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Impact of chronic nicotine on sciatic nerve injury in the rat

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Abstract

Chronic nicotine exposure and the immune response to peripheral nerve injury has not been investigated thoroughly. Rats were exposed to chronic nicotine or saline followed by chronic constriction injury (CCI) of the sciatic nerve. Mechanical sensitivity was measured at various time points and the immune response was investigated at 21 days post-CCI. Chronic nicotine increased mechanical hypersensitivity, microglia activation, and the production of IL-1 β , but not the number of immune cells at the site of injury. These results suggest that chronic nicotine increases mechanical hypersensitivity following peripheral nerve injury through a mechanism that may involve an increased production and release of central and peripheral cytokines.

Keywords

Rodent; macrophages; T cells; neuropathic pain; smoking; microglia

1. Introduction

Smoking is associated with an increased incidence and severity of several chronic pain conditions including low back pain (Thomas et al., 1999), diabetic neuropathy (Eliasson, 2003), and fibromyalgia (Yunus et al., 2002). However, the impact of chronic nicotine exposure on neuropathic pain resulting from nerve injury is unknown. Overwhelming evidence has shown that peripheral and central immune responses contribute to the development and maintenance of neuropathic pain following peripheral nerve injury. Injury to a peripheral nerve increases the number of immune cells at the site of injury and produces glial activation within the spinal cord (Colburn et al., 1999; Bendszus and Stoll, 2003; Moalem et al., 2004). In the periphery, loss of macrophages or mature T cells reduces behavioral hypersensitivity and Wallerian degeneration following peripheral nerve injury (Liu et al., 2000; Moalem et al., 2004). Inhibition of glial activation within the spinal cord similarly reduces injury-induced behavioral hypersensitivity (Raghavendra et al., 2003; Clark et al., 2007). Despite the frequency of smoking amongst the general population, the impact chronic nicotine on the immune response to peripheral nerve injury has not been investigated.

In the rodent, chronic nicotine administration reduces the inflammatory response to immunogenic challenges (McAllister et al., 1998; Kalra et al., 2004) and increases mechanical hypersensitivity resulting from peripheral nerve injury (Josiah and Vincler, 2006). Both immune cells and neurons express nicotinic acetylcholine receptors (nAChRs). Macrophages

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express $\alpha 7^*$ nAChRs while lymphocytes express a variety of nAChR subunits including the $\alpha 7$ and $\alpha 9\alpha 10$ subunits (Sato et al., 1999; Peng et al., 2004). Acetylcholine released from the vagus nerve and locally from lymphocytes reduces pro-inflammatory cytokine production following LPS administration (Wang et al., 2003). However, the impact of this cholinergic system on the development of chronic pain states has not been investigated.

Previous results from our laboratory show that chronic systemic nicotine administration via osmotic minipump produces a dose-dependent mechanical hypersensitivity that correlates with an increased phosphorylation of cAMP response element binding protein (CREB) in the spinal cord (Josiah and Vincler, 2006). In the presence of chronic nicotine, the mechanical hypersensitivity following spinal nerve ligation is increased in an additive fashion. The current studies were undertaken to examine the impact of chronic nicotine on neuropathic pain using a model that is more dependent upon the activation of immune cells, chronic constriction nerve injury of the sciatic nerve.

2. Methods

Male Sprague-Dawley rats (200-300 g; Harlan) were used for these studies. All animals were housed in pairs 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.

Rats ($n=8-12$ /group) were implanted subcutaneously with Alzet osmotic minipumps (Model 2ML4; 2.5 l/hr) calculated to deliver 8.6 mg/kg/day of nicotine (free base concentration; Sigma-Aldrich, USA) or 0.9% saline under general halothane anesthesia (2-3% halothane in 100% oxygen). This dose of nicotine has been shown to produce serum nicotine and cotinine levels consistent with moderate to heavy smokers (Josiah and Vincler 2006). Seven days after minipump implantation, 1 group of saline- and 1 group of nicotine-treated rats underwent loose ligation of the left sciatic nerve as described previously (Bennett and Xie, 1988), but with slight modifications. Briefly, rats were anesthetized with halothane (2-3% halothane in 100% oxygen), the left sciatic nerve was exposed at mid-thigh level and two 4-0 chromic gut sutures were loosely ligated around the sciatic nerve approximately 1 mm apart. The incision was closed with 4-0 silk suture.

