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LACK OF ANALGESIC EFFICACY OF SPINAL ONDANSETRON ON THERMAL AND MECHANICAL HYPERSENSITIVITY FOLLOWING SPINAL NERVE LIGATION IN THE RAT

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Abstract

The balance between descending inhibition and facilitation is thought to be disturbed in chronic pain states. Increased facilitation by spinally released serotonin has been suggested by demonstration that mechanically evoked neuronal responses of wide dynamic range neurons are inhibited by 5-HT₃ receptor antagonists in rats following spinal nerve ligation (SNL) but not sham operation. Despite these physiologic data, the effects of spinal 5-HT₃ receptor blockade on behavioral hypersensitivity and neurochemical alterations in spinal serotonergic system have not been thoroughly investigated following spinal nerve ligation in the rat. To test this, we acutely injected intrathecal ondansetron in rats between 14 and 30 days after SNL and assessed effects on thermal and mechanical hypersensitivity. We also determined the density of serotonergic nerve fibers, serotonin content and the levels of 5-HT₃ receptors within the spinal cord at this time point. Intrathecal ondansetron (1, 3, 10, 30, and 100 µg) produced no effect on behavioral measures of thermal or mechanical hypersensitivity whereas intrathecal morphine (1 µg) and gabapentin (200 µg) partially reversed thermal and mechanical hypersensitivity following SNL. In addition, SNL did not alter the density of serotonergic fibers or 5-HT₃ receptor immunoreactivity or spinal tissue content of 5-HT within the dorsal horn. These results do not support anatomic plasticity of descending serotonergic pathways or tonic 5-HT₃ receptor activity in maintaining hypersensitivity after nerve injury and in contrast to previous studies fail to demonstrate an anti-hypersensitivity effect of intrathecal injection of the 5-HT₃ receptor antagonist ondansetron following peripheral nerve injury. Importantly, behavioral measures of mechanical hypersensitivity assess threshold responses whereas physiological studies of mechanically evoked neuronal responses involve application of suprathreshold stimuli. Thus, suprathreshold or more intense stimuli may be

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necessary to recruit descending serotonergic facilitatory drive required to observe the inhibitory effects of ondansetron on spinal neuronal excitability and behavioral hypersensitivity.

Keywords

rostral ventromedial medulla; neuropathic pain; monoaminergic; serotonin transporter; neuroplasticity

1. Introduction

Descending serotonergic pathways have been implicated in both inhibitory and facilitatory modulation of spinal neuronal excitability and pain related behaviors in various acute and persistent pain states. Intrathecal administration of the 5-HT₃ receptor antagonist ondansetron inhibits spontaneous pain behaviors and neuronal activity following intraplantar formalin injection (Green et al., 2000; Suzuki et al., 2002; Svensson et al., 2006), reverses mechanical allodynia in rodent models of central neuropathic pain due to spinal cord injury (Chen et al., 2009; Oatway et al., 2004) and reverses chronic opioid induced hyperalgesia (Vera-Portocarrero et al., 2007). However, studies examining the contribution of 5-HT₃ receptor activation to behavioral hypersensitivity following peripheral nerve injury have produced equivocal results. Physiologic studies suggest that 5-HT₃R activation has facilitatory effects on responses of spinal cord neurons following L5 and L6 spinal nerve ligation (SNL) (Suzuki et al., 2004a). In behavioral pharmacological studies, blockade of spinal 5-HT₃ receptors has been reported to inhibit (Dogrul et al., 2009) or have no effect (Okazaki et al., 2008) on mechanical hypersensitivity following SNL in rats. Similarly, in two randomized placebo controlled crossover studies, patients with chronic neuropathic pain administered intravenous ondansetron reported transiently reduced ongoing pain intensity (McCleane et al., 2003) or reported no effect on ongoing pain intensity or dynamic mechanical allodynia (Leffler et al., 2008). The primary goal of the current study was to thoroughly examine the ability of ondansetron to reverse thermal and mechanical hypersensitivity following L5/6 SNL in rats. This included examination of a large range of doses on several behavioral endpoints using a controlled, blinded study design with both negative and positive controls and across two laboratories with extensive experience in preclinical examination of neuropathic behavior and the effects of analgesics. We hypothesized that spinal ondansetron would strongly inhibit behavioral measures of mechanical hypersensitivity.

The vast majority (95%) of serotonergic nerve fibers in the spinal cord originate from distinct nuclei within the rostral ventromedial medulla (RVM) and terminate in the dorsal and ventral horn at all segmental levels of the spinal cord (Steinbusch, 1981). The effects of serotonin in the spinal cord are mediated by at least seven serotonin receptor subtypes (5-HT₁₋₇) most of which have some role in nociceptive processing (Millan, 2002). Unlike other serotonin receptors, which are G-protein coupled, 5-HT₃ receptors are ligand gated cation channel predominantly localized on presynaptic terminals of a population of predominantly myelinated (A δ) and a subpopulation of unmyelinated (C) primary afferent fibers within the superficial dorsal horn (Conte et al., 2005; Kidd et al., 1993; Maxwell et al., 2003; Zeitz et al., 2002). There is also anatomical evidence in rats for the localization of 5-HT₃ receptors on terminals of excitatory interneurons (Conte et al., 2005; Maxwell et al., 2003) and in mice on inhibitory GABAergic interneurons (Huang et al., 2008). Neurochemical plasticity in descending serotonergic nerve fibers within the spinal cord has been documented following spinal cord injury (Hains et al., 2002; Oatway et al., 2004) and dorsal rhizotomy (Laporte et al., 1995; MacDermid et al., 2004; Polistina et al., 1990); however whether plasticity of serotonergic nerve fibers occurs following more distal

