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Peripheral nerve injury alters spinal nicotinic acetylcholine receptor pharmacology

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Abstract

Nicotinic acetylcholine receptors are widely expressed in the rat spinal cord and modulate innocuous and nociceptive transmission. The present studies were designed to investigate the plasticity of spinal nicotinic acetylcholine receptors modulating mechanosensitive information following spinal nerve ligation. A tonic inhibitory cholinergic tone mediated by dihydro- β -erythroidine- (DH β E) and methyllycaconitine- (MLA) sensitive nicotinic acetylcholine receptors was identified in the normal rat spinal cord and cholinergic tone at both populations of nicotinic acetylcholine receptors was lost ipsilateral to spinal nerve ligation. The administration of intrathecal nicotinic acetylcholine receptor agonists reduced mechanical paw pressure thresholds with a potency of epibatidine = A-85380 >> nicotine > choline in the normal rat. Following spinal nerve ligation, intrathecal epibatidine and nicotine produced an ipsilateral antinociception, but intrathecal A-85380 and choline did not. The antinociceptive response to intrathecal nicotine was blocked with the $\alpha 7^*$ and $\alpha 9\alpha 10^*$ -selective nicotinic acetylcholine receptor antagonist, MLA, and the $\alpha \beta$ heteromeric nicotinic acetylcholine receptor antagonist, DH β E. The antinociceptive effects of both intrathecal nicotine and epibatidine were mediated by GABA_A receptors. Spinal [3 H]epibatidine saturation binding was unchanged in spinal nerve-ligated rats, but spinal nerve ligation did increase the ability of nicotine to displace [3 H]epibatidine from spinal cord membranes. Spinal nerve ligation altered the expression of nicotinic acetylcholine receptor subunits ipsilaterally, with a large increase in the modulatory $\alpha 5$ subunit. Taken together these results suggest that pro- and antinociceptive populations of spinal nicotinic acetylcholine receptors modulate the transmission of mechanosensitive information and that spinal nerve ligation-induced changes in spinal nicotinic acetylcholine receptors likely result from a change in subunit composition rather than overt loss of nicotinic acetylcholine receptor subtypes.

Keywords

neuropathic pain; antinociception; nicotine; nociception

1. Introduction

A preponderance of behavioral evidence supports the importance of spinal nicotinic acetylcholine receptors in the transmission of nociceptive stimuli. Intrathecal administration of nicotinic acetylcholine receptor agonists increases blood pressure and heart rate, and

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produces agitation, nociceptive behaviors (e.g., vocalizations), and antinociception to thermal stimuli (Khan et al., 1994b;Khan et al., 1994a;Khan et al., 1997). Careful pharmacological studies conducted over the past several years have shown that different nicotinic acetylcholine receptor subtypes, located on distinct spinal structures, are responsible for each of these responses (Khan et al., 2001;Khan et al., 2004;Rashid and Ueda, 2002). Importantly, the nociceptive and antinociceptive responses can be attributed to different nicotinic acetylcholine receptor subtypes (Rueter et al., 2000).

In the rat central nervous system, eight α ($\alpha 2$ - $\alpha 7$, $\alpha 9$ - $\alpha 10$) and three β ($\beta 2$ - $\beta 4$) subunits have been identified (Le Novère et al., 2002;Léna et al., 1999;Lips et al., 2002). Heterologous expression and knockout experiments have identified numerous heteropentameric combinations as well as some homopentameric combinations ($\alpha 7$, $\alpha 9$) of these subunits. The most prevalent nicotinic acetylcholine receptors in rat brain are the $\alpha 4\beta 2^*$ and $\alpha 7^*$ receptors (the asterisk indicates the native subunit composition is unknown (Lukas et al., 1999;Marks et al., 1986). In contrast, spinal cord nicotinic acetylcholine receptors have been far less characterized. Radioligand binding studies support the presence of several distinct populations of nicotinic acetylcholine receptor subtypes in the spinal cord (Khan et al., 1997;Khan et al., 1994b).

In the spinal cord, nicotinic acetylcholine receptors are expressed on primary afferents (Genzen and McGehee, 2003;Miao et al., 2004;Roberts et al., 1995;Khan et al., 2004;Li et al., 1998), descending noradrenergic (Li et al., 2000) and serotonergic (Cordero-Erausquin and Changeux, 2001) fibers presynaptically, as well as postsynaptically on spinal inhibitory and excitatory neurons (Cordero-Erausquin et al., 2004;Genzen and McGehee, 2005;Bradaia and Trouslard, 2002b;Bradaia and Trouslard, 2002a). Previous studies suggest that the $\alpha 4\beta 2^*$ and $\alpha 7^*$ nicotinic acetylcholine receptors on primary afferent C-fibers are likely responsible for the nociceptive responses while an $\alpha 3\beta 4^*$ or a previously undescribed nicotinic acetylcholine receptor may be responsible for the antinociceptive properties (Khan et al., 2001;Rueter et al., 2000).

