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The influence of neuropathic pain on nAChR plasticity and behavioral responses to nicotine in rats

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Abstract

Tobacco smoking is particularly evident in individuals experiencing chronic pain. This complex relationship is poorly understood at both molecular and behavioral levels. Here we describe experiments aimed at understanding whether a chronic pain state induces neuroadaptations into the brain or peripheral nerves that involve nicotinic acetylcholine receptors (nAChRs) and whether these neuroadaptations directly lead to increased vulnerability to nicotine addiction or to the development of coping strategies to relieve pain symptoms. We found that ligation of the rat L5 spinal nerve (SNL) led to a dramatic downregulation in the mRNA expression levels of all nAChR subunits examined in dorsal root ganglia and a time-dependent downregulation of discrete subunits particularly in the cingulate cortex and the amygdala. SNL and sham-operated rats showed minor or no changes in patterns of acquisition and motivation for nicotine taking. SNL rats also showed similar vulnerability to nicotine seeking as sham animals when reinstatement was induced by nicotine-associated cues but failed to reinstate lever pressing when relapse was induced by nicotine priming. SNL and sham rats were equally sensitive to nicotine-induced anxiety-like behavior and antinociception; however, nicotine produced a potent and long-lasting antiallodynic effect in SNL rats. These results demonstrate that chronic pain leads to plasticity of nAChRs that does not directly facilitate nicotine addictive behaviors. Instead, nicotine potently decreases allodynia, an effect that could lead to increased nicotine consumption in chronic pain subjects.

Keywords

allodynia; nAChRs; nicotine; self-administration; reinstatement; chronic pain

Introduction

Chronic pain is a critical and increasing health problem that affects about 30% of adults in the United States [13; 27] and accounts for over \$125 billion in terms of health care costs and loss of productivity [1]. Similarly, tobacco dependence is a highly prevalent condition that is

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Conflict of Interest Statement

The authors declare no conflict of interest.

the leading preventable causes of morbidity and mortality in the United States, accounting for 443,000 deaths each year and greater than \$190 billion in annual medical expenses and loss of productivity [15].

An association between chronic pain and cigarette smoking has been revealed in numerous epidemiological and clinical studies. For example, smokers have been found more likely than nonsmokers to experience chronic pain [47; 59] and smokers with chronic pain indicated their pain as more intense than nonsmokers, a condition associated with occupational and social impairment [55]. Based on this evidence, a causal relationship between these two pathological conditions has been hypothesized with smoking thought to be a risk factor for chronic pain. However, nicotine, the main psychoactive ingredient of tobacco, has analgesic properties particularly in animal studies [16; 48], therefore being somewhat counterintuitive as a risk factor for pain. This may be because antinociceptive effects of nicotine result from acute or short-term nicotine administration whereas chronic nicotine exposure produces tolerance to nicotine-induced antinociception [4; 20], ultimately leading to increased pain awareness. Similarly, withdrawal from tobacco smoking increases pain sensitivity that could, in turn, further encourage smoking [15; 46]. Therefore, the interaction between chronic pain and smoking might be viewed from a double perspective such that not only is smoking a risk factor for greater pain but also pain may exacerbate smoking behaviors.

The causes of this comorbidity are largely unknown. Smokers may be motivated to use tobacco in an attempt to avoid or relieve the pain [55; 59] or to “self-medicate” the negative affective state associated with chronic pain, rather than the pain per se [7; 21; 28; 42; 59]. Alternatively, desire for positive reinforcement may motivate smoking among individuals with chronic pain [15]. However, these assumptions result from cross-sectional studies and clinical evidence that did not directly examine causality of the comorbidity. Furthermore, from a mechanistic standpoint little information is available on the neurobiological substrates that may be responsible for the co-occurrence of nicotine and chronic pain problems.

Although animal models have been used to demonstrate the acute analgesic effect of nicotine, or chronic hyperalgesic effects, preclinical research has rarely examined pain-smoking co-occurrence. Specifically, an animal study that clearly establishes a causal relationship of how chronic/neuropathic pain influences nicotine consumption and other behavioral responses to nicotine has been missing. Here, we describe experiments examining whether chronic pain produces neuroadaptive changes in mRNA expression levels of nicotinic acetylcholine receptors (nAChRs). Then, we determine whether pain directly increases nicotine consumption, motivation for nicotine taking and vulnerability to relapse. Finally, we examine the possibility that the frequent pain-smoking association results from altered responses to the effects of nicotine (e.g., antiallodynic responses, anxiety-like behavior) in subjects in a chronic pain state.

