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# Knock down of the α5 nicotinic acetylcholine receptor in spinal nerve-ligated rats alleviates mechanical allodynia

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**Abstract**

Nicotinic acetylcholine receptor (nAChR) agonists are known to alleviate neuropathic and inflammatory pain via activation of a heterogeneous population of receptors. However, the function of nAChRs in the maintenance of neuropathic pain is not known. Spinal nerve ligation (SNL) increases the spinal expression of the α5 nAChR subunit ipsilateral to injury. The α5 subunit is unique because it modifies numerous characteristics of existing functional nAChRs, but it does not form functional nAChRs when expressed alone or with β nicotinic subunits. Because there are no α5 subunit selective ligands, we used antisense oligonucleotides (ODNs) to assess the contribution of the α5 subunit to the maintenance of mechanical allodynia following SNL. Intrathecal antisense oligonucleotides were administered to SNL rats after the development of mechanical allodynia (10–12 days post-SNL). I.t. antisense specifically reduced α5 immunoreactivity (α5-IR) by 50–70% in the outer laminae of the dorsal horn and moderately alleviated mechanical allodynia. Furthermore, using the phosphorylation of cAMP response element-binding protein (pCREB) as a general marker of neuronal activation, a significant increase in pCREB immunoreactivity was observed in SNL rats. Treatment of SNL rats with α5-antisense significantly reduced pCREB immunoreactivity. These results suggest that the increased expression of the α5 nAChR subunit following SNL contributes to spinal CREB phosphorylation and the maintenance of mechanical allodynia.


*Keywords:* Antisense oligonucleotides; Neuropathic pain; Peripheral nerve injury

## 1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels known to contribute to nociceptive and antinociceptive signaling within the rat spinal cord (Bannon et al., 1998; Colquhoun and Patrick, 1997; Cordero-Erausquin et al., 2000; Decker et al., 1998; Kesingland et al., 2000; Damaj et al., 1998; Lindstrom et al., 1995; Marubio et al., 1999; Yaksh and Rudy, 1976). Although a multitude of nAChR subunits exist within the rat nervous system (α2–α10 and β2–β4; Bitner and Nikkel, 2002; Dineley-Miller and Patrick, 1992; Dominguez et al., 1994; Flores et al., 1992; Flores et al., 1996; Genzen et al., 2001; Goldner et al., 1997; Hill et al., 1993; Khan et al., 1996b; Khan et al., 1997; Le Novere et al., 1996; Skok et al., 1999; Voitenko et al., 2001; Wada et al., 1989; Wada et al., 1990; Winzer-Serhan and Leslie, 1997; Zhang et al., 1998), numerous studies have shown that the α4β2* receptor is critical for antinociception at the supraspinal level (Bitner et al., 2000; Nuseir et al., 1999; Marubio et al., 1999) while a non-α4β2* receptor (Rueter et al., 2000; Khan et al., 2001) plays a more important role in the spinal cord.

Although most nicotinic subunits can be combined to form ligand-activated channels using different combinations of α and β subunits, the α5 nAChR subunit does not form functional receptors when expressed alone or with a β subunit in *Xenopus oocytes* (Ramirez-Latorre et al., 1996; Yu and Role, 1998; Gerzanich et al., 1998). Rather, the function of the α5 nAChR subunit is limited to a

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modulatory role, altering numerous characteristics of the nicotinic receptor (Girod et al., 1999; Nelson and Lindstrom, 1999; Girod et al., 2000; Wang et al., 2002). Heterologous expression of the α5 nAChR with the α4β2 increases acetylcholine-evoked macroscopic currents and increases nicotinic channel conductance (Ramirez-Latorre et al., 1996). The coexpression of α5 with either the α3β4 or α3β2 nAChR increases receptor desensitization, increases calcium permeability, and specifically increases ACh sensitivity of the α3β2 nAChR (Gerzanich et al., 1998).

Recently, we observed an increased expression of the α5 nAChR subunit in the outer laminae of the dorsal horn following spinal nerve ligation (SNL; Vincler and Eisenach, 2001). This increase in α5 immunoreactivity (α5-IR) was confined to fibers in the outer laminae (LI–LII), suggesting that it is expressed on small diameter primary afferent fibers. Functional nAChRs have been identified previously on primary afferent neurons (Gillberg and Wiksten, 1986; Gillberg and Askmark, 1991; Roberts et al., 1995; Wonnacott, 1997; Khan et al., 2001; Puttfarcken et al., 1997; Vizi and Lendvai, 1999), and the presence of α5 mRNA has been identified in rat dorsal root ganglia (Genzen et al., 2001).