Peripheral nerve injury produces a variety of changes within the spinal cord both ipsilaterally and contralaterally, including changes in the expression of nicotinic acetylcholine receptors (Yang et al., 2004). Transection of the sciatic nerve greatly upregulates transcripts for the $\alpha 5$ and $\beta 2$ nicotinic acetylcholine receptor subunits within the spinal cord dorsal horn (Yang et al., 2004). Spinal nerve ligation also increased the numbers of cells expressing the $\alpha 3$ subunit and the number of fibers expressing the $\alpha 5$ subunit (Vincler and Eisenach, 2004). The behavioral implications of injury-induced changes in spinal nicotinic acetylcholine receptors have not been investigated thoroughly. Partial sciatic nerve injury in the mouse results in an increased spinal antinociceptive potency of nicotinic agonists and a loss of cholinergic-stimulated GABAergic inhibitory tone at $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors (Rashid and Ueda, 2002;Rashid et al., 2006). In the rat, tibial nerve transection results in a novel, antinociceptive effect of spinal nicotinic acetylcholine receptor agonists by increasing spinal glycinergic transmission (Abdin et al., 2006).

The current series of studies were undertaken to further examine the role of spinal nicotinic acetylcholine receptors modulating the transmission of nociceptive mechanical stimuli and to define injury-induced changes in nicotinic acetylcholine receptor function that may underlie changes in spinal nicotinic acetylcholine receptor pharmacology.

2. Materials and Methods

2.1. Animals

All animals used in this study were male Sprague-Dawley rats (200–250g; Harlan, IN), housed in pairs prior to surgery and individually post-catheter implantation with free access to food and water. Protocols and procedures were approved by the Animal Care and Use Committee (Wake Forest University Health Sciences, Winston-Salem, NC).

Surgical preparations

Intrathecal catheter implantation: Lumbosacral intrathecal catheters were implanted as described previously (Storkson et al., 1996), with slight modifications (Milligan et al., 1999). Catheters consisted of PE-10 tubing stretched to reduce the overall diameter. Briefly, under halothane anesthesia, an incision was made in the skin of the lower back and a sterile 20 G needle was used as a guide cannula and was inserted between the L5 and L6 vertebrae. A tail flick confirmed entry into the intrathecal space. The stretched PE10 catheter containing a guide wire was gently fed through the needle until the catheter extended 3 cm beyond the tip of the needle to reach the lumbar enlargement. The needle and guide wire were gently removed. A loosely tied knot was made in the catheter and three sutures were used to hold the catheter in place. A small fistula (a modified 1cc syringe hub (Milligan et al., 1999)) was sutured to the muscle surface and the catheter was fed through the fistula. The remaining externalized catheter was coiled into the fistula and the rubber plug sealed with a small amount of silicon sealant. The dead space of the catheter ranged from 7–10 μ l and, therefore, all drug administrations were followed by a 10 μ l saline flush.

Spinal nerve ligation: Rats underwent spinal nerve ligation as described previously (Kim and Chung, 1992). Under halothane anesthesia (2–3% halothane in 100% oxygen), the left L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with 6.0 silk suture. The incision was closed and the animals returned to their home cages for 12–14 days post-ligation to allow for the development of mechanical allodynia.

2.2. Behavioral Testing

All behavioral testing was conducted 12–14 days post-surgery between the hours of 9:00 AM and 4:00 PM. Paw withdrawal thresholds were determined for left and right hind paws using the Randall-Selitto paw pressure technique (Randall and Selitto, 1957). The Analgesy-meter (Ugo Basile, Italy) uses a conical Teflon applicator to apply a constant rate of increasing pressure (16g per second) to the hind paws. The cut-off pressure was set at 250g. Prior to experimental testing, animals were first subjected to 4 training sessions prior to spinal nerve ligation to stabilize baseline responses (Taiwo et al., 1989). Each hind paw was tested 2 times with a 5 minute intertrial interval. In spinal nerve-ligated rats, the mean paw withdrawal thresholds for the ipsilateral and contralateral hind paws was compared to determine the presence of mechanical hypersensitivity. Mechanical hypersensitivity was defined as the presence of at least a 40% decrease in paw withdrawal thresholds for the ipsilateral hind paw.

For pharmacological testing, all drugs were dissolved in sterile 0.9% saline and administered intrathecally in a volume of 10 μ l. Paw withdrawal thresholds were measured at 5, 10, 15, 30, and 60 minutes following the intrathecal administration of agonists and antagonists. When administered in combination, the antagonists were administered 10 minutes prior to agonists. Data are expressed as the mean paw withdrawal thresholds $\pm$ S.E.M. in grams or as a percentage of pre-drug baseline responses (% Baseline = Post-drug paw withdrawal threshold/Pre-drug paw withdrawal threshold $\times$ 100).

