997). Studies in the rat showed that serotonergic neurons of caudal raphe nuclei are a heterogeneous population based on differences in somatic and dendritic morphology (Gao and Mason 1997, 2000). Zhang et al (2006) reported that serotonergic cells in the rostral ventromedial medulla (RVM) of the neonatal rat differed from non-serotonergic cells in their firing patterns, and serotonergic neurons were proposed to be heterogeneous

in terms of their spontaneous discharges, passive membrane and action potential properties, suggesting these serotonergic neurons might play different physiological roles even in the same anatomical region.

Microinjection of EAA or GABA into the PPR was effective in inducing locomotor-like activity which was disrupted by 5-HT_7 receptor antagonists when applied to the spinal cord, suggesting that locomotor-like activity evoked by electrical stimulation is due to the activation of the PPR neurons rather than axons of passage. Moreover, the results provide evidence that 5-HT neurons of the PPR may have glutamate and GABA receptors. Using the same preparation, Atsuta et al (1991) reported that NMDA, GABA antagonists and SP could induce airstepping when applied to the brainstem bath. Previous studies have showed that 5-HT decreases the spontaneous activity of serotonergic neurons of the caudal raphe, an effect achieved by serotonergic autoreceptors (Trulson and Frederickson 1987; Koyama and Kayama 1993), likely somatodendritic 5-HT_{1A} receptors (Li and Bayliss 1998). These local modulatory mechanisms play an important role in feedback autoregulation of serotonergic neurons by 5-HT. Since 5-HT neurons in the caudal raphe nuclei developmentally originate from the same progenitors (Hendricks et al. 2003; Pattyn et al. 2003), it can be speculated that 5-HT neurons in the PPR may have similar membrane receptors. This issue may be clarified by immunohistochemistry and physiological studies at the single cell level. New genetic approaches are making it possible to visualize 5-HT cells via targeted transgene expression of EYGFP (enhanced yellow fluorescence protein) in mice (Scott et al. 2005). We are using this strain of mice to examine the physiological properties of the PPR 5-HT neurons and synaptic inputs on these cells.

It must be pointed out that neurons other than those containing 5-HT in the PPR are activated with stimulation in the PPR. There are several different types of neurons in the PPR including glutamate, peptide and GABA releasing neurons (Helke et al 1989; Dean et al 1993; Holmes et al. 1994; Nicholas et al 1992; Skagerberg and Bjorklund 1985).We cannot rule out a probable contribution of the glutamatergic system or peptides to the initiation of PPR-induced locomotor-like activity, given the fact that some serotonergic neurons coexpress these substances.

The experiments performed in the neonatal rat led us to propose that functional 5-HT_7 receptors located in the low thoracic and upper lumbar cord involve play an important role in the activation of locomotor networks. This notion was strengthened by experiments conducted in neonatal 5-HT_7 $^{-/-}$ mice and in adult mice. These results are consistent with previous observations that many cells in the thoraco-lumbar segments are both *c-fos* immunoreactive and 5-HT_7 receptor positive (Jordan and Schmidt 2002; Schmidt and Jordan 2000). The essential role of 5-HT_7 receptors in 5-HT-induced locomotion has been demonstrated by other groups (Hochman et al 2001; Madriaga et al 2004). Furthermore, 5-HT_7 receptors also participate in the modulation of NMDA-induced locomotor rhythm (Pearlstein et al 2005) and contribute to recovery of locomotion after spinal cord transection in mice (Landry et al 2006).