To investigate the impact of an increase in the α5 subunit on mechanical allodynia following SNL, we used antisense oligonucleotides (ODNs). Furthermore, we examined the postsynaptic phosphorylation of cAMP response element-binding protein (pCREB) in the outer laminae to correlate spinal neuronal activation to mechanical allodynia. The relevance of CREB phosphorylation to hypersensitive states following peripheral nerve injury or inflammation has been well demonstrated (Ji and Rupp, 1997; Anderson and Seybold, 2000; Ma and Quirion, 2001), and it has been suggested that pCREB is a sensitive marker for neuronal activity following noxious stimulation (Ji and Rupp, 1997). An increase in peripheral stimulation, either by nerve injury (Ma and Quirion, 2001) or inflammation (Ji and Rupp, 1997; Anderson and Seybold, 2000), leads to an increase in CREB phosphorylation in the spinal cord dorsal horn generally with a time course consistent with the appearance of nociceptive behaviors. Based on the localization of the α5 subunit in SNL rats, we hypothesized that an increased expression of α5 on primary afferent fibers would increase the presynaptic release of excitatory amino acids, increase the postsynaptic phosphorylation of CREB, and contribute to mechanical allodynia.

## 2. Materials and methods

Male Sprague–Dawley rats (200–300 g; Harlan) were used for this study. All animals were housed in pairs prior to intrathecal catheter implantation and had free access to food and water. All experiments were performed in accordance with the regulations of Wake Forest University School of Medicine Animal Care and Use Committee.

### 2.1. Surgical procedures

Fifty animals underwent spinal nerve ligation (SNL), 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 10–12 days postligation to allow for the development of mechanical allodynia.

Antisense- and missense-treated rats were implanted with an intrathecal catheter, as described previously (Yaksh and Rudy, 1976), with slight modifications (Sakura et al., 1996) 7 days following SNL. Indwelling catheters were constructed with 32 G polyurethane tubing connected to Tygon tubing (0.01 ID) via a Tygon tubing cuff (0.02 ID). All catheter joints were fused using cyclohexanone (Sigma). Briefly, under halothane anesthesia, the 32 G polyurethane catheter containing a guide wire (ReCathCo, LLC) was inserted through a puncture of the atlanto-occipital membrane and advanced caudally 7.5 cm so that the tip of the catheter was at the level of the lumbar enlargement. Animals were examined for neurological deficits following surgery, and any animals exhibiting such deficits were immediately euthanized. Animals were housed individually following surgery and allowed to recover for 3–5 days prior to use.

### 2.2. Behavioral testing

All behavioral tests prior to oligonucleotide (ODN) treatment were conducted on days 10–12 postsurgery between the hours of 1:00 and 4:00 PM. Behavioral testing following oligonucleotide (ODN) treatment was conducted 1 h after the last intrathecal administration. Paw withdrawal thresholds (PWT) were determined for left and right hind paws using von Frey filaments (Chaplan et al., 1994) and the Randall–Selitto paw pressure technique (Randall and Selitto, 1957). Behavioral tests were performed on separate groups of rats. 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 ODN treatment to stabilize baseline responses (Taiwo et al., 1989). 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. Regardless of the behavioral test used, each hind paw was tested three times with a 5-min intertrial interval. The mean PWT for the ipsilateral and contralateral hind paws was compared to determine the presence of mechanical allodynia. Mechanical allodynia was defined as the presence of at least a 20% decrease in PWT for the ipsilateral hind paw.

### 2.3. Oligonucleotide treatment

Antisense (5′-CTC TTG ATG GTG CAG CTG-3′) and missense, the same bases rearranged (5′-GAC GTA GTG GTT GCT TCC-3′), 18-mer oligonucleotides (ODNs) overlapping the 5′ translation initiation region (nucleotides −6 to 12) of the rat α5 nAChR subunit were synthesized by the Wake Forest University School of Medicine DNA synthesis laboratory and purified by HPLC. The bases were unmodified because phosphorothioate-modified ODNs have exhibited some toxicity (Bitner et al., 2000). Both the antisense and missense sequences were checked for homology using GenBank. Rats received intrathecal bolus injections of 5 nmol antisense or missense in 20 μl of saline twice daily (injections 10–12 h apart) for 3.5 days for a total of 35 nmol ODNs.