Methods

Animals

Male Sprague-Dawley rats (200–225 g at their arrival), obtained from Charles River (Portage, MI) were used throughout the study. Rats were housed two per cage in a room with a reverse 12-hour light/12-hour dark cycle (lights off at 7:30 AM). All behavioral experiments were conducted during the dark phase of the cycle. Animals were acclimated for seven days with water and chow (Teklad Diets, Madison, WI) provided *ad libitum* and handled for three days before the experiments were conducted. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All methods used were pre-approved by the Institutional Animal Care and Use Committee at the Torrey Pines Institute for Molecular Studies (Port Saint Lucie, FL). The following experimental groups were used (see Figure 1): one batch of animals (Group 1, N=30) was exposed to Spinal Nerve Ligation (SNL) and used for initial assessment of mechanical allodynia and thermal nociception 1 week following surgery. The same rats were used to examine the effect of nicotine on anxiety-like responses in the Elevated Plus-Maze (EPM) 4 weeks following surgery. Within this group, nicotine-naïve SNL and sham rat samples of L4 and L5 Dorsal Root Ganglia (DRGs, N=6 per group) were assessed for nAChR mRNA expression at 4-week time point. Another batch of rats subjected to SNL or sham surgery (N=8–10 per group, Group 2) was used to determine long-term allodynia. A new cohort of rats (N=24, Group 3) was employed for measurements of $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR mRNA levels in various brain regions at 1 and 4 weeks following surgery. A new cohort of SNL-operated and sham-operated rats (N=21, Group 4) was employed for nicotine self-administration and cue-induced reinstatement studies, whereas additional rats (N=14, Group 5) were used for nicotine prime-induced reinstatement. Group 6 was used to examine the effects of nicotine on pain responses in SNL vs. sham (N=7–8 per group).

Drugs

(–)-Nicotine hydrogen tartrate salt was purchased from Sigma (St. Louis, MO). Solution of nicotine for intravenous (iv) or subcutaneous (sc) injection was obtained by dissolving (–)-Nicotine hydrogen tartrate in 0.9% saline and the pH adjusted to 7.0–7.4 with 5 M sodium hydroxide. Nicotine doses are reported as free base concentration.

SNL

SNL was induced by a procedure similar to that previously described [31]. Prior to surgery, animals were given buprenorphine (0.07 mg/kg) to reduce post-surgical pain. Under isoflurane anesthesia (Vetamac, Inc., Rossville, IN), rats were placed in a prone position and a longitudinal incision (~1.5 cm in length) on the dorsal midline was made, using the level of the iliac crests as the midpoint of the incision. At the mid-sacral region, a stab incision was made to expose the left paraspinal muscles. The paraspinal muscles were then separated all the way down from the level of the L5 spinous process to the sacrum. Muscle retractors were used to maintain clear vision of the L6 transverse process. Then the L-6 transverse process was carefully removed to identify visually the L4 and L5 spinal nerves. The left L5 spinal nerve was isolated and individually tightly ligated (twice) with 6-0 silk. Muscles were

then sutured using absorbable suture and the skin closed with non-absorbable suture. In sham-operated rats the L-6 transverse process was removed but the L5 spinal nerve was not ligated.

Von Frey testing

Rats were placed in suspended chambers with wire mesh bottoms for 15 minutes to habituate before testing. A series of calibrated von Frey filaments were applied perpendicular to the plantar surface of the ipsilateral hind paw until the filament buckled. The up-down method was used to determine the 50% withdrawal threshold [8]. Assessment of mechanical allodynia was routinely carried out in all the animals used throughout this study. Effect of nicotine was determined in rats subjected to ligation of the L5 spinal nerve or sham surgery whose baseline scores for the 50% paw withdrawal threshold were determined before drug administration in each animal. After baseline measures were taken, all rats received sc injection of nicotine 0.0, 0.15 or 0.5 mg/kg and force (g) was assessed in each animal 5, 30 and 180 min later. All rats received 0.15 mg/kg dose on testing trial 1, 0.5 mg/kg on testing trial 2 and vehicle on testing trial 3. After each drug testing trial, rats were left undisturbed in their home cages for 3–4 days. Von Frey testing was conducted by an experimenter blind to group and drug treatment.

Plantar test

Thermal nociception was assessed by measuring hind paw withdrawal latency in response to radiant heat using a plantar test apparatus (Stoelting, Wood Dale, IL) according to the method used by Hargreaves et al. [24]. Briefly, each rat was placed into a compartment enclosure on a glass surface. A mobile heat source was positioned under the plantar surface of the hind paw and activated with a light beam. A digital timer automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. The mean withdrawal latency (seconds) for the left hind paw was determined from the average of three trials separated by a 10-min interval to prevent thermal sensitization [29]. Plantar test was conducted by an experimenter blind to group assignment.