2.1. Behavioral Testing

All behavioral tests were conducted between the hours of 9:00 AM and 4:00 PM. No differences in baseline paw withdrawal thresholds (PWT) were noted during these hours. 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 Teflon plinth to apply a constant rate of increasing pressure (16g per second) to the hind paws. The cut-off pressure was 250g. For the Randall-Selitto test, animals were first subjected to 4 training sessions to stabilize baseline responses (Taiwo et al., 1989). Hind paws were alternately tested 3 times with a 5 minute inter-trial interval.

Paw withdrawal thresholds (PWT) were measured prior to osmotic pump implantation and these values were considered baseline measurements. Paw withdrawal thresholds were measured 2, 5, and 7 days post-pump implantation. One group of saline-treated and 1 group of nicotine-treated rats underwent CCI surgery following behavioral testing on day 7 of nicotine/saline exposure and paw withdrawal thresholds were measured 2, 6, 8, 10, 13, 15, 17, and 21 days after CCI. The experimenter performing behavioral testing was blind to nicotine or saline treatment. The mean PWT for the ipsilateral and contralateral hind paws was compared in CCI rats 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.

2.2. Immunohistochemistry

Following behavioral testing on Day 21 post-CCI (Day 28 post-minipump implantation), rats were deeply anesthetized with pentobarbital and perfused transcardially with 0.01M PBS + 1% sodium nitrite followed by 4% paraformaldehyde (400 mL). The lower lumbar spinal cord and left (injured) and right (uninjured) sciatic nerves were removed and post-fixed in 4% paraformaldehyde (2-3 hours) followed by 30% sucrose (48-72 hours). Tissue was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, USA) and cut transversely at 16 μ m (sciatic nerve) or 40 μ m (spinal cord) on a Leica CM3000 cryostat.

Immunohistochemistry was performed using standard biotin-streptavidin techniques. For all immunohistochemistry, spinal cord or sciatic nerve sections were washed in 0.01M phosphate-buffered saline + 0.15% Triton-X 100 (PBS+T) and incubated in 0.3% H₂O₂ (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 serum for 1 hour. Sections were incubated with primary antibodies to CD2 (1:1000, Serotec), CD68 (ED1) (1:1000, Serotec), Iba-1 (1:1000, DAKO), calcitonin gene-related peptide (CGRP; 1:1000, Peninsula Laboratories), or pCREB (1:1000, Cell Signaling Technology) primary antibodies overnight at 4°C. Sections were washed in PBS+T, incubated in biotinylated goat anti-rabbit (pCREB, CGRP, Iba-1) or anti-mouse (CD2, ED1) antibody (Vector Laboratories) for 1 hour at room temperature, washed in PBS+T and incubated for 1 hour in streptavidin linked horseradish peroxidase (ABC Elite Kit, Vector Laboratories). Antibodies were visualized using the enhanced glucose-nickel-diaminobenzidine method. Images were captured on a Leica Axioplan2 light microscope at 10X magnification. Positively labeled objects were identified for automated counting using SigmaScan Pro 5 at a preset intensity threshold. For spinal cord sections, labeling was examined in every 4th slice in laminae I-II with 6-10 slices examined per animal. For sciatic nerve slices, 4 non-consecutive slices were quantified for CD2 and ED1 staining.

2.3. Cytokine measurement

The ligated and uninjured sciatic nerves and lower lumbar spinal cord (L4-L6) were removed by rapid dissection 28 days after chronic nicotine or saline exposure (21 days post-CCI where appropriate). Tissue samples were homogenized on ice in 50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Igepal CA-630 with a protease inhibitor cocktail at a 1:100 dilution (Mammalian Cell Lysis Kit, Sigma, Saint Louis, MO) and subject to centrifugation at 1500 $\times$ g for 10 min at 4°C. Two samples of supernatant were used immediately for cytokine measurement. A fourplex, bead-based rat cytokine immunoassay kit (Lincoplex) was used for the simultaneous detection of TNF α , IL-1 β , IL-6 and IL-10 concentrations, following the manufacturer's instructions. Multi-wavelength fluorescence and cytokine concentrations were determined with a luminometer (Luminex 100 system; Luminex, TX) and Bio-Rad software.