### 2.4. Immunocytochemistry

Two hours after behavioral testing (3 h after the last ODN administration), rats were deeply anesthetized with pentobarbital and perfused transcardially with 0.01 M PBS+1% sodium azide followed by 4% paraformaldehyde (400 mL). The placement of the tip of the intrathecal catheter was verified, and the lower lumbar spinal cord was removed and postfixed in 4% paraformaldehyde (2–3 h) followed by 30% sucrose (48–72 h). Tissue was imbedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, USA) and cut transversely into 40-μm sections on a Leica CM3000 cryostat.

Immunocytochemistry (ICC) was performed on free-floating sections using standard biotin–streptavidin techniques. For all immunocytochemistry, spinal cord sections were washed in 0.01 M phosphate-buffered saline+0.15% Triton-X 100 (PBS+T) and incubated in 0.3% $H_2O_2$ (15 min). Following further washes in PBS+T, sections were incubated in 50% alcohol (45 min), washed in PBS+T, and blocked with 1.5% normal goat (pCREB) or rabbit (α5 nAChR) serum for 1 h. Sections were incubated in either pCREB (1:1000; Cell Signaling Technology) or α5 nAChR (1:1000; Santa Cruz) primary antibodies overnight at 4 °C. Sections were washed in PBS+T, incubated in the appropriate biotinylated secondary antibodies (Vector Laboratories) for 1 h at room temperature, washed in PBS+T, and incubated for 1 h in streptavidin-linked horseradish peroxidase (ABC Kit, Vector Laboratories). Antibodies were visualized using the enhanced glucose–nickel–diaminobenzidine method. Images were captured on a Leica Axioplan2 light microscope at 10× magnification. Positively labeled nuclei (pCREB) were identified for automated counting using SigmaScan Pro 5 at a preset intensity threshold. For α5 nAChR subunit quantification, the number of immunoreactive pixels in lamina I–II was determined using a preset intensity threshold (Sigma Scan Pro 5). Labeling was examined in laminae I–II with 6–10 slices examined per animal. Two antisense-treated rats that did not exhibit significant α5 knockdown were excluded from behavioral and pCREB analyses. No missense-treated rats exhibited altered α5 immunoreactivity, and therefore, all were included in subsequent analyses.

### 2.5. Statistical analysis

Behavioral data are presented as the mean±the standard error and were analyzed using Student's *t*-test or one-way ANOVA where appropriate. Immunocytochemical data were analyzed using Student's *t*-test and one-way ANOVA where appropriate.

## 3. Results

### 3.1. Spinal nerve ligation

Using von Frey filament testing following SNL (Fig. 1A), paw withdrawal threshold decreased ipsilateral to

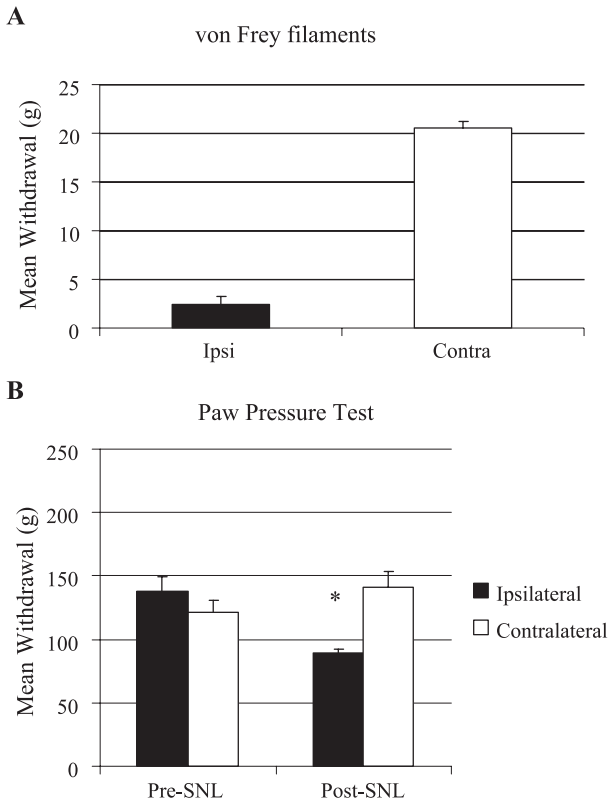


Fig. 1. Hind paw withdrawal thresholds 10–12 days postspinal nerve ligation. (A) Hind paw withdrawal thresholds of the ipsilateral (solid bar) and contralateral (open bar) paws as measured by von Frey filaments 10–12 days post-SNL. Data are presented as the mean paw withdrawal pressure±S.E.M. for six rats. *$p<0.05$ compared to contralateral side. (B) Hind paw withdrawal thresholds of the ipsilateral (solid bar) and contralateral (open bar) paws as measured by the paw pressure test. Data are presented as the mean paw withdrawal pressure±S.E.M. for six rats.

injury (2.5±0.7 g), while the contralateral paw exhibited no change in threshold (21±7 g).