Tail flick assay

Nicotine-induced analgesia was assessed by tail flick assay with a thermal stimulator that uses radiant heat (Stoelting, Wood Dale, IL). During testing, the focused beam of light was applied to the animal's tail (~1.5 cm from the end) and tail flick latency (s) was recorded. A 15 s cutoff time was scheduled to avoid tissue damage. Baseline values for tail-flick latency were determined before drug administration in each animal. After baseline measures were taken, all rats received injection of nicotine 0.0, 0.5 or 1.0 mg/kg (sc) and tail flick latency was assessed in each animal 5, 10 and 30 min later. All rats received 0.5 mg/kg dose on testing trial 1, 1.0 mg/kg on testing trial 2 and vehicle on testing trial 3. After each drug testing trial rats were left undisturbed in their home cages for 3–4 days. Tail flick assay was conducted by an experimenter who was blind to group and drug treatment.

Quantitative polymerase chain reaction (qPCR)

A cohort of rats was employed for time-dependent measurements of $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR mRNA levels. Two groups of 8 rats underwent SNL surgery, whereas the remaining group was sham operated. Brains from SNL and sham-operated rats were collected and snap-frozen with isopentane all at the same time 1 week and 4 weeks after surgeries. Specifically, 4 sham rats matched time point 1 week and 4 sham rats matched the 4-week time point. The brains were sliced on a cryostat, and bilateral punches (300 μ m thickness, 1–2 mm diameter) were collected from the cingulate cortex (CgCx), nucleus accumbens (NAcc), medial habenula (MHb), amygdala, ventral tegmental area (VTA) and periaqueductal gray (PAG). Anatomical representation of the dissected brain regions is shown Figure S1. $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR mRNA levels were examined in L4 and L5 in DRGs that were dissected from a separate group of SNL and sham rats 4 weeks following nerve injury. Total RNA was extracted and purified using RNeasy MiniKit (Qiagen, Valencia, CA). cDNA reverse transcription was performed using Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA). mRNA expression levels were determined by qPCR using a QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) according to manufacturer's protocol on the iCycler IQ5 multicolor real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Gene-specific primers were designed using NCBI primer-BLAST (Table 1). Amplification efficiency of each primer pair was determined based on the slope of standard curves generated by 1:10 serial dilutions of a cDNA sample pooled from each region examined (Table 2). Melting curve analysis (60–95 °C) was performed to verify reaction specificity for each PCR product. Only reactions with appropriate amplification efficiencies and melting curves were included for analysis. The expression of all investigated genes was normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated according to the relative quantification ($-C_t$) method [33]. GAPDH was chosen as reference gene due to previous evidence showing stable expression in DRGs between sham and nerve-injured animals [6]. The expression stability of GAPDH for each region examined was also assessed. Expression levels of GAPDH did not differ within the group of sham rats and between sham, SNL-1 week and SNL-4 week groups. The average value ($-C_t$) of the sham group was used for normalization of SNL group data. Data are reported as fold change relative to sham.

Iv catheter implantation

In operant self-administration and reinstatement experiments SNL and iv catheterization surgeries were conducted simultaneously. Catheters were implanted as previously described [10; 11]. Following ligation of L5 nerve an incision was made to expose the right jugular vein and a catheter made from silicon tubing (I.D. =0.020 inches, O.D. =0.037 inches) was subcutaneously positioned. After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk. The distal end of the catheter was attached to a threaded cannula guide bent at a 90° angle that protruded from the rat's back. The cannula was capped with plastic tubing and covered with threaded lightweight aluminum hood. To maintain patency for the duration of the experiment, catheters were flushed daily with 0.2 mL of heparin (1000 UPS U/ml)-containing saline solution, which also contained 0.7 mg/ml enrofloxacin.

Apparatus

Self-administration experiments began 1 week after surgery and were conducted in operant conditioning chambers (Med Associates, Inc., St. Albans, VT) enclosed in lit, sound attenuating, ventilated environmental cubicles. Each chamber was equipped with two retractable levers located in the front panel. Chambers were also equipped with auditory stimuli presented via a speaker and visual stimuli located above the levers (cue lights). Infusions occurred by means of syringe pumps (Med Associates, Inc., St. Albans, VT) and liquid swivels (Instech Solomon, Plymouth Meeting, PA), connected to plastic tubing protected by a flexible metal sheath for attachment to the external catheter terminus. An infusion pump was activated by responses on one (active) lever, while responses on the second (inactive) lever were recorded but did not result in any programmed consequences. Activation of the pump resulted in a delivery of 0.1 ml of nicotine. A microcomputer controlled the delivery of reinforcers, presentation of auditory and visual stimuli, and recording of the behavioral data.