peripheral nerve injury has not been thoroughly investigated. The secondary aim of the current study was to quantify alterations in the density of serotonergic nerve fibers and 5-HT₃ receptor immunoreactivity in the spinal cord, comparing control to SNL conditions. We postulated that the state dependent facilitation previously observed following SNL occurs in part due to increased serotonergic fiber sprouting or upregulation of 5-HT₃ receptors on primary afferent fibers within the spinal cord of SNL rats.

2. Results

2.1 Effects of intrathecal ondansetron on mechanical hypersensitivity associated with L5/6 SNL

Rats that underwent L5/6 SNL developed mechanical hypersensitivity by fourteen days after surgery as indicated by reduced paw withdrawal thresholds elicited using von Frey filaments (Figure 1A) and the Randall Selitto paw pressure device (Figure 1B) compared to pre SNL baseline values in all groups examined (Data not shown). The high efficacy 5-HT₃ receptor selective antagonist, ondansetron was used to assess the contribution of 5-HT₃ receptor activation on the mechanically evoked behavioral hypersensitivity associated with peripheral nerve injury. Intrathecal ondansetron (1, 3, 10, 30, and 100 µg) administered to rats beginning 14 days after SNL failed to reverse mechanical hypersensitivity between 30 minutes and two hours post administration compared to saline treated rats (Figure 1A, B). Intrathecal morphine (1 µg) administered at a dose that has previously been shown to reduce mechanical hypersensitivity following SNL attenuated mechanical hypersensitivity compared to saline treated rats in both behavioral assays (Figure 1A, B). In order to verify reproducibility of these data, the effects of ondansetron on mechanical allodynia were confirmed by an independent laboratory using a single dose of ondansetron (30 µg) administered to SNL rats. Intrathecal administration of gabapentin (200 µg) was administered as a positive control (Figure 1C). In additional experiments, we examined the efficacy of another clinically available 5-HT₃ receptor antagonist on mechanical hypersensitivity following L5/6 SNL. Acute intrathecal administration of the 5-HT₃ receptor antagonist, dolasetron (40 µg) 14 days after SNL failed to reverse mechanical hypersensitivity assessed using von Frey filaments (Post SNL baseline: $4.15 \pm 0.2g$, 30 min: $4.89 \pm 0.7g$, 60 min: $5.49 \pm 1.4g$, 120 min: $3.60 \pm 0.4g$, $P=0.522$).

2.2 Effects of intrathecal ondansetron on thermal hypersensitivity associated with L5/6 SNL

Rats that underwent L5/6 SNL developed a significant thermal hyperalgesia by 14 days after surgery as evidenced by reduced latency to withdrawal from a radiant heat source focused on the paw ipsilateral to ligation (Figure 2A, 15.9 ± 0.7 s latency before SNL versus 9.4 ± 0.3 s latency after SNL, $P<0.001$). Withdrawal latencies were not significantly reduced in the contralateral paw (Figure 2B, 14.6 ± 0.6 s latency before SNL versus 16.0 ± 0.6 s latency after SNL, $P=1.0$). Intrathecal ondansetron (1, 3, 10, 30 µg) administered between 14 and 30 days following L5/6 SNL did not attenuate thermal hypersensitivity in ipsilateral paw (Figure 2A) or alter withdrawal latency in contralateral paw (Figure 2B) at any dose examined when compared to saline treated values at the same time point or compared to post SNL baseline values within treatment groups ($P>0.05$, Two way repeated measures ANOVA, Bonferroni post hoc analysis). Intrathecal morphine (1 µg) administered 14 days following L5/6 SNL attenuated thermal hypersensitivity 30, 60 and 90 minutes post administration in the ipsilateral paw (Figure 2A) and increased withdrawal latency at 30 and 60 minutes in the contralateral paw (Figure 2B) when compared to saline treated values at the same time point. Within the morphine treated group of rats, thermal withdrawal latencies were significantly increased at 30, 60, 90 and 120 minutes in the ipsilateral paw and 30 and

60 minutes in the contralateral paw compared to postSNL baseline values ($P < 0.05$, Two way repeated measures ANOVA, Bonferroni post hoc analysis).