A similar result was observed using the Randall–Selitto paw pressure test. Spinal nerve ligation resulted in a significant decrease of paw withdrawal threshold (PWT) from 138±11 (pre-SNL) to 89±3 g, 10–12 days post-ligation (Fig. 1B). The mean PWT of the contralateral side was not different than normal rats or presurgery baselines (141±12 g).

### 3.2. PCREB immunoreactivity and spinal nerve ligation

In the normal rat, pCREB immunoreactivity (pCREB-IR) was confined to outer laminae, and few nuclei were labeled (83±24 nuclei; Figs. 2A and 3). Following SNL, pCREB-IR increased bilaterally in the outer laminae (LI–II). The increase in pCREB-IR was significantly greater ipsilateral to ligation compared to the contralateral side (612±34 nuclei vs. 474±26 nuclei, $p<0.05$; Fig. 3).

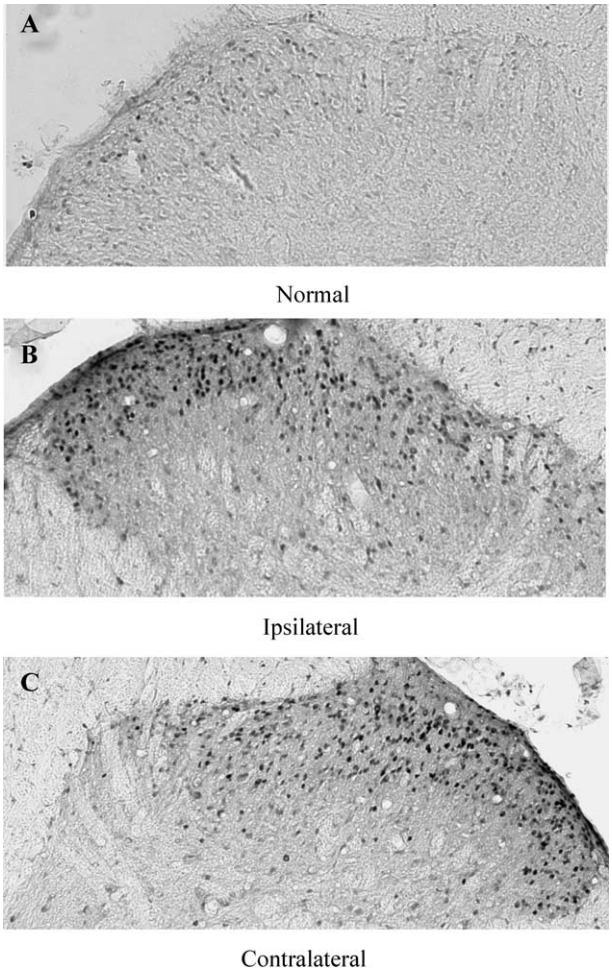


Fig. 2. Phosphorylated CREB immunoreactive nuclei in the dorsal horn of normal and spinal nerve-ligated rats. (A) Representative image of pCREB immunoreactivity in the outer laminae of the normal rat. Representative images of pCREB immunoreactivity in SNL rats ipsilateral (B) and contralateral (C) to ligation.

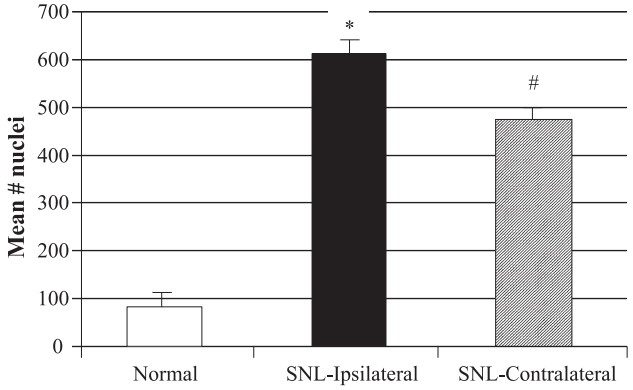


Fig. 3. Phosphorylated CREB immunoreactive nuclei in normal and spinal nerve-ligated rats. pCREB immunoreactivity was quantified in the outer laminae of the dorsal horn in normal rats (open bar) and SNL rats ipsilateral (solid bar) and contralateral (hatched bar) to ligation. Data are presented as the mean number of immunoreactive nuclei±S.E.M. for six normal and six SNL rats. * $p<0.05$ compared to normal rats. #$p<0.05$ compared to both normal rats and SNL-ipsilateral.