Nicotine self-administration

Rats were assigned to two groups. One group was L5 ligated and implanted with iv catheter, the other group was sham operated and implanted with the catheter. Following recovery from surgery, all animals were trained to self-administer nicotine 30 µg/kg/infusion using a fixed ratio-1 (FR-1) [20 s time out (TO)] schedule during daily 2-h sessions conducted across 5 days per week. Active lever presses that resulted in nicotine infusion simultaneously resulted in the presentation of a cue-light located above the active lever and a tone (7 kHz, 70 dB) conditioning stimulus. After 7 days on this reinforcement schedule, the task was switched to FR-3 for 6 additional 2-h sessions and then to progressive ratio (PR) schedule that better measures motivational properties rather than rate of drug intake. The objective of the PR schedule was to escalate the response ratios until animals no longer respond. For the PR procedure, the response requirement for successive injections was 3, 6, 9, 12, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, 268, etc. [11; 44]. The PR schedule used a 20 s TO following each drug infusion. The variable examined in FR experiments was the number of infusions earned in 120 min, whereas the parameter measured in PR tasks was the break point, which was defined as the highest ratio completed before a 60 min period during which no injections were earned and lasted a maximum of 4 h. Following the PR task all rats were allowed to lever press for a nicotine dose of 60 µg/kg/infusion under the FR-3 schedule for 6 additional sessions. Subsequently, the PR task was carried out to measure motivation for the intake of 60 µg/kg/infusion nicotine dose. Responses to the inactive lever were recorded throughout all experimental phases and served as a measure of non-specific motor behavior. Doses of nicotine to be instrumentally self-administered were the most rewarding dose (30 µg/kg) and a rather aversive dose (60 µg/kg) corresponding to the apex and the descending limb of the dose-response curve according to our pilot data (Figure S2) and previous literature [54].

Cue-induced reinstatement of nicotine seeking

Following assessment of self-administration of two nicotine doses under FR and PR schedules, the same rats began extinction (EXT) training, during which all programmed

consequences were removed from lever pressing. Thus, neither nicotine nor cue-light and tone were available for 12 consecutive 60 min daily sessions. Reinstatement was induced by presentation of light-tone cues but not nicotine following an active lever press. These reinstatement sessions were repeated every third day for a total of 4 reinstatement sessions. The parameter measured was the total number of responses to the lever previously associated with nicotine delivery. Responding on the inactive lever was also recorded throughout the experiment.

Nicotine prime-induced reinstatement of nicotine seeking

L5 ligated and sham-operated rats, implanted with the iv catheters were initially trained to self-administer nicotine (30 µg/kg/infusion) in daily 2-h self-administration sessions under an FR-1 schedule (7 days, 5 days a week) and then under an FR-3 schedule (6 days). Active lever presses that resulted in nicotine infusion resulted in the presentation of a cue-light located above the active lever. An intermittent tone was sounded throughout the session. Following 13 sessions the dose of nicotine was increased as described above. Rats were allowed to self-administer for 6 additional 2-h FR-3 sessions. Responses were then extinguished over 12 consecutive daily sessions. During 1-h EXT sessions, the lever presses were no longer associated with nicotine delivery while, unlike for cue-induced reinstatement, cue-light and the intermittent tone were presented to allow for their concomitant EXT. Reinstatement sessions were then carried out under conditions identical to that of EXT [11]. After the last EXT session, animals were systemically pre-treated with increasing doses of nicotine (0.0, 0.05, 0.15 or 0.3 mg/kg, sc) administered 10-min prior to reinstatement sessions. All rats received 0.05 mg/kg dose on testing session 1, 0.15 mg/kg on testing session 2, 0.3 mg/kg on testing session 3 and vehicle on testing session 4. Reinstatement sessions were repeated every third day. Two 1-h EXT sessions were carried out between testing sessions. Responses to the inactive lever were recorded and served as a measure of non-specific motor behavior.

EPM

To measure anxiety-like responses, the EPM test was used, as previously described [9]. In brief, the apparatus was made of black plastic with two open arms (50 × 10 cm) and two closed arms (50 × 10 × 45 cm) connected by a 10 × 10 cm central area. The maze was 50 cm above the floor and placed in a spacious room. Testing was performed under a source of red light. Thirty minutes after nicotine (0.5 mg/kg) or saline injection [39] each rat was placed in the center of the maze, facing a closed arm and the 5-min test procedure began. The percent of time spent exploring the open arms and the number of open arm entries were used as measures of anxiety-like behavior, whereas the number of entries into the closed arms was used as an indicator of general motor activity. An entry into an arm was defined as the animal placing all four paws over the line marking that area. The apparatus was cleaned with tap water between each rat performance. All animals used were naive to the EPM.