2.4. Statistical Analysis

Behavioral data are presented as the mean $\pm$ the standard error and were analyzed using two-way repeated measures ANOVA followed by Tukey Test where appropriate. Immunohistochemical data and cytokine measurements were analyzed using Student's t-test.

3. Results

3.1. Chronic nicotine exacerbates CCI-induced mechanical hypersensitivity

Rats were implanted with Alzet osmotic minipumps to subcutaneously deliver 8.6 mg/kg/day nicotine or saline. Chronic nicotine treatment significantly reduced paw withdrawal thresholds within 7 days of administration compared to saline (Figure 1). Following seven days of nicotine

or saline exposure, 1 group each of saline- and nicotine-treated rats underwent chronic constriction injury of the sciatic nerve. Chronic constriction injury reduced paw withdrawal thresholds in both saline- and nicotine-treated groups. In the CCI groups, two-way repeated measures ANOVA revealed a significant effect of nerve injury [$F(7,121)=69.2$; $p < 0.001$] and of nicotine treatment [$F(1,121)=23.1$; $p < 0.001$], but no interaction was noted.

3.2. Chronic nicotine does not alter immune cell recruitment

As reported by others previously, CCI significantly increased the number of macrophages (ipsilateral: 108 ± 11 vs. contralateral: 22 ± 2 cells) and T cells (ipsilateral: 53 ± 12 vs. contralateral: 10 ± 1 cells) in the ligated sciatic nerve compared to the unligated, contralateral nerve [$F(1,15)=60$; $p < 0.001$] (Figure 2). However, chronic nicotine treatment beginning 7 days prior to CCI did not alter injury-induced macrophage (ED1 positive) recruitment (Figure 2A). The contralateral sciatic nerve in chronic saline- and chronic nicotine-treated rats also displayed equal numbers of ED1-immunoreactive macrophages.

Chronic constriction injury also increased the number of CD2-immunoreactive T cells when compared to the uninjured, contralateral side [$F(1,14)=11$; $p < 0.01$]. However, chronic nicotine treatment did not alter the recruitment of T cells to the site of nerve injury (Figure 2B).

3.3. Chronic nicotine alters cytokine production in the sciatic nerve

Twenty-eight days of chronic nicotine administration significantly increased the amount of IL-1 β , but not IL-6 in the sciatic nerve (Figure 3). Chronic constriction injury also significantly increased the production of both IL-1 β and IL-6 in the sciatic nerve 21 days post-ligation. Although the presence of chronic nicotine increased IL-1 β production when administered alone, IL-1 β production was not further increased following CCI. The production of TNF α and IL-10 were largely undetectable across treatment groups.

3.4. Chronic nicotine activates spinal cord microglia

In the spinal cord, chronic nicotine itself, significantly increased the activation of microglia, measured by Iba-1 immunoreactivity [$F(1,10)=9$; $p < 0.05$] (Figure 4 and Figure 5A and 5B). CCI also produced an increase in the number of Iba-1 immunoreactive microglia in chronic saline- and chronic nicotine-treated rats both ipsilateral and contralateral to ligation (Figure 5C and 5D). Nicotine-treated CCI rats tended to have a greater number of Iba-1 immunoreactive pixels, although this was only significant on the contralateral side [$F(1,14)=5.5$; $p < 0.05$].

The activation of spinal cord microglia was confirmed by measuring spinal cord content of cytokines following 28 days of chronic nicotine administration, 21 days post-CCI, or chronic nicotine administration followed by CCI. Chronic nicotine significantly decreased spinal cord content of IL-1 β but did not alter the content of IL-6 (Figure 6). Although CCI produced a small decrease in spinal cord IL-1 β content, this decrease was not statistically significant. The combination of CCI + nicotine significantly reduced IL-1 β content in the spinal cord; the amount of IL-6 was not changed. Similar to what was found in the sciatic nerve, largely undetectable levels of spinal cord TNF α and IL-10 were observed.