### 3.3. Knock down of the α5 nAChR subunit

Following SNL, α5 immunoreactivity was increased 99±18% in outer laminae ipsilateral to injury (Fig. 4A,C) compared to the contralateral side (Fig. 4B,D). Treatment of SNL rats with α5 antisense significantly reduced the number of α5 immunoreactive pixels by 63±9% ($p<0.001$) when compared to missense-treated SNL rats (Fig. 5B). The α5-immunoreactivity of SNL rats treated with α5 missense did not differ from untreated SNL rats (Fig. 5C).

### 3.4. α5 knock down and pCREB immunoreactivity

Knock down of the α5 nAChR in the SNL rat decreased pCREB-IR nuclei in the outer laminae ipsilateral to ligation (Fig. 6). Missense-treated rats also showed a small reduction in pCREB-IR when compared to untreated SNL rats. However, the decrease in pCREB-IR was significantly greater in antisense-treated when compared to missense-treated ($p<0.005$).

### 3.5. α5 knock down and mechanical allodynia

Rats receiving α5 antisense demonstrated a significant alleviation of mechanical allodynia as measured by von Frey filaments (Fig. 7A). Ten to twelve days post-SNL, rats exhibited mechanical allodynia, with PWTs being reduced to 20±1% of baseline values. Antisense treatment of SNL rats increased PWTs to 41±7% of baseline values ($p=0.01$). Successful knock down of the α5 subunit in the L4–L6 spinal cord was determined by immunocytochemistry, and only those rats where knock down was successful were included in the behavioral analyses. Most antisense-treated rats (8 of 10) in which the tip of the catheter was in the L4–L6 region of the spinal cord exhibited a significant degree of knock down.

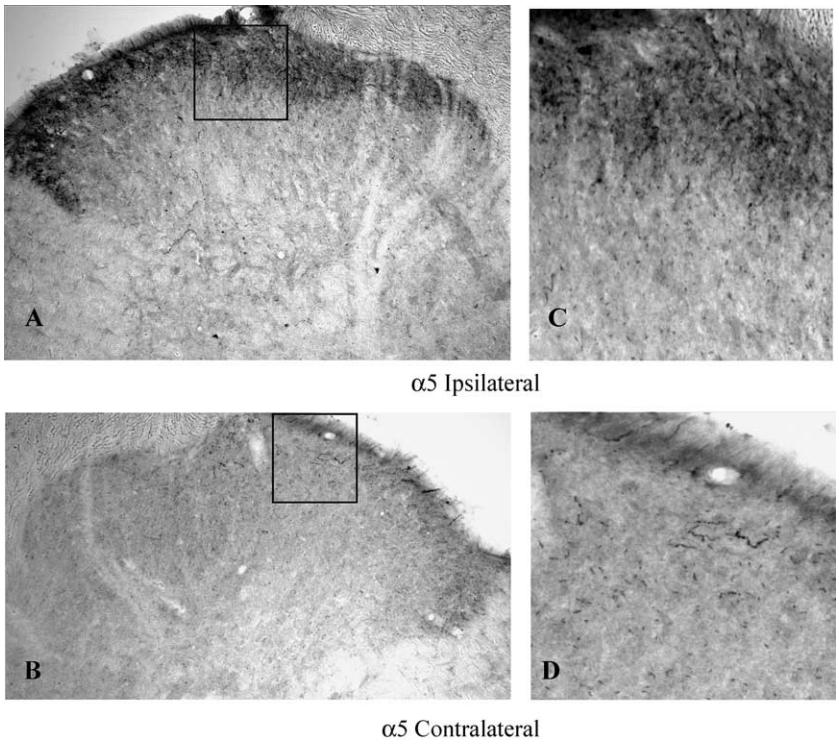


Fig. 4. Expression of the α5 nAChR subunit in the spinal nerve-ligated rat. The expression of the α5 nAChR subunit is increased ipsilateral to ligation (A and C). There is no increase of α5 on the contralateral side (B and D).