Data analysis

A t-test was used to compare thermal nociception in sham-operated vs. neuropathic rats and qPCR data regarding the use of pooled sham groups and the DRGs. The non-parametric Mann-Whitney U test was used to compare responses to mechanical stimuli between the two

rat groups and PR data, as recommended [8; 44]. The Wilcoxon test was used to analyze responses to mechanical stimuli following nicotine administration. Gene expression data were analyzed by means of analysis of variance (ANOVA). To compare each gene of each brain structure/tissue independently, a one-way ANOVA that used “time” as a between-subject factor was used. Acquisition, maintenance of nicotine self-administration and EXT patterns were analyzed by two-way repeated measures ANOVA that used the between-factor “group” and the within-factor “session”. Mixed factorial ANOVA was also used to analyze reinstatement sessions. Tail flick data were analyzed by three-way ANOVA, considering the between-subject factor “group”, and “treatment” and “time” as repeated measures. EPM data were analyzed by means of a two-way ANOVA where rat “group” and “treatment” were between-subject factors. Correlations among pain variables were estimated by regression analysis. Accepted level of significance was $p < 0.05$. When appropriate, analyses of behavioral data were followed up by Newman-Keuls post hoc tests while qPCR data were followed by Bonferroni post hoc tests.

Results

Experiment 1: SNL-induced mechanical allodynia and heat hyperalgesia

Rats of Group 1 were tested for development of mechanical allodynia (using von Frey filaments) and thermal hyperalgesia (using the Hargreaves plantar test) 1 week following unilateral ligation of the L5 spinal nerve or sham surgery. L5 SNL induced marked allodynia to mechanical stimulation of the affected paw, as determined by a response to von Frey filaments [$p < 0.05$; (Figure 2A)], an effect that was accompanied by a weak but significant hyperalgesic response to thermal stimuli of the paw [$t_{(13)} = 2.3$, $p < 0.05$; (Figure 2B)]. Rats examined for response to mechanical allodynia were tested the following day for thermal hyperalgesia, and vice versa. Overall, results obtained in rats that were not naïve to pain assays (von Frey: $p < 0.01$; Hargreaves: $t_{(28)} = 2.3$, $p < 0.05$) were similar to results obtained in the same rats that were naïve to pain assays. There was no correlation between measures of mechanical allodynia and thermal hyperalgesia in SNL or sham-operated rats, possibly due to the limited extent of thermal sensitivity ($R^2 = 0.1$ and $R^2 = 0.0$, Figure S3). Mechanical allodynia persisted up to 8 weeks as demonstrated by a significant effect of ligation in Group 2 rats at each time point examined [$p < 0.05$ and $p < 0.01$ (Figure 2C)].

Experiment 2: mRNA expression levels of nAChR subunits in SNL rats

Group 3 rats were employed to examine gene expression levels that encode for nAChR subunits in a network of brain structures densely populated in nAChRs with particular attention to regions forming the reward circuitries (e.g., VTA, NAcc, MHb) and those modulating emotions, stress and pain processes (e.g., amygdala, CgCx, PAG). The analysis included various subunits considered relevant in modulating the addictive properties of nicotine (e.g., $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 2$ and $\beta 4$ [23]). As $\alpha 7$ homomeric nAChR subunit is a target particularly important for supraspinal analgesia, mood and anxiety disorders [41; 50], $\alpha 7$ nAChR mRNA levels were specifically assessed in the PAG, amygdala and CgCx. Since symptoms associated with neuropathic pain (e.g., mood disorders, anxiety) might be precipitated in a time-dependent fashion, gene expression of nAChR subunits in the brain was tested at 1 and 4 weeks following nerve injury. mRNA levels in DRGs was examined in

a separate group of rats (Group 1) 4 weeks following nerve injury. We found that gene expression levels of all nAChR subunits examined, including $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ were dramatically downregulated in L5 DRGs of SNL rats as compared to sham-operated animals, with $\alpha 3$ and $\beta 4$ mRNA levels being nearly undetectable. T tests revealed the following values ($\alpha 3$: $t_{(10)} = 4.3$, $p < 0.01$; $\alpha 4$: $t_{(10)} = 5.4$, $p < 0.001$; $\alpha 5$: $t_{(10)} = 3.7$, $p < 0.01$; $\beta 2$: $t_{(10)} = 2.5$, $p < 0.05$; $\beta 4$: $t_{(10)} = 4.6$, $p < 0.01$; $\alpha 7$: $t_{(10)} = 5.6$, $p < 0.001$). The decrease in nAChR subunit expression in L4 DRGs did not achieve statistical significance ($\alpha 3$: $t_{(10)} = 1.8$, NS; $\alpha 4$: $t_{(10)} = 1.7$, NS; $\alpha 5$: $t_{(10)} = 1.9$, NS; $\beta 2$: $t_{(10)} = 2.1$, NS; $\beta 4$: $t_{(9)} = 1.6$, NS; $\alpha 7$: $t_{(9)} = 2.1$, NS).