2.3. Radioligand Binding

Spinal cord tissue preparation—The dorsal half (normal rats) or ipsilateral dorsal quadrant (spinal nerve-ligated rats) of the L4-L6 spinal cord tissue was placed in 10 volumes (w/v) of ice-cold hypotonic buffer (14.4 mM NaCl, 0.2 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgSO₄, 2.0 mM HEPES, pH 7.5) and homogenized using a Kinematica polytron. Homogenized samples were centrifuged at 25,000g for 15 minutes. The pellet was resuspended in hypotonic buffer and again centrifuged. The resuspension/centrifugation cycle was repeated two more times. The resulting pellet was stored frozen at –80°C under fresh hypotonic buffer until ready for use.

Radioligand Binding—At the time of assay, the pellet was thawed and resuspended with Tris-HCl buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, pH 7.5) supplemented with 0.1 mM PMSF and 5 mM iodoacetamide. The assay mixture consisted of 200 µg of membrane protein in a final incubation volume of 60 µl. Incubations were carried out in a cold room on a gentle shaker for 60 minutes. Assays were initiated with the addition of the membrane suspension with rapid mixing to polypropylene tubes containing tritiated ligands. Incubations were terminated by the addition of 3 ml of ice-cold assay buffer followed by rapid filtration through Whatman GF/B filter papers previously equilibrated with 0.5% polyethyleneimine at 4°C using a Brandel cell harvester. Samples were then washed four times with 4 ml of ice-cold assay buffer (Tris-HCl buffer supplemented with 10 µM atropine sulfate). The filters were then placed in counting vials, mixed vigorously with scintillation fluid and counted the next day in a Beckman Coulter LS 6500 liquid scintillation counter. All assays were done in triplicate and protein was assayed by the Bradford protein assay.

For saturation binding, final concentrations of (±)-[³H]epibatidine (Perkin-Elmer, USA) varied between 0.02 nM to 4.3 nM; stock solutions are prepared in assay buffer. Nonspecific binding is determined by including 40 µM nicotine. For competition binding, a concentration of 0.8 nM (±)-[³H]epibatidine was used with various concentrations of nicotine or cytosine.

2.4. Western Blotting

The lower lumbar (L4-L6) spinal cord was removed and the dorsal half (normal rats) or ipsilateral dorsal quadrants (spinal nerve ligated rats) were homogenized in lysis buffer (Mammalian Cell Lysis Kit, Sigma). Protein concentrations were quantified using a Bio-Rad Protein Assay Kit, aliquoted (50 µg), and stored at –80° C until use. Proteins were separated using SDS-PAGE electrophoresis in a 10% acrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. Membranes were blocked with 5% dehydrated milk and incubated with primary antibody generated against the α4 (rabbit polyclonal, Santa Cruz Biotechnology; 1:500), α5 (goat polyclonal, Santa Cruz Biotechnology, Inc.; 1:100), α7 (goat polyclonal, Santa Cruz Biotechnology; 1:500), and β2 (rabbit polyclonal, Santa Cruz Biotechnology; 1:100) nicotinic acetylcholine receptor subunits overnight at 4° C. Membranes were washed with 0.01 M PBS and incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. Protein bands were visualized with a chemiluminescence detection system (Amersham) and exposed to autoradiography film. Membranes were then stripped using Reblot Western Blot Recycling Kit (Millipore) and re-probed with an antibody to β-actin (Cell Signaling; 1:1000). β-Actin was visualized as described above. Protein band density was measured using SigmaScan Pro 5.

2.5. Materials

Nicotine hydrate tartrate salt, (±)-epibatidine dihydrochloride, 3-(2(S)-Azetidinylmethoxy) pyridine HCl (A-85380), choline chloride, (–)-bicuculline methiodide, dihydro-β-erythroidine, methyllycaconitine, and all chemical components of buffers were purchased from Sigma. [³H]-Epibatidine was purchased from Perkin-Elmer.

2.6. Statistics

Behavioral pharmacology was analyzed using one-way or two-way repeated measures ANOVA where indicated. Radioligand binding curves were calculated by nonlinear regression and were compared between groups using the four parameter Hill equation in Prism 4 (GraphPad).