Mechanical allodynia was also alleviated as measured by paw withdrawal to pressure (Analgesy-meter). Spinal nerve ligation reduced PWTs ipsilateral to injury to 58±3% of baseline values prior to antisense treatment (Fig. 7B). Antisense treatment significantly increased PWTs to 73±5% of baseline values ($p$<0.05). Treatment of SNL

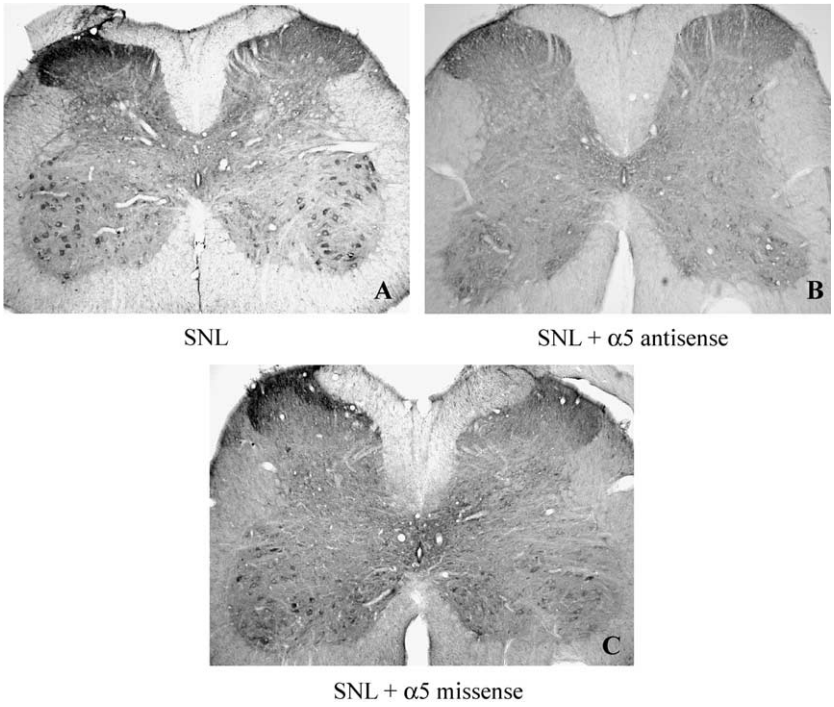


Fig. 5. Knock down of the α5 nicotinic acetylcholine receptor subunit. α5 immunoreactivity is increased 10–12 days post-SNL, ipsilateral to ligation (A, ipsilateral side is on left). (B) α5 immunoreactivity in the antisense-treated SNL rat is reduced on the ipsilateral dorsal horn (left dorsal horn) and in the ventral motoneurons. (C) α5 immunoreactivity in the missense-treated SNL rats is not reduced.

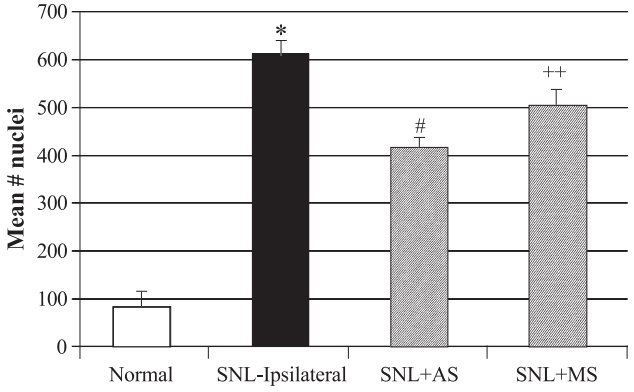


Fig. 6. Effects of α5 antisense and missense treatment on pCREB immunoreactivity. pCREB immunoreactive nuclei were quantified in laminae I–II in normal rats (open bar), untreated SNL rats (black bar), SNL rats treated with α5 antisense (striped bar), and SNL rats treated with α5 missense (dotted bar). Data are presented as the mean number of immunoreactive nuclei±S.E.M. for six rats per group. *$p$<0.05 compared to normal rats. #$p$<0.05 compared to both normal and SNL-ipsilateral rats. ++$p$<0.005 compared to SNL+AS-treated rats.

rats with missense did not significantly alter mechanical allodynia (64±4% of baseline values).