3.5. Chronic nicotine and CREB phosphorylation

Chronic nicotine alone significantly increased the number of pCREB immunoreactive nuclei in the outer laminae (LI-II) of the spinal cord dorsal horn. Chronic constriction injury also increased the number of pCREB positive nuclei ipsilateral to ligation (Figure 7A). Spinal cords of rats treated with chronic nicotine and undergoing CCI displayed significantly more pCREB nuclei than rats treated with nicotine alone [$F(1,11)=19$; $p < 0.005$].

3.6. Chronic nicotine and spinal cord CGRP

Chronic nicotine has been reported previously to contribute to neurogenic inflammation by increasing the release of CGRP in the oral mucosa (Dussor et al., 2003). Therefore, we examined CGRP immunoreactivity in the spinal cord of chronic nicotine-treated rats. As shown in Figure 7B, chronic nicotine treatment did not alter CGRP immunoreactivity in the rat spinal cord.

4. Discussion

Our results extend previous findings from our laboratory showing chronic nicotine-induced increases in mechanical hypersensitivity following peripheral nerve injury. In contrast to previous reports on the immunosuppressive effects of nicotine, chronic nicotine administration did not suppress nerve injury-induced macrophage and T cell recruitment to the peripheral nerve. Chronic nicotine actually increased IL-1 β production in the sciatic nerve, but did not increase IL-6, TNF α , or IL-10. In the spinal cord, chronic nicotine and CCI individually, increased the activation of microglia. However, only chronic nicotine decreased IL-1 β content in the spinal cord. Chronic constriction injured rats treated with nicotine tended to have higher microglial activation, but this increase was only significant on the contralateral side and appeared to reflect the nicotine-induced activation of microglia in normal rats. The phosphorylation of CREB in the spinal cord was also increased with nicotine alone and pCREB immunoreactivity was further increased in nicotine-treated rats following CCI.

The chronic nicotine-induced mechanical hypersensitivity we observed in the present study is consistent with our previous findings (Josiah and Vincler, 2006). Our previous data showed a dose-dependent reduction in paw withdrawal thresholds that persisted across 21 days. Furthermore, spinal nerve-ligated rats treated with nicotine showed a mechanical hypersensitivity that was greater than rats treated with nicotine alone or than saline-treated SNL rats (Josiah and Vincler, 2006). In the current studies, CCI produced a much greater degree of mechanical hypersensitivity than did spinal nerve ligation. Therefore, the additive effects of chronic nicotine were less pronounced. Nonetheless, CCI rats treated with chronic nicotine were significantly more sensitive to mechanical pressure than CCI rats treated with chronic saline.

Although chronic nicotine has been shown to be immunosuppressive to overt immunogenic challenges, the number of macrophages and CD2-expressing T cells recruited to the site of nerve injury was not reduced by nicotine treatment. This suggests that the presence of chronic nicotine does not alter nerve injury-induced immune cell recruitment. However, we measured only 1 time point, 21 days after CCI, in the current study. Therefore, it remains possible that nicotine may have altered immune cell recruitment at an earlier time point. This possibility seems less likely however, because the behavioral hypersensitivity following CCI developed in a parallel fashion in saline- and nicotine-treated rats. The recruitment of macrophages and T cells to the site of nerve injury has been shown to be necessary for the development of mechanical hypersensitivity (Liu et al., 2000; Moalem et al., 2004). Therefore, if nicotine suppressed macrophage or T cell recruitment at an earlier time point, less, not more, mechanical hypersensitivity would have been the result.

Equivalent numbers of macrophages and T cells at the site of nerve injury in saline and nicotine-treated rats, however, says nothing about the functioning of these cells. Chronic nicotine is known to reduce T cell activation by interfering with intracellular signaling (Kalra et al., 2000). Our results show an increase in IL-1 β content in the sciatic nerve with chronic nicotine treatment alone. However, few T cells are present in an uninjured sciatic nerve, suggesting a more predominant role of resident macrophages in this response. Chronic nicotine treatment in hyperlipidemic mice significantly increases serum TNF α , IL-1 β , and keratinocyte-derived

chemokine and upregulates nuclear factor κ B (NF- κ B) target genes such as cyclooxygenase-2 (Lau et al., 2005). Our results in rats suggest that chronic nicotine likely activates resident macrophages in the sciatic nerve resulting in an increased production of IL-1 β and mechanical hypersensitivity.