In the brain, nAChR mRNA changes occurred particularly in two brain structures, the amygdala and the CgCx (Figure 3). In the amygdala, overall ANOVA displayed reduced expression of $\alpha 3$ [$F_{(2,18)} = 9.2$, $p < 0.01$], $\beta 2$ [$F_{(2,18)} = 10.0$, $p < 0.01$] and $\beta 4$ [$F_{(2,18)} = 5.5$, $p < 0.05$] nAChR subunits. Post hoc comparisons revealed that these changes occurred in a time-dependent manner ($p < 0.05$ for $\beta 4$ and $p < 0.01$ for $\alpha 3$ and $\beta 2$ at 4 weeks). Expression of nAChRs mRNAs containing $\alpha 3$ and $\beta 2$ subunits was also downregulated in the CgCx: [$F_{(2,21)} = 4.7$, $p < 0.05$] and [$F_{(2,21)} = 4.9$, $p < 0.05$], respectively. Similar to effects observed in the amygdala, effects in the CgCx were time-dependent and reached significance at 4 weeks ($p < 0.05$). In addition, mRNA levels of $\alpha 5$ nAChR was decreased in the VTA of SNL rats [$F_{(2,20)} = 5.4$, $p < 0.05$] both at 1 and 4 weeks following SNL ($p < 0.05$). Ct mean values and statistics for each gene of 1-week versus 4-week sham groups indicated no differences within the sham groups in each brain region (Table S1). Altogether, the observed SNL-induced changes of various nAChR mRNA subunits in DRGs, amygdala and CgCx suggest that plasticity of nAChRs could play an important role in the modulation of somatosensory as well as affective component of neuropathic pain.

Experiment 3: Nicotine self-administration and cue-induced reinstatement studies

To investigate whether the presence of chronic neuropathic pain influences operant responding for nicotine, acquisition of nicotine lever pressing under a FR-1 schedule was initially examined for Group 4 SNL and sham rats. We found (Figure 4A) that the two groups similarly acquired the operant task [main effect of “group” $F_{(1,19)} = 2.1$, NS; main effect of “session” $F_{(6,114)} = 16.4$, $p < 0.001$; “group x session” interaction $F_{(6,114)} = 0.8$, NS]. Figure 4B shows patterns of nicotine (30 $\mu\text{g/kg/inf}$)-maintained responding for SNL and sham-operated groups. Similar to acquisition, overall ANOVA indicated no difference between the two groups [“group” $F_{(1,19)} = 2.7$, NS; “session” $F_{(5,95)} = 10.3$, $p < 0.001$; “group x session” interaction $F_{(5,95)} = 1.1$, NS]. For self-administration under PR schedule we found that SNL did not influence either number of infusions [$F_{(1,19)} = 2.4$, NS], total responses [$F_{(1,19)} = 2.8$, NS] or break point [$p = 0.2$, (Figure 4C)] as compared to sham animals. In contrast, operant self-administration under FR-3 for the high nicotine dose of 60 $\mu\text{g/kg/inf}$ was significantly decreased in SNL rats compared to sham (Figure 4D). Indeed, overall ANOVA showed effect of “group” [$F_{(1,19)} = 5.0$, $p < 0.05$] accompanied by a barely significant “group x session” interaction [$F_{(5,95)} = 2.3$, $p = 0.05$]. However, PR responding was not different between SNL and sham groups in all the dependent variables examined (number of infusions: [$F_{(1,19)} = 2.0$, NS]; total responses: [$F_{(1,19)} = 2.3$, NS]; break point: [$p = 0.2$], Figure 4E). Altogether, these results suggest that rats with neuropathic pain show minor or no

changes in nicotine-taking behavior and motivational responses compared to rats that are not in pain.

There was no difference in the inactive lever responding between ligated and non-ligated animals throughout all operant measures: acquisition [“group” $F_{(1,19)}=0.3$, NS; “session” $F_{(6,114)}=0.6$, NS; interaction $F_{(6,114)}=1.0$, NS], maintenance of 30 $\mu\text{g/kg}$ nicotine [“group” $F_{(1,19)}=0.3$, NS; “session” $F_{(5,95)}=0.6$, NS; interaction $F_{(5,95)}=0.1$, NS], PR for 30 $\mu\text{g/kg}$ dose [$F_{(1,19)}=1.1$, NS], maintenance of 60 $\mu\text{g/kg}$ nicotine [“group” $F_{(1,19)}=0.0$, NS; “session” $F_{(5,95)}=0.6$, NS; interaction $F_{(5,95)}=0.5$, NS], PR for 60 $\mu\text{g/kg}$ dose [$F_{(1,19)}=0.0$, NS]. Active/inactive lever ratios for acquisition and maintenance of nicotine (30 and 60 $\mu\text{g/kg}$) self-administration are shown in Figure S4.