## 4. Discussion

Our results confirm a role for the α5 nAChR subunit in the spinal cord of the nerve-injured rat. As we reported previously, ligation of spinal nerves L5 and L6 induces an up-regulation of the α5 nAChR subunit in the outer lamina of the spinal cord dorsal horn ipsilateral to injury. Knock down of the α5 subunit in SNL rats moderately alleviates mechanical allodynia, suggesting that the presence of the α5 nAChR subunit contributes to this condition. Furthermore, α5 antisense treatment in SNL rats decreases pCREB immunoreactive nuclei in the outer laminae, suggesting that the α5 subunit functions to enhance ACh-evoked excitatory amino acid release.

Knock down of the α5 subunit was confirmed using immunocytochemistry (ICC). This technique has been used recently by a number of laboratories to demonstrate the efficiency of protein knock down (Bitner et al., 2000; Wang et al., 2002). Although other methods are often used to detect receptor knock down, we decided to use ICC for several reasons. First, the α5 subunit does not participate in ligand binding directly but can alter ligand affinity. Therefore, the use of radioligand binding would be inappropriate since changes in receptor number would be difficult to interpret. Second, the α5 subunit is increased in a specific anatomical region (L4–L6) of the ipsilateral spinal cord following SNL, and it is in this area that nociceptive fibers from the affected hind paw terminate. By using ICC, we were able to determine that the α5 subunit was specifically diminished in this area of interest. Third, by using ICC, we were able to demonstrate that the administration of ODNs did not result in a general neurotoxicity because α5 immunoreactivity was identical in α5 missense-treated and untreated SNL rats. Furthermore, treatment with α5 antisense did not alter the expression of other nAChR subunits as measured by ICC (i.e., α3, α4, α7, β2, or β4; data not shown).

The finding that SNL increased CREB phosphorylation in the outer laminae of the dorsal horn is consistent with previous reports. An increase in CREB phosphorylation has been described following partial sciatic nerve ligation (Ma and Quirion, 2001) and formalin-induced inflammation (Ji and Rupp, 1997; Anderson and Seybold, 2000). The phosphorylation of CREB is thought to contribute to long-term changes in the spinal cord resulting in hyperalgesia and allodynia (Anderson and Seybold, 2000).

Our results showing a decrease in pCREB-IR in α5 antisense-treated SNL rats suggests that the inclusion of the α5 nAChR subunit with nAChRs on primary afferent fibers enhances ACh-evoked release of excitatory neurotransmitters and peptides. The dependence of CREB phosphorylation on the release of glutamate from primary afferents (Ji and Rupp, 1997) and the presence of several nAChR subtypes on primary afferent neurons have been shown previously (Gillberg and Wiksten, 1986; Gillberg and Askmark, 1991; Roberts et al., 1995; Wonnacott, 1997;

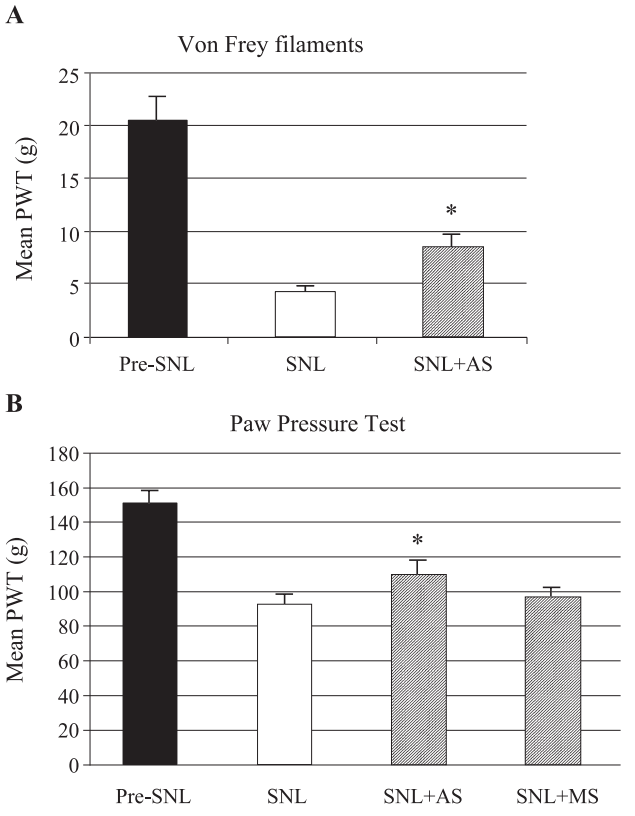


Fig. 7. α5 antisense treatment increased PWTs of spinal nerve-ligated rats. (A) Paw withdrawal evoked by von Frey filaments was increased in α5 antisense-treated SNL rats ($n$=5). (B) Paw withdrawal thresholds to paw pressure in SNL, α5 antisense-treated rats, and α5 missense-treated rats ($n$=6). *$p$<0.05 compared to untreated SNL rats.