3. Results

3.1. Pro- and antinociceptive populations of spinal nicotinic acetylcholine receptors modulate the transmission of nociceptive mechanical stimuli

To assess the functionality of spinal nicotinic receptors in the modulation of mechanosensitive stimuli, normal rats were administered 2 commonly used nicotinic acetylcholine receptor antagonists intrathecally and paw withdrawal thresholds to mechanical pressure were measured. Baseline paw withdrawal thresholds ranged from 135 to 149 g and data were normalized to baseline paw withdrawal thresholds for each rat prior to drug administration. Both nicotinic acetylcholine receptor antagonists tested dose-dependently reduced paw withdrawal thresholds (Fig. 1A). Dihydro- β -erythroidine (DH β E), which functionally blocks $\alpha\beta$ heteromeric nicotinic acetylcholine receptors with some preference for the $\alpha 4\beta 2$ subtype, dose-dependently reduced paw withdrawal thresholds following the administration of 2, 10, 28, or 140 nmol [$F(4,28)=8.4$, $P < 0.001$]. Intrathecal methyllycaconitine (MLA), which preferentially blocks $\alpha 7$ and $\alpha 9\alpha 10$ nicotinic acetylcholine receptors, dose-dependently reduced paw withdrawal thresholds following the administration of 1, 11, or 57 nmol [$F(3,20)=14$, $P < 0.0001$]. The greatest reductions in paw withdrawal thresholds following DH β E and MLA was observed at 15 min with a complete return to baseline levels at 60 min (data shown in Figs. 2C and 2D).

Intrathecal administration of nicotinic acetylcholine receptor agonists also reduced paw withdrawal thresholds to mechanical pressure in normal rats with a rank order potency of A-85380 = epibatidine $\gg$ nicotine $>$ choline (Fig. 1B). The reduction of paw withdrawal thresholds was dose-dependent for each agonist. A-85380, which is selective for $\beta 2$ -containing nicotinic receptors, significantly reduced paw withdrawal thresholds [$F(3,32)=17.5$, $P < 0.001$]. Intrathecal administration of epibatidine [$F(6,43)=4.1$, $P < 0.005$] and nicotine [$F(4,25)=6.2$, $P < 0.005$], which are potent agonists at heteromeric nicotinic acetylcholine receptors, also produced dose-dependent reductions in nociceptive thresholds. Choline, an $\alpha 7^*$ selective nicotinic receptor agonist, also produced significant mechanical hypersensitivity [$F(3,17)=6.6$, $P < 0.01$]. The peak effect of nicotinic acetylcholine receptor agonists ranged from 5–15 min with significant hypersensitivity remaining at 45 min at the highest doses administered (data not shown).

3.2. Spinal nerve ligation alters nicotinic acetylcholine receptor pharmacology

Rats underwent spinal nerve ligation and mechanical hypersensitivity developed 14 days post-ligation. Paw withdrawal thresholds decreased from 143 ± 5 g at baseline to 73 ± 4 g in the ipsilateral hind paw 14 days post-spinal nerve ligation. No change in baseline paw withdrawal thresholds was observed on the contralateral side. Intrathecal epibatidine significantly increased paw withdrawal thresholds on the ipsilateral hind paw at 0.036 pmol and 0.36 pmol while these same doses produced no or pro-nociceptive effects, respectively, on the contralateral side (Fig. 2A). At a higher dose of epibatidine (360 pmol), however, a pro-nociceptive effect was observed bilaterally. Similar to epibatidine, intrathecal nicotine produced antinociceptive effects ipsilateral to spinal nerve ligation at lower doses (2.2 and 6.5 nmol) while these same doses produced no significant effect on the contralateral side. This antinociceptive effect could be overcome at higher doses, with 22 nmol reducing paw withdrawal thresholds equally on the ipsilateral and contralateral sides. Conversely, the $\alpha 7$ and

$\alpha 9\alpha 10$ nicotinic acetylcholine receptor agonist, choline, did not produce antinociception. Intrathecal choline produced either no effect or reduced paw withdrawal thresholds equally in the ipsilateral and contralateral hind paws. Similarly, intrathecal A-85380 failed to produce antinociception in spinal nerve-ligated rats (Fig. 2B), although the pharmacology was modified compared to normal rats.

Spinal nerve ligation produced significant mechanical hypersensitivity ipsilateral to ligation with mean paw withdrawal thresholds ranging from 84 – 95 g 14 days post-ligation (Figs. 2C and 2D). The intrathecal administration of DH β E (10 nmol) to spinal nerve-ligated rats significantly reduced paw withdrawal thresholds on the hindpaw contralateral to ligation [$F(5,47)=3.7$, $P < 0.01$] but had no effect on the ipsilateral hind paw (Fig. 2C). MLA (11 nmol) produced a similar effect when administered intrathecally to spinal nerve-ligated rats (Fig. 2D), significantly reducing paw withdrawal thresholds contralateral to spinal nerve ligation [$F(5,22)=2.9$, $P < 0.05$], but not ipsilaterally.

3.3. Spinal nicotinic acetylcholine receptor agonist antinociception is mediated by GABA

The antinociceptive effects of intrathecal epibatidine (0.36 pmol) and nicotine (2.2 nmol) ipsilateral to spinal nerve ligation were antagonized by intrathecal administration of the GABA_A receptor antagonist bicuculline (Fig. 3A). Although bicuculline has been reported to produce behavioral hypersensitivity following intrathecal administration previously, this dose of bicuculline (0.3 μ g/10 μ l) did not alter paw withdrawal thresholds in normal or spinal nerve-ligated rats.