2.3 Effects of SNL on density and distribution of serotonergic nerve terminals in the spinal cord of rats

Serotonergic nerve fibers undergo plastic changes following dorsal rhizotomy, however alterations in the distribution of serotonergic fibers has not to our knowledge been examined following L5/6 SNL. Therefore, we examined the distribution of serotonergic nerve fibers using an antibody against the serotonin transporter (5-HTT). 5-HTT is a transmembrane protein highly expressed in the spinal cord whose distribution parallels serotonergic innervation (Sur et al., 1996). In normal rats, 5-HTT-IR was present throughout the spinal cord with particularly dense nerve fibers present in superficial laminae (I-IIo) and to a slightly lesser degree in deeper laminae. There was a distinct band within laminae III with a low density of 5-HTT-IR similar to previous reports (Marlier et al., 1991; Polistina et al., 1990; Zhang et al., 1993). 5-HTT was present on axons with varicosities typical of monoaminergic nerve terminals (Figure 3). The pattern of 5-HTT-IR was similar in the dorsal spinal cord of rats 14 days following SNL; however the intensity of labeling was slightly but not significantly reduced in SNL rats (Figure 3, Table 1). The values of 5-HTT IR were not significantly different between normal and SNL rats at L4 or L5/6 spinal levels.

2.4 No change in serotonin content with the spinal cord of SNL rats

As a more quantifiable method to examine changes in serotonergic nerve fiber density, we measured serotonin content in the spinal cord dorsal horn. We assayed the ipsilateral and contralateral dorsal lumbar (L4-6) spinal cord from SNL and normal rats for serotonin content as well as that of its primary metabolite 5-HIAA. Within SNL rats the mean difference in 5-HT and 5-HIAA levels were not significantly different between ipsilateral and contralateral dorsal quadrants (5-HT: 771 ± 106 pg/mg ipsilateral vs. 856 ± 98 pg/mg contralateral; mean diff = -85 pg/mg; 95% CI of difference in means: -438 to 268, $p = 0.58$) and (5-HIAA: 1050 ± 142 pg/mg ipsilateral vs. 1120 ± 136 pg/mg contra; mean diff = -76 pg/mg; 95% CI of difference in means -556 to 405; $p = 0.71$). The mean difference in ipsilateral 5-HT levels were not significantly different between SNL and normal rats (771 ± 106 pg/mg SNL vs. 882 ± 22.6 pg/mg normal; mean diff = -111; 95% CI of difference in means -418 to 197; $p = 0.41$). The mean differences in ipsilateral 5-HIAA levels were not significantly different between SNL and normal rats (1050 ± 142 pg/mg SNL vs. 895 ± 35.8 pg/mg normal; mean diff = 151; 95% CI of difference in means -207 to 509; $p = 0.34$).

2.5 Lack of alterations in 5-HT3 receptor-IR in the spinal cord of rats following SNL

We used a previously characterized antibody against the A subunit of the 5-HT3 receptor (Conte et al., 2005) to examine alterations in the density of 5-HT3 receptor following peripheral nerve injury. In normal rats 5-HT3 receptor -IR was present as an intense band of labeling predominantly in laminae I-II (Figure 4) of the spinal cord. Sparse labeling was present in fibers in deep laminae (III-VI). Similar to previous reports, occasional 5-HT3 receptor positive neuronal cell bodies were present in laminae I-II dorsal horn (data not shown). There were no detectable levels of 5-HT3 receptor in motor neurons or dorsal horn neurons within deeper laminae (data not shown). Because of the dense innervation and nearly undetectable levels of 5-HT3R IR within deeper laminae, we focused our quantification of immunohistochemical results to laminae I-II. Fourteen days following SNL 5-HT3 receptor immunoreactive levels were not significantly different compared to levels in normal rats at L4 and L5/6 level of the spinal cord (Figure 4, Table 2).

3. Discussion

The primary finding in the current study was that spinal delivery of the highly selective 5-HT₃ receptor antagonist ondansetron between 14 and 30 days following L5/6 spinal nerve ligation in the rat failed to reduce the thermal or mechanical hypersensitivity that accompanies this neuropathic pain state. Secondly, we used immunohistochemical and biochemical approaches to assess alterations in the spinal serotonergic system. We didn't observe alterations in spinal serotonin content, density of serotonergic terminals, or distribution of 5-HT₃ receptors following SNL. These results were unanticipated and surprising considering the recent interest in 5-HT₃ receptor antagonists as potential analgesics (Greenshaw and Silverstone, 1997; McCleane et al., 2003; Suzuki et al., 2004b; Thompson and Lummis, 2007) and several preclinical studies that demonstrate a facilitatory role for spinal 5-HT₃ activation in the setting of tissue injury (Svensson et al., 2006) and central neuropathic pain states (Chen et al., 2009; Oatway et al., 2004; Suzuki et al., 2004a). Failure of intrathecal ondansetron to alter hypersensitivity across three behavioral endpoints and two laboratories led us to abandon neurotoxicity screening of this compound for intrathecal study in humans under NIH grant GM48085.

It is well known that descending pathways that originate in the rostral ventral medulla (RVM) are essential for maintaining mechanical hypersensitivity and spinal neuronal excitability that accompanies a variety of persistent pain states including neuropathic pain (Porreca et al., 2002; Suzuki et al., 2002; Vera-Portocarrero et al., 2006). A component of descending facilitation following peripheral nerve injury is likely due to enhanced serotonergic drive originating in the RVM and subsequently activating spinal 5-HT₃ receptors (Marinelli et al., 2002; Suzuki et al., 2004a). The most compelling evidence for this comes from *in vivo* electrophysiological studies. Suzuki and colleagues reported that pharmacological blockade of 5-HT₃ receptors with spinal ondansetron strongly inhibits mechanical and to a lesser degree thermal evoked neuronal responses of WDR neurons in deep laminae (V-VI) of rats 14 days following SNL compared to sham operated rats. This enhanced 5-HT₃ receptor activation relies on a spinobulbar circuit involving neurokinin 1 (NK1) receptor bearing neurons within the superficial aspects of the spinal cord (Suzuki et al., 2004a) as ablation of spinal NK1 neurons abolishes the inhibitory effects of ondansetron. Similar physiological evidence for enhanced serotonergic facilitation of spinal excitability has been observed following injection of formalin in the hindpaw (Suzuki et al., 2002), bone cancer (Donovan-Rodriguez et al., 2006), and osteoarthritis (Rahman et al., 2009).