Khan et al., 2001; Puttfarcken et al., 1997; Vizi and Lendvai, 1999).

The finding that α5 missense application resulted in a decrease in CREB phosphorylation ipsilateral to SNL when compared to the ipsilateral untreated SNL rats was surprising because no detectable reduction in α5 immunoreactivity was observed in these rats. The reduction in pCREB-IR in missense-treated rats correlated with the slight reduction of mechanical allodynia, but neither of these values was significantly different than untreated SNL rats. This suggests that although CREB phosphorylation is correlated with the degree of mechanical allodynia, there is a range in the number of pCREB positive cells required before any changes in overt behavior can be detected. The results of missense oligonucleotide application suggest that the activity of spinal cord neurons was reduced nonspecifically. Despite this difference in pCREB-IR between untreated SNL and missense-treated SNL rats, a greater reduction of CREB phosphorylation was observed in the antisense-treated SNL rats, and this correlated with the knockdown of α5 expression and a greater reduction of mechanical allodynia.

Although we did not confirm the presence of α5 on primary afferent neurons, the increased expression of α5 following SNL was confined to fibers in the outer laminae. The results of these studies suggest that the inclusion of the α5 subunit, possibly on primary afferent fibers, increases the sensitivity of nAChR for ACh and thereby enhances ACh-evoked excitatory neurotransmitter release. Consistent with this hypothesis, the inclusion of the α5 subunit with the α4β2 nAChR increased ACh-evoked macroscopic currents in a heterologous expression system (Ramirez-Latorre et al., 1996).

The results of our behavioral studies confirm a role for the α5 subunit in the maintenance of mechanical allodynia. Previous behavioral studies have demonstrated that different populations of spinal nAChRs are responsible for the pro- and antinociceptive effects of intrathecal nicotinic agonists (Khan et al., 2001; Khan et al., 1998). The intrathecal administration of nicotine, cytisine, and epibatidine evokes both glutamate and aspartate release, and the pronociceptive effects of A-85380 can be antagonized with an NMDA antagonist (Khan et al., 1996a; Khan et al., 2001). Our results suggest that the increased expression of the α5 subunit contributes to an increased release of glutamate from primary afferent fibers, which contributes to the mechanical allodynia following SNL.

In contrast, Rashid and Ueda (2002) have noted a neuropathy-specific analgesic action of intrathecal nicotinic agonists in the mouse. This does not necessarily contradict our observations because nicotinic agonists have been shown to excite both inhibitory and excitatory spinal cord neurons via distinct populations of nAChRs (Cordero-Erausqin et al., 2004). Because the α5 subunit modulates the functioning of existing nAChRs, our results could be explained by either an increase in the activity of the excitatory pronociceptive population of nAChRs or a decrease in the activity of inhibitory antinociceptive nAChRs. Our results and those of Rashid and Ueda (2002) suggest that the α5 subunit increases the activity of excitatory pronociceptive nAChRs because the knockdown of this subunit produces a modest antinociception and a reduction of CREB phosphorylation. Rashid and Ueda (2002) demonstrated an increase in the sensitivity of the inhibitory antinociceptive nAChRs in neuropathic mice, and this was attributed to the loss of a tonic cholinergic-stimulated GABA release. If the function of the up-regulated α5 subunit was to increase the sensitivity of the inhibitory antinociceptive population of nAChRs as a compensatory mechanism, then the knockdown of this subunit would have produced the opposite effects (e.g., increased mechanical allodynia and CREB phosphorylation). Thus, it seems that the up-regulation of the α5 subunit seems limited to the pronociceptive population of nAChRs.

In conclusion, these data support a role for the α5 nAChR subunit in the maintenance of mechanical allodynia in nerve-injured rats. The up-regulation of the α5 subunit following SNL clearly alters the characteristics of the existing nAChR subtype and may represent a unique way in which the activity of nAChRs is modified. Conceivably, it is more efficient for a neuron to regulate the expression of one gene (e.g., the α5 subunit) than to regulate the expression for two or more subunit genes (e.g., the α4 and β2 genes). The identity of the subunits associated with the α5 subunit may be helpful for the targeting of therapeutic nicotinic agonists for the treatment of chronic pain and is currently under investigation.

## Acknowledgement

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