The number of lever presses during EXT sessions was also assessed. SNL and sham rats decreased pressing for the lever previously associated with nicotine delivery over time, but no significant differences between SNL and sham-operated rats were observed [“group” $F_{(1,19)}=1.9$, NS; “session” $F_{(11,209)}=27.6$, $p<0.001$; interaction $F_{(11,209)}=1.2$, NS (Figure 4F)].

Next, we investigated whether chronic pain alters nicotine-seeking behavior. Environmental cues previously associated with nicotine lever pressing trigger nicotine-seeking behavior. As shown in Figure 5A, a robust increase of responding on the nicotine-associated lever was observed when previously nicotine-paired stimuli (cue-light and tone) were reintroduced following EXT. During repeated reinstatement tests, responses at the active lever were significantly increased over the last EXT day levels in both SNL and sham groups (main effect of “session” [$F_{(4,76)}=26.0$, $p<0.001$], main effect of “group” [$F_{(1,19)}=2.5$, NS], interaction [$F_{(4,76)}=1.4$, NS]). Post hoc analysis conducted on the main effect of “session” revealed significant difference from EXT collapsed across “group” suggesting successfully induced nicotine-seeking behavior in all reinstatement sessions for both SNL and sham-operated rats ($p<0.001$) and no difference between Sham and SNL animals. Responses at the inactive lever were not different between groups (main effect [$F_{(1,19)}=1.9$, NS]; “group x session” interaction [$F_{(4,76)}=0.1$, NS]).

Experiment 4. Nicotine self-administration and drug prime-induced reinstatement studies

Vulnerability to nicotine seeking was then investigated following administration of various priming doses of nicotine as drug priming also triggers nicotine-seeking behavior. In a separate group of SNL and sham rats (Group 5), operant responding for 30 $\mu\text{g/kg}$ nicotine was similar [“group” $F_{(1,12)}=0.0$, NS; “session” $F_{(5,60)}=0.8$, NS; interaction $F_{(5,60)}=0.6$, NS]. Lever pressing for 60 $\mu\text{g/kg}$ was also similar for SNL and sham groups (Figure S5) in contrast to the small difference shown in Figure 4D [“group” $F_{(1,12)}=0.2$, NS; “session” $F_{(5,60)}=2.0$, NS; interaction $F_{(5,60)}=0.2$, NS].

During EXT both groups also showed similar patterns of responding [“group” $F_{(1,12)}=1.6$, NS; “session” $F_{(11,132)}=3.4$, $p<0.001$; interaction $F_{(11,132)}=1.1$, NS].

In the reinstatement phase, in which various priming doses of nicotine were administered to trigger relapse-like behavior, overall ANOVA revealed significant “group” x “session”

interaction [$F_{(4,48)} = 3.2, p < 0.05$], suggesting that response to nicotine produced a different outcome in SNL vs. sham animals. On post hoc analysis, reinstatement of lever pressing was only observed in sham-operated rats following administration of nicotine priming doses of 0.15 and 0.3 mg/kg ($p < 0.001$ for both), whereas responses at the nicotine-paired lever were not modified in SNL rats as compared to the last EXT day levels at any of the nicotine doses examined (Figure 5B). Responses at the inactive lever were not different between groups and did not change within sessions (“group” [$F_{(1,12)} = 1.2$, NS], “group x session” interaction [$F_{(4,48)} = 1.6$, NS]).

Experiment 5: Effect of nicotine on anxiety-like behavior in SNL vs. sham

EPM results obtained in nicotine-naïve rats 4 weeks after surgery indicated nicotine-induced anxiogenic activity in SNL as well as sham rats (Group 1). As shown in Figure 6A, overall ANOVA conducted for % open arm time revealed a main effect of “nicotine” [$F_{(1,26)} = 4.3, p < 0.05$] accompanied by a trend toward a difference in basal anxiety (main effect of “group” [$F_{(1,26)} = 3.5, p = 0.07$]) with no interaction between the two factors [$F_{(1,26)} = 0.3$, NS]. Open arm entries were comparable between groups (main effect of “group” [$F_{(1,26)} = 1.9$, NS], main effect of “nicotine” [$F_{(1,26)} = 3.8$, NS] and interaction [$F_{(1,26)} = 0.1$, NS], Figure 6B). Nicotine also produced reduced locomotor behavior as described by reduced closed arm entries [$F_{(1,26)} = 6.5, p < 0.05$]. There was no effect of “group” [$F_{(1,26)} = 0.0$, NS] or interaction [$F_{(1,26)} = 0.1$, NS], Figure 6C.