A facilitatory role of spinal 5-HT₃ receptor activation on pain related behaviors has also been described in several persistent pain states (Zeititz et al., 2002). Depletion of serotonergic nerve fibers reduces the delayed (phase II) nocifensive pain behaviors that occur following injection of formalin into the hindpaw (Svensson et al., 2006) as well as thermal and mechanical hypersensitivity associated with central (Oatway et al., 2004) and peripheral neuropathic pain states (Rahman et al., 2006). Transgenic knockout and pharmacological studies using 5-HT₃ receptor subtype selective antagonists have begun to address the 5-HT receptor subtypes responsible for these behavioral facilitatory effects. Spinal ondansetron in rats (Svensson et al., 2006; Suzuki 2002) or genetic deletion of 5-HT₃ receptors in mice (Zeititz 2005) reduced phase II formalin responses. Additionally, administration of ondansetron in the formalin model reduced spinal levels of phosphorylated ERK providing evidence that 5-HT₃ receptor activation contributes to sensitization of spinal cord neurons (Svensson et al., 2006). Spinal ondansetron administered acutely (Oatway et al., 2004) or chronically (Chen et al., 2009) has also been shown to effectively reduce mechanical allodynia in rats with spinal cord injury as well as thermal and mechanical allodynia that develops in rats following chronic opioid administration (Vera-Portocarrero et al., 2007).

Despite these positive finding studies, preclinical studies examining the effects of ondansetron on symptoms of neuropathic pain have produced conflicting results. Recently, Dogrul and colleagues reported that acute spinal ondansetron (10 μ g) produced a sustained attenuation of thermal and mechanical hypersensitivity following L5/6 SNL in rats. In contrast to these findings, Okazaki and colleagues reported that spinal administration of the 5-HT₃ receptor antagonist CGP35348 had no effect on established mechanical allodynia in rats (Okazaki et al., 2008). Furthermore, mice deficient in 5-HT₃ receptors develop a similar degree of mechanical allodynia as wild type mice following partial sciatic nerve injury and peripheral inflammation (Zeitz et al., 2002). These studies suggest that spinal facilitatory behavioral effects are not universally observed in all persistent pain states or consistently in neuropathic models of peripheral nerve injury. In the current study, we addressed this controversy by examining more than one 5-HT₃ receptor selective antagonist in a battery of behavioral tests. We observed that spinal ondansetron (1, 3, 10, 30, and 100 μ g) and dolasetron (40 μ g) did not reverse thermal or mechanical hypersensitivity when administered acutely between 14-30 days following SNL.

Several explanations for the differences between our findings and those reported by Dogrul and colleagues are possible. Both studies examined ondansetron using a similar dose, drug formulation, and method of administration. In the study by Dogrul spinal ondansetron was administered at 7 days post SNL while in our study we initiated ondansetron between 14 -30 days following SNL. It is possible that 5-HT₃ mediated facilitatory effects are time dependent and resolve between 14 and 30 days post SNL. However, physiological studies demonstrate that enhanced serotonergic drive is still present 14 days following SNL (Suzuki et al., 2004a). Secondly, Dogrul et al. do not comment whether behavioral assays were conducted in a blinded manner. In the current study, all behavioral assays were conducted by an investigator blind to treatment and negative results were replicated using blinded conditions in a second laboratory. In both laboratories, positive controls were included which showed clear efficacy. Unintended experimenter bias has been suggested to have an impact on behavioral outcomes in preclinical studies. Notably, negative and/or positive outcomes of pharmacological studies examining the efficacy of spinal opioids in preclinical models of neuropathic pain closely paralleled the consensus on the efficacy of spinal opioids reported simultaneously in clinical studies (Eisenach and Lindner, 2004). It is also possible that subtle differences in surgical procedures may impact behavioral outcomes between labs. There is considerable variability in the extent of nerve injury and onset of mechanical hypersensitivity in the L5/6 SNL model (Chung et al., 2004). In the study by Dogrul the baseline levels of mechanical hypersensitivity as measured using von Frey filaments are slightly lower than baseline values in the current study (1.6 ± 0.7 gm vs. 4.1 ± 0.7 gm). Thus, the degree of mechanical hypersensitivity may be important for recruiting descending serotonergic drive sufficient to observe an inhibitory effect of ondansetron. Likewise, the degree of nerve injury may have a pronounced impact on spinal plasticity of the serotonergic system.