Our observation that chronic nicotine increases the activation of spinal cord microglia by Iba-1 immunostaining and reduces spinal cord IL-1 β content also suggests a pro-inflammatory, rather than anti-inflammatory, role of chronic nicotine. Although the reduced spinal cord content may be interpreted as chronic nicotine-induced suppression of IL-1 β production, a recent study suggests that the decreased content may be due to an increased release of IL-1 β (Clark et al., 2006). Spinal cord slices treated with LPS show an immediate release of stored IL-1 β mediated by p38 MAP kinase (Clark et al., 2006). Moreover, mechanical hypersensitivity induced by intrathecal LPS was blocked with soluble receptors to IL-1 β suggesting that the acute release of spinal IL-1 β can contribute to mechanical hypersensitivity. Of course, we administered nicotine chronically so a direct comparison isn't valid. It remains plausible, however, that the presence of nicotine presents a more prolonged stimulus that results in a more tonic release of IL-1 β .

In rat cultured microglia, nicotine pre-treatment has been reported to reduce LPS-induced production of pro-inflammatory cytokines (Shytle et al., 2004; DeSimone R. et al., 2005). Our results are in contrast to these in vitro observations and suggest that the administration of chronic systemic nicotine itself increases the activation of spinal cord microglia. Although in the previous studies by Shytle et al. (2004) and DeSimone et al. (2005), the effects of chronic nicotine alone on pro-inflammatory cytokine production were not examined making a more direct comparison difficult. Cultured microglia express the $\alpha 7^*$ nAChR and stimulation of this receptor prior to microglial activation dose-dependently reduces LPS-induced release of TNF α , but has little or no effect on the release of nitric oxide, IL-10, and IL-1 β . However, chronic systemic nicotine reduced spinal cord IL-1 β content in the current studies. These discrepancies are likely due to differences in experimental conditions including purified cultured microglia versus microglia in vivo and differences in the stimuli responsible for microglial activation (direct LPS activation in vitro versus a host of in vivo activators).

Peripheral nerve injury has been reported previously to increase cytokine production at the site of injury (George et al., 1999; Ma and Quirion, 2005) and in the spinal cord (Winkelstein et al., 2001; Lee et al., 2004) and our results are largely consistent with these previous observations. We observed small or undetectable levels of TNF α in normal sciatic nerve and 21 days post-CCI. The content of TNF α in the sciatic nerve increases dramatically 12 hours following CCI, but rapidly drops thereafter and is not different than sham CCI sciatic nerve by day 15 (George et al., 1999). Partial sciatic nerve ligation increases the number of IL-6 immunoreactive macrophages and our results show a large increase in sciatic nerve content of IL-6 21 days post-CCI. In the spinal cord, previous studies show a small increase in IL-6 mRNA remains 28 days post-CCI whereas IL-1 β mRNA is no different than sham CCI at 14 days (Lee et al., 2004). Our results show an insignificant reduction in IL-1 β and IL-6 spinal cord content 21 days post-CCI which is consistent with IL-1 β mRNA production, and suggests an increase in IL-6 release.

Although nicotine itself increased IL-1 β production in the sciatic nerve and increased the activation of spinal cord microglia, nicotine did not alter nerve injury-induced changes in peripheral or cytokine production. The effects of systemic chronic nicotine administration on overt nerve injury have not been studied extensively. Repeated administration of once daily bolus subcutaneous injection of nicotine spared spinal cord white matter and tended to improve motoric recovery following spinal cord contusion injury (Ravikumar et al., 2005). The current

studies did not monitor motor functioning nor did we assess neurodegeneration at the site of peripheral nerve injury.

We have reported previously that chronic nicotine and spinal nerve ligation increase CREB phosphorylation in the spinal cord (Josiah and Vincler, 2006). Moreover, SNL rats treated with chronic nicotine had significantly greater pCREB immunoreactive nuclei than rats subjected to either condition alone. The number of pCREB positive nuclei was inversely correlated with paw withdrawal threshold (i.e., the higher the number of pCREB nuclei, the lower the paw withdrawal threshold). Our current data concur with these previous observations; CCI rats treated with nicotine had significantly greater pCREB positive nuclei than nicotine-treated rats. Our results are also in agreement with a recent report showing an increase in CREB phosphorylation following CCI (Song et al., 2005).