To determine which populations of nicotinic acetylcholine receptors contribute to the antinociceptive effects of intrathecal nicotine on the ipsilateral hind paw, nicotinic acetylcholine receptor antagonists were administered intrathecally 10 mins prior to intrathecal nicotine. Figure 3B shows the antinociceptive effect of 2.2 nmol intrathecal nicotine ipsilateral to spinal nerve ligation. Similar to the rats in Figure 2, intrathecal MLA (11 nmol.) and DH β E (10 nmol) do not alter paw withdrawal thresholds ipsilateral to SNL 15 minutes post-administration (Fig. 3B, white bars). Pretreatment with MLA and DH β E, however, completely blocked the antinociceptive effect of intrathecal nicotine (Fig. 3B, gray bars).

3.4. Spinal nerve ligation-induced changes in epibatidine- and nicotine-sensitive nicotinic acetylcholine receptors in the rat spinal cord

Because spinal nerve ligation reduced the pronociceptive responses of intrathecal epibatidine and nicotine, we investigated whether changes in epibatidine and nicotine binding sites in the ipsilateral dorsal horn could underlie these effects. Saturation radioligand binding of [³H] epibatidine was performed in spinal cord membranes from the dorsal half (normal rats) or ipsilateral dorsal quadrant (spinal nerve-ligated rats) of the lower lumbar (L4-L6) spinal cord. Epibatidine binding was saturable in normal and spinal nerve ligation spinal cord membranes with 2 binding sites being observed (Fig. 4A and Table 1). Apparent K_D values of 0.079 ± 0.05 nM and 1.74 ± 0.1 nM for the higher and lower affinity sites in the normal rat, respectively, were calculated by nonlinear regression. Spinal nerve ligation did not alter [³H]epibatidine binding [$F(4,52)=1.17$, $P > 0.05$] or the percentage of [³H]epibatidine binding sites in the higher affinity population (Table 1).

Competition radioligand binding of [³H]epibatidine (0.8 nM) by nicotine was performed to identify spinal nerve ligation-induced changes in nicotine-sensitive nicotinic acetylcholine receptor populations. Nicotine bound 2 sites rat spinal cord membranes of normal rats with IC_{50} s of 16.2 ± 1.5 and 531.6 ± 1.4 for the higher and lower affinity sites, respectively (Fig. 4B and Table 2). Spinal nerve ligation significantly altered nicotine binding [$F(1,68)=7.2$, $P < 0.0001$], significantly reducing the IC_{50} s of both the higher and lower affinity sites (Table 2).

3.5. Spinal nerve ligation-induced changes in spinal nicotinic acetylcholine receptor subunit expression

Changes in the expression of individual nicotinic acetylcholine receptor subunit proteins by Western blotting following spinal nerve ligation has not been reported previously. Spinal nerve ligation significantly altered the expression of $\alpha 4$, $\alpha 5$, and $\beta 2$ nicotinic acetylcholine receptor subunits, but had no effect on the expression of the $\alpha 7$ subunit when compared to levels of expression in normal rat spinal cord (Fig. 5). The expression of the $\alpha 4$ subunit was significantly decreased ipsilateral to spinal nerve ligation, while the expression of the $\alpha 5$ and $\beta 2$ subunits was significantly increased.

4. Discussion

The results of these studies show that multiple populations of spinal nicotinic acetylcholine receptors function to facilitate and inhibit the transmission of nociceptive mechanical stimuli in the normal rat spinal cord. A variety of nicotinic acetylcholine receptor agonists reduced paw withdrawal thresholds in a dose-dependent manner while the nicotinic acetylcholine receptor antagonists DH β E and MLA blocked a tonic cholinergic antinociceptive tone within the normal rat spinal cord. Spinal nerve ligation differentially altered nicotinic acetylcholine receptor pharmacology resulting in the appearance of a novel unilateral antinociceptive effect of intrathecal epibatidine and nicotine, but not A-85380 or choline, and the loss of apparent cholinergic tone at DH β E- and MLA-sensitive nicotinic acetylcholine receptors. The antinociceptive effects of intrathecal epibatidine and nicotine could be blocked with the GABA_A receptor antagonist, bicuculline, supporting a role for nicotinic acetylcholine receptor-evoked GABA release in SNL rats. The antinociceptive effects of intrathecal nicotine in spinal nerve-ligated rats were antagonized by both DH β E and MLA, nicotinic acetylcholine receptor subtypes that were identified as antinociceptive in normal rats. This suggests that although the inhibitory cholinergic tone is lost ipsilateral to SNL, the receptors themselves are still present. These spinal nerve ligation-induced changes in behavioral pharmacology could not be explained by changes in the number of [³H]epibatidine binding sites or changes in affinity. However, nicotine-sensitive sites were altered by spinal nerve ligation. The underlying mechanism of this spinal nerve ligation-induced change in nicotine affinity may be the large upregulation of the modulatory $\alpha 5$ nicotinic acetylcholine receptor subunit.