Multiple factors may impact the net effect of 5-HT₃ receptor activation in the spinal cord including plasticity of serotonergic terminals, alterations in 5HT₃ receptor distribution, as well as the nature and intensity of peripheral stimuli (Tyce and Yaksh, 1981). Following dorsal rhizotomy sprouting of serotonergic terminals has been observed in segmental regions of the spinal cord corresponding to deafferented terminals but not in regions with intact afferents (Polistina et al., 1990; Ramer et al., 2007). Following chronic constriction injury to the sciatic nerve bilateral increases in serotonin content were observed between seven and 14 days postoperatively (Satoh and Omote, 1996) coinciding with the development of mechanical hypersensitivity in this model. Following injury to the spinal cord an increase in the density of serotonergic terminals is observed rostral to the lesion site which has been causally linked to mechanical hypersensitivity in corresponding dermatomes (Oatway et al.,

2004). In a recent study, spinal levels of 5-HT and its metabolite transiently decreased in the dorsal ipsilateral lumbar spinal cord of L5/6 SNL rats seven days postoperatively but recovered to baseline levels by 28 days. These alterations coincided with decreased inhibitory effects of 5-HT on C fiber evoked responses of WDR neurons (Liu et al., 2010). It is unclear why both increases and decreases in descending serotonergic system have been described following nerve injury, however this may be due to differences in magnitude or type of peripheral nerve injury as spinal cord injury, dorsal rhizotomy at multiple spinal segments and chronic constriction of the sciatic nerve involve injury to a larger percentage of afferents innervating the spinal cord compared to L5/6 SNL.

In our study, we did not observe alterations in serotonin content or density of serotonergic terminals consistent with the negative behavioral effects of ondansetron (14 days post SNL). We also did not observe alterations in the density of 5-HT₃ receptors using immunohistochemical approaches. One limitation of our approach is that we do not discriminate between alterations of 5-HT₃ receptor on terminals of primary afferent fibers or populations of interneurons. Although a recent study in model of osteoarthritis provides evidence that 5-HT₃ receptor mRNA was unchanged in the dorsal root ganglia of rats with osteoarthritis despite enhanced physiological effects of ondansetron in this model (Rahman et al., 2009) suggesting alterations in 5-HT₃ receptor on primary afferents are not required for enhanced serotonergic drive.

Our behavioral results also disagree with physiological results that demonstrate facilitatory effects of 5-HT₃ receptor activation on mechanically evoked neuronal responses of deep dorsal horn WDR neurons in SNL animals (Suzuki et al., 2004a). When comparing physiological results to behavioral outcome measures it is important to consider the differences between these outcome measures. Notably, behavioral measures of mechanical hypersensitivity assess threshold responses whereas physiological studies of mechanically evoked neuronal responses involve application of suprathreshold stimuli. Suprathreshold or more intense stimuli may be necessary to recruit descending serotonergic facilitatory drive required to see an inhibitory effect of ondansetron on neuronal activity. Thus, behavioral assessment of withdrawal thresholds employed in the current study may not be sensitive enough to detect facilitatory effects of 5-HT₃ receptors in the spinal cord.

Another possibility for the lack of behavioral effect compared to physiologic studies may be due to dual effects of 5-HT₃ receptor activation on distinct population of neurons within the spinal cord dorsal horn resulting in a net lack of effect on behavioral outcomes. Previous electrophysiological studies assessing the effects of ondansetron on neuronal responses following SNL focused on WDR neuron responses in deeper laminae (V-VI). It should be noted that deep WDR neurons only comprise a subpopulation of ascending pathways that contribute to the behavioral manifestation of mechanical hypersensitivity (Keller et al., 2007; Ossipov et al., 2000; Sun et al., 2001). Several recent functional studies demonstrate that the dominant effect of 5-HT₃ receptor activation in the spinal cord is anti-nociceptive by promoting GABAergic inhibitory synaptic transmission (Alhaider et al., 1991; Giordano, 1991; Giordano and Schultea, 2004). Specifically, it has been reported in rats that activation of 5-HT₃ receptors in the spinal cord mediates the release of GABA but not glycine or glutamate (Kawamata et al., 2003). Recently, Fukushima and colleagues using transgenic GAD67-GFP reporter mice and *in vitro* spinal cord slice physiology demonstrated that 5-HT₃ receptor activation induces outward currents in GABAergic interneurons and increases evoked and spontaneous inhibitory postsynaptic currents in a GABA_A receptor dependent manner. Interestingly, intrathecal administration of the selective 5-HT₃ receptor agonist chlorophenylbiquanide (m-CPBQ) in rats with L5 SNL reverses established mechanical allodynia in a GABA_A dependent manner (Okazaki et al., 2008) suggesting that spinal 5-HT₃ receptor activation in the context of a neuropathic pain state still possesses inhibitory

effects on nociceptive processing. It is not clear if similarly endogenous spinal 5-HT₃ receptor activation has analgesic effects in the setting of nerve injury. In the current study this appears not to be the case as spinal ondansetron did not further reduce thermal and mechanical withdrawal thresholds following SNL. However, reduced withdrawal latency to noxious thermal stimuli has been observed in naïve mice following spinal administration of ondansetron (Scott et al., 2006). Peripheral nerve injury results in decreased inhibitory GABAergic tone in neurons within the superficial spinal cord in part due to transient disruption of chloride homeostasis (Miletic and Miletic, 2008). Downregulation of the potassium chloride cotransporter KCC2 in superficial postsynaptic neurons has been shown to shift GABA_A mediated signaling from hyperpolarizing to depolarizing leading to increased neuronal excitability (Coull et al., 2003; Price et al., 2005). This may in part explain enhanced facilitation at the physiological level previously observed in SNL rats; however it is unclear how this would impact behavioral outcomes at the systems level. Future studies are warranted to examine the effects of endogenous 5-HT₃ receptor activation on GABAergic nociceptive transmission and on distinct populations of dorsal horn neurons in both normal and SNL rats.