We show in the second section that 5-HT failed to induce locomotor-like activity in almost all 5-HT_7 $^{-/-}$ mice. Instead, uncoordinated or ipsilateral synchronous ventral root discharges were observed, which were blocked by a 5-HT_{2A} receptor antagonist. The results indicate that 5-HT_7 receptors play a critical role in the production of 5-HT-induced locomotion in neonatal mice, and activation of 5-HT_{2A} receptors mainly

contributes to the non-locomotor activity. The role of 5-HT_{2A} receptors in the control of locomotion has been previously proposed by several authors (Cazalets et al 1992; MacLean et al 1998; Beato and Nistri 1998; Madriaga et al 2004). Whelan and his colleagues reported that a 5-HT_2 receptor agonist, α-methyl-5-HT was able to elicit locomotor rhythm in the neonatal mouse (Whelan et al 2000; Madriaga et al 2004). *In Situ* hybridization and immunohistochemistry show that 5-$HT_{2A/2C}$ mRNA receptors are detected in laminae VII, VIII and IX (Fonseca et al 2001; Pompeiano et al 1994), with densely labeled 5-HT_{2A} receptors on the soma of motoneurons (Cornea-Hébert et al 1999; [illegible]tor-like activity was induced by 5-HT in one 5-HT_7 $^{-/-}$ mouse. Thus, 5-HT receptor subtypes involved in the initiation of locomotion needs to be further examined. In addition, it remains to be determined whether there is upregulation of other 5-HT receptor subtypes in 5-HT_7 $^{-/-}$ mice.

5-HT receptors on motoneurons can account for some of the effects observed in these experiments. The ability of ketanserin and spiperone to block PPR evoked locomotion when applied onto the caudal segments of the spinal cord may be due to the blockage of 5-HT_{2A} receptors on motoneurons. There is ample evidence that 5-HT_{2A} receptors mediate 5-HT-induced increases in excitability of motoneurons (Jackson and White 1990; Takahashi and Berger 1990; Wang and Dun 1990; Schmidt and Jordan 2000; Perrier and Hounsgaard 2003; Harvey et al 2006). Other 5-HT receptors on motoneurons include 5-HT_{1A} (Kheck et al 1995), 5-HT_{2C} and 5-HT_3 (Fonseca et al 2001). In turtle motorneurons, activation of 5-HT_{1A} receptors contributes to the excitatory effect of serotonin on spinal motoneurons by inhibition of a TASK-1 potassium channel leading to

depolarization and increased input resistance (Perrier et al 2003). SK (small conductance potassium) channels are inhibited by activation of 5-HT_{1A} receptors when coexpressed in *Xenopus laevis* oocytes (Grunnet et al. 2004). This would increase motoneuron excitability by decreasing the AHP. Daily injections of a 5-HT_{1A} agonist improved motor recovery in chronic spinal rats (Antri et al 2003). However, hyperpolarization of spinal motoneurons via 5-HT_{1A} receptors has also been reported (Wang and Dun 1990). In complete paraplegic mice, a 5-HT_3 agonist improved hindlimb movement (Guertin and Steuer 2005). The action of a 5-$HT_{1A/7}$ agonist to improve locomotor recovery in paraplegic mice has been demonstrated (Niitsu et al 1995), and there is one report of 5-HT_7 receptors on motoneurons (Doly et al 2005). The same distribution has been observed in the 5-HT_7 receptor knockout mouse, however, casting doubt on the validity of this finding (Jordan and Gibbs, unpublished). In summary, 5-HT has pronounced excitatory effects on motoneurons, mediated by 5-$HT_{1A, 2A, 2C}$, and possibly 5-$HT_{3, 7}$ receptors. The work presented in this thesis supports a role for the 5-HT_{2A} receptor in the control of motoneuron excitability.

It is possible that 5-HT_7 receptor involvement in relay of sensory information from the moving limb could account for some of our observations in the adult mouse after administration of SB-269970. 5-HT_7 receptors are found in dorsal root ganglion cells, where they tend to modulate Ih (Cardenas et al. 1999). There is also immunohistochemical evidence for 5-HT_7 receptors on dorsal horn neurons and on presynaptic terminals (Doly et al 2005), but as pointed out above, there is some doubt as to the specificity of the antibody used in that study. 5-HT_7 receptors mediates effects on

primary afferent synapses appear to be facilitatory (Hochman et al 2001). It is well known 5-HT has strong effects on transmission in certain sensory pathways (see General Introduction), and actions at presynaptic sites or postsynaptically on sensory relay neurons may account for the potent action of SB-269970 in the adult walking mouse. A role for 5-HT_7 receptors in relay of sensory information from the cauda equina has been suggested by Gordon and Whelan (2006) to explain their finding that a 5-HT_7 agonist interferes with locomotor activity produced by cauda equina stimulation. More detail regarding the contribution of 5-HT_7 receptors to the control of afferent feedback from the moving limb is required before a definitive answer to the question of whether there is a sensory component to the effects observed in this thesis. The similarity between the effects observed in vitro and in vivo in the experiments reported here suggest that most if not all the effects of SB-269970 can be attributable to actions on the locomotor network elements.