Our studies show the presence of an inhibitory cholinergic tone in the spinal cord of the normal rat, similar to the cholinergic regulation of thermal stimuli reported in the mouse cord (Rashid and Ueda, 2002; Rashid et al., 2006). In the mouse spinal cord, this tone is mediated by $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors, but not $\alpha 7^*$ nicotinic acetylcholine receptors (Rashid et al., 2006). Similar to these observations, the intrathecal administration of DH β E in the current studies dose-dependently produced mechanical hypersensitivity suggesting the presence of a population of $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors that function to inhibit the transmission of noxious mechanical stimuli. However, DH β E is, at best, only moderately selective for $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors and the concentration of this antagonist at spinal $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors following intrathecal administration is unknown. The selective nature of DH β E for $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors in this model is unclear, and, therefore, we limit our interpretation of the effects of DH β E to that of an $\alpha\beta$ heteromeric nicotinic acetylcholine receptor. In addition to the effects of DH β E, we observed pronociceptive effects of MLA at doses that produced no effect in the mouse spinal cord (Rashid et al., 2006). Differences in spinal nicotinic acetylcholine receptor pharmacology between rats and mice have been noted previously (Damaj et al., 2000; Khan et al., 2001).

The intrathecal administration of nicotinic acetylcholine receptor agonists in the normal rat spinal cord has been reported previously to produce a short-lived thermal antinociception and a more prolonged touch-evoked hypersensitivity (Khan et al., 2001). Our results demonstrate

that the intrathecal administration of nicotine, epibatidine, A-85380, and choline only reduced paw withdrawal thresholds to mechanical pressure with the expected potencies. Previous studies have shown that the nocifensive behaviors (e.g., vocalization) of intrathecal nicotinic acetylcholine receptor agonists are mediated via glutamate release following the direct activation of nicotinic acetylcholine receptors on primary afferent C-fibers (Khan et al., 1998; Khan et al., 1996). However, mechanical stimulation is transmitted to the spinal cord by A δ and A β fibers which express nicotinic acetylcholine receptor subtypes distinct to those localized on C-fibers (Rau et al., 2004). The dependence of the pro-nociceptive effect of intrathecal nicotinic acetylcholine receptor agonists on mechanical paw withdrawal thresholds on the release of glutamate was not examined in our studies.

Peripheral nerve injury altered spinal endogenous inhibitory tone with an apparent loss of cholinergic tone at $\alpha\beta$ heteromeric (DH β E-sensitive) and $\alpha 7^*$ or $\alpha 9\alpha 10^*$ (MLA-sensitive) nicotinic acetylcholine receptors. The antinociceptive populations of nicotinic acetylcholine receptors stimulated by intrathecal nicotinic agonists following spinal nerve ligation appear to be comprised of the same antinociceptive nicotinic acetylcholine receptors under tonic cholinergic regulation in the normal rat spinal cord. Nicotine-induced antinociception ipsilateral to spinal nerve ligation was mediated by a population of $\alpha\beta$ heteromers (DH β E-sensitive) and $\alpha 7^*$ or $\alpha 9\alpha 10^*$ (MLA-sensitive) nicotinic acetylcholine receptors, and was dependent upon the release of GABA. The contribution of spinal GABA correlates with previous findings in the mouse spinal cord (Rashid et al., 2006), but contrasts with the findings of Abdin et al. (2006) in the rat who reported that the neuropathy specific effects of intrathecal nicotinic agonists were dependent on glycine, but not GABA release (Abdin et al., 2006). The difference between the studies of Abdin et al. (2006) and the current study is likely due to the intensity of the mechanical stimuli used. Spinal GABA $_A$ and glycine receptors inhibit tactile allodynia (Sivilotti and Woolf, 1994; Sorkin et al., 1998), but only GABA $_A$ receptors inhibit hypersensitivity to high intensity stimuli (Sorkin et al., 1998).

Similar to our observations with nicotinic antagonists, spinal nerve ligation altered the behavioral pharmacology of nicotinic agonists. Nicotine and epibatidine produced antinociception on the hind paw ipsilateral to spinal nerve ligation but produced pronociceptive effects contralaterally. These results are similar to those of Rashid and Ueda (2002) and Abdin et al. (2006) who observed a neuropathy-specific antinociceptive effect of intrathecal nicotine and epibatidine following peripheral nerve injury. In contrast, no antinociceptive effect of intrathecal choline was observed, despite using a lower dose than Abdin et al. (2006). The previous studies by Abdin et al. (2006) measured antinociception using von Frey filaments while the current studies utilized a more intense mechanical stimulus, paw pressure. Choline is a fairly weak antinociceptive agonist, exhibiting an analgesic ED $_{50}$ to thermal stimuli of approximately 0.47 μ M and this analgesic effect is likely overcome at higher stimulus intensities such as paw pressure (Damaj et al., 2000). The failure A-85380 to elicit antinociception in spinal nerve-ligated rats is likely due to the stimulation of a distinct population of spinal nicotinic acetylcholine receptors. Epibatidine and A-85380 produce antinociception to thermal stimuli that is mediated via separable populations of spinal nicotinic acetylcholine receptors localized at different preterminal sites (Khan et al., 2001). The antinociceptive effects of intrathecal A-85380 rely on the release of norepinephrine whereas the antinociceptive effects of epibatidine are not dependent on norepinephrine release (Khan et al., 2001).