Conclusions

In the current study, descending serotonergic inputs acting via 5-HT₃ receptor did not contribute to thermal or mechanical hypersensitivity following SNL. Examination of the spinal cord revealed no significant changes in the density of serotonergic nerve fibers or serotonin content in the dorsal horn of SNL rats compared to naïve rats. Additionally, the 5-HT₃ receptor levels in the spinal cord dorsal horn were not different between SNL and naïve rats. These results agree with some more limited behavioral studies and suggest that spinal blockade of 5-HT₃ receptor is insufficient to inhibit behavioral measures of mechanical hypersensitivity following experimental SNL.

4. Experimental Procedure

4.1 Animal preparation and surgery

Male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN, USA), weighing 200–250 g, were used. All studies conformed to the Wake Forest University Guidelines on the Ethical use of animals, and studies were performed under Animal Care and Use Committee approval. Animals were housed under a 12-h light–dark cycle, with food and water ad libitum. Spinal nerve ligation (SNL) was performed as previously described (Yaksh and Rudy, 1976). In brief, animals were anesthetized with inhalational isoflurane (2%) in oxygen, the transverse processes of lower lumbar and upper sacral vertebrae were exposed, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were identified and tightly ligated using 5–0 silk suture.

Seven days following SNL, some animals were anesthetized with inhalational isoflurane (2%) for insertion of an intrathecal catheter as previously described (Ali et al., 1996). Animals were placed prone in a stereotaxic frame and a small incision was made at the back of the neck. A small puncture was made in the atlanto-occipital membrane of the cisterna magnum and a polyethylene catheter, 8.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. The rostral end of the catheter was exteriorized at the top of the head and the wound was closed with sutures. After implantation of the intrathecal catheters, rats were housed individually with free access to food and water. Animals were allowed 7 days to recover from the surgery prior to behavioral analysis. Animals showing signs of motor dysfunction (forelimb or hindlimb paralysis) following insertion of intrathecal catheters were excluded from the study.

4.2 Drug administration

Spinal nerve ligated rats were acutely administered various doses (1, 3, 10, 30 μ g, i.t.) of two clinically available 5-HT₃ receptor antagonists ondansetron hydrochloride (Sigma-Aldrich or Zofran® GlaxoSmithKline) and dolasetron mesylate injection (40 μ g, i.t., 20 mg/ml solution, Anzemet®, Sanofi Aventis, U.S. LLC, Italy) between 14–30 days post surgery after behavioral measures of mechanical hypersensitivity were established. Morphine (1 μ g), gabapentin (200 μ g) or vehicle (0.9 % saline) was administered in control animals. Vehicle for all drugs including ondansetron hydrochloride, dolasetron mesylate and morphine was 0.9% saline solution. Stock solutions of ondansetron, dolasetron and morphine were prepared the day of behavioral experiments at a concentration required to deliver 10 μ l drug solution followed by 10 μ l saline intrathecally to ensure equal volume of injection between experiments. The range of doses of ondansetron used in the current study are based on intrathecal doses of ondansetron required to elicit analgesic or anti-allodynic effects in several preclinical models of acute (Ali et al., 1996; Svensson et al., 2006) and persistent pain states (Oatway et al., 2004; Vera-Portocarrero et al., 2007). Additionally, *in vitro* studies in NG 108-15 cells report a lower binding affinity for dolasetron versus ondansetron (pK_i 7.60 versus 8.39) (Wong et al., 1995). Due to the lower inhibitor antagonist potency of dolasetron versus ondansetron we used a higher intrathecal dose of dolasetron for this study (Boeijinga et al., 1992; Thompson and Lummis, 2007).

4.3 Behavioral analysis

In all instances the person conducting the behavior was blinded to the dose and treatment group. Paw withdrawal thresholds (PWT) to mechanical stimuli were determined using von Frey filaments (Chaplan et al., 1994) and the Randall–Selitto paw pressure device (Randall and Selitto, 1957). The different behavioral tests were performed on separate groups of rats. We used calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied to the plantar surface of the paw to measure hypersensitivity to light touch (innocuous mechanical stimuli) following SNL. Filaments were applied to the bending point for 6 s, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up–down statistical method (Taguchi et al., 2005). We also examined withdrawal threshold to paw pressure (Randall–Selitto test) as this testing paradigm allows assessment of hypersensitivity to noxious mechanical stimuli as well as hyposensitivity. The other distinguishing characteristic of these behavioral assays is that von Frey filaments engage predominantly cutaneous afferents while Randall Selitto paw pressure test engages cutaneous and deep tissue afferent fibers (Taguchi et al., 2005) allowing us to discriminate intensity and neuronal population dependent effects of 5-HT₃ receptor blockade. The Analgesy-meter (Ugo Basile, Italy) uses a Teflon plinth to apply a constant rate of increasing pressure (16 g/s) to the hind paws. The cut-off pressure was set at 250 g. For the Randall–Selitto test, animals were first subjected to four training sessions 10–12 days post-SNL and 2–3 days immediately prior to drug treatment to stabilize baseline responses. No such training was used for von Frey filament testing, although rats were acclimated to the testing apparatus at least 30 min prior to testing.