Adult 5-HT_7 $^{-/-}$ mice do not exhibit any appreciable deficiency of locomotion (Hedlund et al 2003). This is the case even in conditionally 5-HT depleted mice (Zhao et al 2006). One explanation is that adaptation takes place in these mice and walk normally over time possibly via sensory feedback and/or exercising during prenatal or postnatal stage. An alternate explanation is that normal locomotion is attributed to redundant or multiple descending command systems (Orlovsky et al 1999), which means that none of the descending pathways plays an indispensable role in the generation of locomotion (Rossignol 2006). Our preliminary results in 5-HT_7 $^{-/-}$ mice showed that the combination of NMDA and dopamine or an increase of the concentration of recording solution was

able to induce the locomotor rhythm, suggesting that the locomotor CPG can still be activated by other neurotransmitter systems. We presume that in wild type animals, multiple command systems might orchestrate and interplay to produce well coordinated locomotor rhythm. If one of these systems is acutely interfered with, normal locomotion will be disrupted, which is the case observed in adult *in vivo* experiments.

How can the uncoordinated locomotion produced by 5-HT in 5-HT_7 $^{-/-}$ or SB-269970 treated mice in vitro and in vivo be explained? And can these observations provide clues to the organization of the mammalian locomotor CPG? One of the most obvious suggestions that arises from the observation that 5-HT produces un-coordinated rhythmic activity in 5-HT_7 knockout mice and in wild-type mice treated with SB-269970 is that the uncoordinated discharges may be due to conditional bursting activity induced by 5-HT in motoneurons, without involvement of the CPG. 5-HT has strong excitatory effects on motoneurons (reviewed in Rekling et al 2000), and they are known to display endogenous rhythmic activity (Hochman et al. 1994). In addition, 5-HT promotes intrinsic membrane voltage oscillations (MacLean and Schmidt 2001; MacLean et al 1998). This is consistent with the fact that the uncoordinated rhythmic activity can be blocked by 5-HT_{2A} antagonists, and motoneurons are rich in the receptors affected by these antagonists. Another possible explanation for the observations is that 5-HT_7 receptors are present on CPG neurons required for coordinating the locomotor rhythm, while certain other rhythm generating neurons can still be active and provide an uncoordinated drive to the motoneurons (Rybak et al 2006; Lafreniere-Roula and McCrea 2005). There is currently no evidence for two such classes of CPG neurons, but clearly the coordinating elements must possess 5-HT_7 receptors. Some of these coordinating

elements may be inhibitory interneurons that account for the proposed reciprocal inhibition at the rhythm generating (Rybak et al 2006; Lafreniere-Roula and McCrea 2005) of the CPG (Rybak et al 2006; Lafreniere-Roula and McCrea 2005). It is possible that 5-HT_7 receptors are limited to inhibitory interneurons of the CPG. This is consistent with the observation that one of the effects of 5-HT_7 receptor blockade in vitro is progressive slowing of the locomotor rhythm, a feature of the disinhibited rhythm produced after blockage of glycine and GABA synapses (Cowley and Schmidt 1995). The locomotor rhythm is also slowed in animals lacking inhibitory V1 interneurons (Gosgnach et al 2006). It is also consistent with the observation that synchronous ventral root activity was produced by 5-HT in wildtype mouse spinal cord treated with SB269970 and in 5-HT_7 knockout mouse spinal cord. Nevertheless, because SB-269970 eventually can completely block rhythmic activity in vitro, it is necessary to conclude that excitatory neurons of the CPG also possess 5-HT_7 receptors.

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