Although the loss of epibatidine binding in the rat spinal cord has been correlated previously with a reduced nociceptive response to intrathecal nicotinic acetylcholine receptor agonists, no changes in epibatidine binding were observed in spinal nerve ligation exhibiting injury-induced antinociception. This suggests that an overt loss of pronociceptive spinal nicotinic acetylcholine receptors does not account for spinal nerve ligation-induced antinociception.

This idea is further supported by our behavioral data showing that the pronociceptive effects of intrathecal nicotine and epibatidine are elicited at higher doses.

Competitive nicotine binding showed a small but significant change in affinity in spinal nerve-ligated rats; a result that is more consistent with a change in nicotinic acetylcholine receptor subunit composition rather than the up- or down-regulation of whole nicotinic acetylcholine receptor subtypes. The increased expression of the modulatory $\alpha 5$ subunit presents a possible mechanism underlying the subtle change in nicotine affinity. Inclusion of the $\alpha 5$ subunit in heteromeric nicotinic acetylcholine receptors alters the affinity of many nicotinic acetylcholine receptor agonists, although the effect logically depends upon the identities of the α and β subunits. Consistent with the current study, the presence of the $\alpha 5$ subunit does not alter epibatidine saturation binding or the affinity of epibatidine for $\alpha 3\beta 2$ or $\alpha 3\beta 4$ nicotinic acetylcholine receptors (Wang et al., 2002; Wang et al., 1996). However, the rate of epibatidine-induced desensitization is increased for both receptors when the $\alpha 5$ is included (Wang et al., 1996). The impact of $\alpha 5$ inclusion on nicotine affinity has not been reported, but inclusion of the $\alpha 5$ subunit in $\alpha 3\beta 4$ nicotinic acetylcholine receptors reduces the EC₅₀ for nicotine from 6.8 to 1.9 μ M. Behaviorally, the increased expression of $\alpha 5$ in the spinal cord contributes to the presence of mechanical hypersensitivity in SNL rats (Vincler and Eisenach, 2005). Therefore, the upregulation of the $\alpha 5$ subunit in the spinal cord of SNL rats may alter the pharmacology of endogenous ACh and exogenous nicotinic acetylcholine receptor agonists. The identification of which spinal nicotinic acetylcholine receptors include the $\alpha 5$ subunit in SNL rats will be critical to a more thorough understanding of the observed changes in behavioral pharmacology.

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Figure 1. Dose-response relationships of intrathecal nicotinic acetylcholine receptor antagonists (A) and agonists (B) on paw withdrawal threshold to mechanical pressure in the normal rat
A) The mean percent of baseline paw withdrawal thresholds $\pm$ S.E.M. following the intrathecal administration of nicotinic acetylcholine receptor antagonists dihydro- β -erythroidine (DH β E) and methyllycaconitine (MLA) are shown. **B)** The mean percent of baseline paw withdrawal thresholds $\pm$ S.E.M. following the intrathecal administration of nicotinic acetylcholine receptor agonists epibatidine, A-85380, nicotine, and choline are shown. Each data point represents the mean $\pm$ S.E.M of 8 animals.

Figure 2. Spinal nerve ligation alters the pharmacology of intrathecal nicotinic acetylcholine receptor agonists and antagonists

A) Intrathecal epibatidine (Epibat) and nicotine (Nic) showed a novel, unilateral antinociceptive response on the hind paw ipsilateral (Ipsi) to ligation at concentrations that produced no effect or pronociceptive responses contralaterally (Contra). **B)** Intrathecal administration of A-85380 and choline failed to produce antinociception in spinal nerve-ligated rats. **C)** Antinociceptive cholinergic tone is lost at spinal DH β E-sensitive nicotinic acetylcholine receptors ipsilateral (Ipsi) to spinal nerve ligated while this tone remains apparent on the contralateral (Contra) hind paw. Changes in paw withdrawal thresholds (PWTs) in grams of spinal nerve-ligated and uninjured (Normal) rats to 10 nmol intrathecal DH β E over time are shown. **D)** Antinociceptive cholinergic tone is lost at spinal MLA-sensitive nicotinic acetylcholine receptors ipsilateral (Ipsi) to spinal nerve ligation but not contralaterally (Contra). Changes in paw withdrawal thresholds (PWT) in grams (g) of spinal nerve-ligated and uninjured (Normal) rats to 11 nmol MLA over time are shown. Data are depicted as the mean percent of baseline paw withdrawal thresholds $\pm$ S.E.M. (**A** & **B**) or mean paw withdrawal thresholds $\pm$ S.E.M. (**C** & **D**) for 8–10 animals per experimental group. In **A** & **B**, significant antinociceptive (# $P < 0.05$) and pronociceptive (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$) effects are indicated. In **C** & **D**, the pronociceptive of DH β E and MLA on the contralateral hind paw in spinal nerve-ligated rats (^ $P < 0.05$, ^^ $P < 0.01$) and in normal rats (** $P < 0.01$, *** $P < 0.005$).