Thermal testing was performed after the rat had been placed in a clear plastic box on a glass surface maintained at 30°C. A calibrated radiant heat source was focused on the hind paw, and the latency to withdrawal was recorded, using a 30-s maximum exposure to avoid tissue injury. Withdrawal latency was measured two times in the ipsilateral and contralateral foot in the middle of the footpad. These two observations were averaged for each animal. Thermal withdrawal latencies were determined between 14 and 30 days post surgery at 30, 60, 90 and 120 minutes after drug administration. Withdrawal latency to thermal stimulation is reported as mean + SEM.

4.4 Tissue preparation for Immunohistochemistry

Fourteen days following L5/6 SNL, rats were anesthetized with sodium pentobarbital (i.p.; 100 mg/kg), the thorax was opened, and fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) was perfused through the left ventricle with a peristaltic pump (20 ml/min). Rats used for immunohistochemical studies did not receive indwelling intrathecal catheters. The spinal cord (L4-L6) and brain were removed, immersed in fixative for 12 hours at 4°C and followed by immersion in 30% sucrose at 4°C for cryoprotection until ready to be sectioned. In order to examine density of serotonergic nerve terminals in the spinal cord, cross sections (40µm) were cut on a cryostat. Every 4th section was incubated over night at 4°C with primary antibody for 5HT (rabbit anti-rat 5-HT, Immunostar, Minneapolis, MN) or serotonin transporter (rabbit anti-rat 5-HTT, 1:1000, Immunostar). To visualize the distribution of 5-HT3R within the spinal cord, we used a rabbit anti-rat 5-HT3 receptor antibody (5-HT3R, Oncogene). Antibodies were diluted in a solution consisting of phosphate buffered saline (PBS, pH 7.4) containing 1% normal donkey serum (NDS) and 0.1% Triton X-100. Sections were washed in 0.1 M PBS solution and incubated rabbit biotinylated secondary antisera (1:500, Jackson ImmunoResearch) for 2 hours at room temperature. The Elite Vectastain ABC kit was used to link the antigen-antibody complex to horseradish peroxidase (HRP; ABC Elite, Vector), which was then visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) histochemistry. Finally, the sections were washed thoroughly in PBS, mounted on plus-slides, air-dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX.

4.5 Image analysis

Sections were examined with brightfield illumination and images were captured with a CCD digital camera attached to the microscope using a 10X objective at a resolution of 1,600 × 1,200 pixels. Images of ipsilateral and contralateral L4 and L5/6 dorsal horn of normal and SNL rats were captured. We examined neurochemistry at the level of L5/6 and L4 corresponding to projection sites of injured neurons or denervated regions of the spinal cord versus projections of adjacent uninjured neurons. A square with a fixed area covering a region of laminae I-II for 5-HT3 receptor (650 µm²) and laminae III-IV for 5-HTT (350 µm²) was randomly positioned in the dorsal horn of the spinal cord sections. In order to conduct regional analysis of the spinal cord, digital templates corresponding to L4 and L5 levels from the electronic version of the rat Paxinos atlas were superimposed on digital spinal cord images of 5-HTT and 5-HT3 receptor immunoreactivity to verify that sampling regions fell within designated boundaries of Rexed laminae (Molander et al., 1984). The number of pixels occupied by IR axons within a defined threshold was measured using image analysis software (SigmaScan, Jandel Scientific Inc., San Rafael, CA, USA). Immunodensity measurements were obtained from a minimum of 5 spinal cord sections/rat and averaged.

4.6 Serotonin content

The ipsilateral and contralateral dorsal lumbar spinal cord s (L4-6) were dissected out, weighed, and then kept at -80°C. Each spinal cord was homogenized in 500 µl of 0.2 M perchloric acid containing 0.1 mM Na₂-EDTA and isoproterenol (0.02 mg/ml) as an internal standard, and centrifuged at 16,350 g at 4°C for 15 min. The supernatants were kept at pH 3.0 by adding 1 M sodium acetate, and filtered through 0.2-µm syringe filter (Millipore Co., Billerica, MA). The contents of serotonin (5-HT), and 5-hydroxyindolacetic acid (5-HIAA) were measured using reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection. 0.1 M phosphate buffer (pH 6.0) containing 50 mg/l Na₂-EDTA, 500 mg/l sodium 1-octanesulphate acid and 17% methanol was used as the mobile phase. Samples (10 µl) were injected into the reverse-phase column (EICOMPAK CA-50DS 2.1F 150 mm, Eicom, Kyoto, Japan), and the flow rate and column temperature

were kept at 0.5 ml/min and 25 C, respectively. The HPLC system consisting of an Eicom 500 series with a graphite electrode (WE-3G, Eicom) at a voltage setting of ± 0.45 V vs. an Ag/AgCl reference electrode was used for measurement of monoamine contents. The detection limit of the assay is under 30 fg per injection (information from Eicom Co.).