In conclusion, the presence of chronic nicotine further increases nerve injury-induced mechanical hypersensitivity and increases IL-1 β production in the periphery without altering the recruitment of macrophages or T cells to the site of injury. In the spinal cord, chronic nicotine itself increased the activation of microglia and decreased IL-1 β content, but did not increase the expression of CGRP or IL-6 content. The phosphorylation of the transcription factor CREB was also increased in nicotine-treated CCI rat, implicating a role of chronic nicotine in central sensitization. Taken together, our findings provide no evidence for an anti-inflammatory role of chronic nicotine in response to overt peripheral nerve injury.

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Figure 1.

Chronic nicotine increases mechanical sensitivity. Rats were treated with 8.6 mg/kg/day chronic nicotine (filled squares and crosses) or saline (open circles and filled triangles) for 28 days. On day 7 post-nicotine/saline treatment, one group of saline (filled triangles) and nicotine-treated (crosses) rats underwent chronic constriction injury (CCI). Mean paw withdrawal thresholds (PWT) in grams (g) $\pm$ S.E.M. to mechanical pressure are shown across 28 days. (n = 6-8/group). * p < 0.01 in CCI rats treated with nicotine versus CCI rats treated with saline.

Figure 2.

Immune cell recruitment following chronic constriction injury of the sciatic nerve. Rats treated for 28 days with 8.6 mg/kg/day chronic nicotine (filled bars) had the same number of macrophages (A) and T-cells (B) as saline-treated (open bars) rats following CCI. The mean number of immunoreactive cells $\pm$ S.E.M. are shown.

Figure 3.

Cytokine content in the sciatic nerve. The mean content $\pm$ S.E.M. of IL-1 β (A) and IL-6 (B) is shown in sciatic nerve from normal rats, 8.6. mg/kg/day nicotine-treated rats (Nicotine), the ipsilateral sciatic nerve in CCI rats (CCI), and the ipsilateral sciatic nerve in CCI rats treated with 8.6 mg/kg/day nicotine (CCI+Nic). * p < 0.05 compared to Normal; # p < 0.05 compared to Nicotine n = 4-6/group

Figure 4.

Chronic nicotine increases Iba-1 immunoreactive microglia in the rat spinal cord. The mean numbers of immunoreactive pixels/area $\pm$ S.E.M. are shown. Iba-1 immunoreactive pixels were quantified in normal saline and nicotine-treated rats and in CCI rats treated with saline or nicotine both ipsilateral (Ipsi) and contralateral (Contra) to ligation. * $p < 0.05$ compared to saline-treated rats in the same group (Normal, Ipsi, or Contra).

Figure 5.

Iba-1 immunoreactivity in the rat spinal cord. Representative images of Iba-1 staining in the rat dorsal horn are shown. Normal rats (Nor) treated with saline (A) or nicotine (B) and CCI rats treated with saline (C) or nicotine (D) are shown. For CCI rats, only the ipsilateral dorsal horn is shown.

Figure 6.

Cytokine content of the spinal cord. The mean content $\pm$ S.E.M. of IL-1 β (A) and IL-6 (B) in the spinal cords of normal rats (Normal) and 8.6 mg/kg/day nicotine-treated rats (Nicotine) and in the ipsilateral half of CCI rats (CCI) and CCI rats treated with chronic nicotine (CCI +Nic). * $p < 0.05$ compared to Normal.

Figure 7.

pCREB and CGRP in the dorsal horn of the rat spinal cord. (A) The mean number of pCREB immunoreactive cells $\pm$ S.E.M. in the outer dorsal horn (laminae I-II) are shown. All rats were treated with nicotine (8.6 mg/kg/day) or saline for 28 days. pCREB immunoreactivity was quantified in rats treated with nicotine alone, in CCI rats treated with saline (CCI-Saline), and in CCI rats treated with nicotine (CCI-Nicotine). * $p < 0.05$ compared to normal rats. # $p < 0.05$ compared to Nicotine rats. (B) CGRP immunoreactivity in the rat spinal cord. The mean number of CGRP immunoreactive pixels/area $\pm$ S.E.M. in laminae I-II are shown. CGRP immunoreactivity was quantified in rats treated with chronic saline or chronic nicotine (8.6 mg/kg/day) for 28 days.