Figure 3. The antinociceptive effects of intrathecal nicotine and epibatidine in spinal nerve-ligated rats

A) The antinociceptive effects of intrathecal nicotine (2.2 nmol) and epibatidine (0.36 nmol) are blocked by pretreatment with the GABA_A receptor antagonist bicuculline (0.3 μg). Significant pronociceptive (* P < 0.05) and antinociceptive (# P < 0.05) effects are shown. ^ P < 0.05 compared to nicotine or epibatidine treatment alone. **B)** The antinociceptive effects of intrathecal nicotine (2.2 nmol) are blocked by pretreatment with DHβE (10 nmol) or MLA (11 nmol) in spinal nerve-ligated rats. * P < 0.05 compared to nicotine administration alone. Data are shown as the mean percent of baseline paw withdrawal thresholds ± S.E.M. for 8–10 animals per treatment group.

Figure 4. Radioligand binding of $[^3\text{H}]$ epibatidine in spinal cord membranes from normal and spinal nerve-ligated rats

A) Saturation radioligand binding of $[^3\text{H}]$ epibatidine in membranes prepared from the dorsal half (Normal) or ipsilateral dorsal quadrant (spinal nerve ligation, SNL) of the lower lumbar spinal cord. Inset is the Scatchard plot showing the presence of high and low affinity sites.

B) Competitive radioligand binding of nicotine displacement of $[^3\text{H}]$ epibatidine in membranes prepared from the dorsal half (Normal) or ipsilateral dorsal quadrant (SNL) of the lower lumbar spinal cord.

Figure 5. Spinal nerve ligation differentially alters nicotinic acetylcholine receptor subunit expression in the dorsal horn of the lower lumbar rat spinal cord

A) The expression of the $\alpha 4$ subunit is significantly decreased ($^{\wedge} P < 0.05$) ipsilateral (Ipsi) to spinal nerve ligation (SNL) compared to the levels of expression in the normal rat spinal cord. The expression of the $\alpha 5$ and $\beta 2$ subunits is significantly increased ($* P < 0.05$) compared to the expression of these subunits in the normal rat spinal cord. Data are expressed as the mean percentage of the levels of expression in normal rats (% Normal) $\pm$ S.E.M. of 4 separate experiments per nicotinic acetylcholine receptor subunit. **B)** Representative Western blots for nicotinic acetylcholine receptor subunit expression in the spinal cords of normal (Nor) rats and SNL rats ipsilateral (Ipsi) and contralateral (Contra) to ligation.

Table 1

[³H]Epibatidine binding to spinal cord membranes

	K _{D1} (nM)	B _{MAX1} (fmol/mg)	K _{D2} (nM)	B _{MAX2}	% High Affinity
Normal	0.079 ± 0.05	1.7 ± 0.6	1.74 ± 0.1	27.5 ± 4	5.8 ± 2
SNL	0.076 ± 0.01	2.6 ± 0.2	2.432 ± 0.7	33.6 ± 2	7.2 ± 1

Mean affinities (K_D) and number of binding sites (B_{MAX}) ± S.E.M. for high and low [³H]epibatidine-sensitive nAChRs in normal and spinal nerve-ligated (SNL) rats. The mean percentage of high affinity sites of total [³H]epibatidine binding sites ± S.E.M. is also shown.

Table 2Displacement of [³H]epibatidine binding to spinal cord membranes by nicotine

	IC ₅₀ (nM)		% Total Binding	
	High affinity	Low affinity	High affinity	Low affinity
Normal	16.2 ± 1.5	531.6 ± 1.4	41.59 ± 7%	58.41 ± 7%
SNL	<i>8.406 ± 1.7</i>	<i>401.8 ± 1.7</i>	43.5 ± 8%	56.5 ± 8%

Mean IC₅₀ ± S.E.M. of nicotine displacement of 0.8 nM [³H]epibatidine to spinal cord membranes from normal and spinal nerve-ligated (SNL) rats. The mean percentage ± S.E.M. of high and low affinity nicotine-sensitive sites in spinal cord membranes from normal and SNL rats. Italicized text indicates statistical significance of $P < 0.05$.