4.7 Statistical analysis

Statistical analysis was conducted using Sigmaplot (Version 11.0, Systat software Inc, San Jose, CA) software. Mechanical withdrawal thresholds were analyzed using nonparametric analyses. Friedman's repeated measures analysis of variance on ranks and Kruskal-Wallis analysis of variance on ranks was conducted for within treatment group and within timepoint comparisons, respectively. Multiple comparisons following Friedman's or the Kruskal-Wallis test were performed using Dunnett's or Dunn's test, respectively. Thermal withdrawal latencies and paw pressure thresholds were analyzed using parametric statistics by Two-way repeated measures ANOVA followed by Bonferroni's pairwise comparisons. Immunocytochemical and biochemical data were analyzed using a Student's t-test for parametric results and Mann Whitney Rank Sum test for nonparametric results where appropriate. The criterion for statistical significance was $P < 0.05$.

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Abbreviations

WDR	wide dynamic range
SNL	spinal nerve ligation
RVM	rostral ventromedial medulla
GABA	gamma amino butyric acid
PAG	periaqueductal gray

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Figure 1.

Spinal blockade of 5-HT₃ receptors with ondansetron does not reverse mechanical hypersensitivity following L5/6 spinal nerve ligation (SNL) in rats. Rats developed mechanical hypersensitivity following SNL as assessed using withdrawal responses to tactile stimulation with von Frey filaments (A) and paw pressure stimuli using Randall Selitto device (B). Rats received acute intrathecal administration of saline (n=12), morphine (1 µg, n=7), or ondansetron (1, 3, 10, 30, 100 µg; n=5-8) between 14 and 30 days post SNL. (C) The ability of spinal ondansetron (30 µg, n=10) to reverse mechanical hypersensitivity in SNL rats was verified in an independent laboratory. Intrathecal gabapentin (200 µg, n=10) and vehicle (n=10) were administered as controls. All values represent the mean $\pm$ SEM. # $P < 0.05$ vs. post SNL baseline within each treatment group * $P < 0.05$ vs. saline within each timepoint.

Figure 2.

Spinal blockade of 5-HT₃ receptors does not reverse thermal hypersensitivity following L5/6 spinal nerve ligation (SNL) in rats. Rats were administered acute intrathecal ondansetron, morphine, or vehicle between 14 and 30 days following SNL. Paw withdrawal latency to radiant heat was determined in ipsilateral (A) and contralateral paws (B). All values represent the mean $\pm$ SEM for 7-12 rats. # $P < 0.05$ vs. post SNL baseline within each treatment group. * $P < 0.05$ vs. saline control within each time point.

Figure 3.

Spinal nerve ligation (SNL) does not alter the density of serotonergic nerve fibers in the spinal cord. Photomicrographs of serotonin transporter immunoreactive (5-HTT-IR) axons at the L4 (right panels) and L5 (left panels) spinal dorsal horn of a normal rat (A,B) or 14 days after spinal nerve ligation, ipsilateral to injury (C,D). Numerous 5-HTT-IR axons are observed throughout the dorsal horn, particularly in laminae I-IIo and laminae III-V. High power photomicrographs depicting 5-HTT-IR in axonal varicosities in spinal cord dorsal horn of normal (inset b) and SNL (inset d) rats. Scale bar = 100 μ m; b,d = 25 μ m.

Figure 4.

Spinal nerve ligation (SNL) does not alter the density or distribution of 5-HT3 receptor in the spinal cord. Photomicrographs of 5-HT3 receptor immunoreactive (5-HT3R-IR) nerve fibers at the L4 (right panels) and L5 (left panels) spinal dorsal horn of a normal rat (A,B) or 14 days after spinal nerve ligation, ipsilateral to injury (C,D). Note the spinal levels of 5-HT3R-IR are predominantly present in superficial aspects of the spinal cord and possess similar distribution in normal and SNL rats. Scale bar = 100 μ m

Table 1

Effect of spinal nerve ligation on 5-HTT density in the dorsal horn (laminae III-V) 14 days after L5/6 SNL

	Normal (n=5)	SNL - ipsilateral (n=5)	SNL - contralateral (n=5)
L4	35.4 ± 2.6	24.7 ± 6.8	24.3 ± 3.8
L5/6	33.0 ± 1.8	26.6 ± 4.7	25.3 ± 4.3

Values are means ± SEM of pixels/1000 in a fixed area of 350 μm^2 .

Table 2 Effect of spinal nerve ligation on 5-HT_{3R} -IR in the dorsal horn (laminae I-II) 14 days after L5/6 SNL

	Normal (n=6)	SNL - ipsilateral (n=6)	SNL - contralateral (n=6)
L4	84.9 ± 3.5	84.3 ± 10.2	78.1 ± 7.4
L5/6	84.1 ± 8.2	73.8. ± 4.6	88.3 ± 7.7

Values are means ± SEM of pixels/1000 in a fixed area of 650 μm^2 .