Experiment 6: Effect of nicotine on pain responses in SNL vs. sham

The possibility that nicotine conferred an antiallodynic effect was examined in Group 6 SNL and sham rats using von Frey filaments. This experiment was carried out beginning 2 weeks post-injury according to results of Figure 2C showing reliable and long-lasting mechanical allodynia following SNL from 1 week up to at least 8 weeks. As expected, rat’s paws were more sensitive to mechanical pain in SNL as compared to sham rats (average withdrawal thresholds at $T = 0, p < 0.05$). Administration of 0.5 mg/kg nicotine potently elicited antiallodynic response in neuropathic rats. Sham controls (Figure 7A) showed a significant antinociceptive response 5 min following nicotine administration ($p < 0.05$), an effect that rapidly faded. Notably, the antiallodynic response was long lasting in SNL rats as it was observed at all time points examined ($p < 0.05$ for all time points, Figure 7B). Furthermore, a lower dose of nicotine (0.15 mg/kg) conferred a significant antiallodynic response in neuropathic rats at 5 min time point, leaving responses of sham controls unaltered.

Group 6 SNL and sham rats were also tested for their response to nicotine’s antinociceptive activity, using the tail flick test, in the same animals used to test the effect of nicotine on tactile allodynia (4 weeks after injury). As shown in Figures 7C and 7D, when analyzing nociceptive response to acute heat pain in the tail flick assay following administration of nicotine, overall ANOVA revealed a main effect of “treatment” [$F_{(2,24)} = 31.3, p < 0.001$] that was accompanied by significant “treatment” x “time” [$F_{(6,72)} = 11.6, p < 0.001$] but not “treatment” x “group” [$F_{(2,24)} = 2.0$, NS] interaction. Interaction of the three factors was not significant [$F_{(6,72)} = 0.4$, NS]. These results indicate that SNL and sham rats showed similar response to nicotine’s antinociceptive effect in the tail flick assay. Post hoc analysis revealed that 1.0 mg/kg was the nicotine effective dose in both rat groups ($p < 0.001$) and this

analgesic response was observed only at 5 and 10 min time points in the sham as well as SNL rat group ($p < 0.001$ for both time points).

Discussion

Directionality of the comorbid relationship between chronic pain and smoking is poorly understood. Although acute nicotine exerts antinociceptive effects in animal models [48], chronic nicotine results in tolerance and reduced pain inhibition [4; 20; 26]. This would translate to a drive to smoke more [15]. Pain can also be a motivator of smoking, as suggested by observational studies showing smoking-pain covariation [55] or cross-sectional studies [25] that do not permit determination of the causality of the two disorders. Accordingly, chronic pain-smoking comorbidity has been described as a positive feedback loop, in which individuals smoke to relieve their pain, smoking worsens the pain, and individuals, in turn, increase smoking [15]. Here, we examine the process by which pain motivates smoking by using animal paradigms that mimic a chronic/neuropathic pain state and smoking behavior in humans.

Ligation of the L5 spinal nerve is a validated rodent model of neuropathic pain [31]. Using this model we replicated previous findings that this manipulation produces thermal hyperalgesia and robust mechanical allodynia that last for over 8 weeks [45; 53]. One goal of the present study was to specifically determine whether this induced chronic pain state led to increased nicotine taking and motivation through positive reinforcement processes. To do so, we examined the impact of SNL on dose-response functions in the operant nicotine self-administration paradigm. We found no or minor changes in patterns of acquisition and maintenance of nicotine-reinforced responses, or motivation to obtain nicotine reinforcement, between SNL and sham-operated animals. Collectively, these results indicate that responses for nicotine reward are not altered in rats with neuropathic pain, an outcome that supports recent work showing unchanged responses for food self-administration following SNL in rats [38] but differs from previous studies in which chronic/neuropathic pain lowers rewarding and reinforcing effects of opiates [34; 40] and increases alcohol drinking [22]. Apparently, the effect of chronic pain on drug-taking behavior is dependent upon the drug under investigation.

We hypothesized that rats in pain could be more vulnerable to relapse into nicotine seeking than control rats. Relapse is an important feature of nicotine addiction and can be modeled in rats using EXT-based reinstatement paradigms [52]. During EXT of nicotine self-administration, in which rats lever press with no scheduled reward, responses were similar in SNL and sham-operated rats. Environmental cues elicited a powerful reinstatement in both groups with repeated reinstatement experiments indicating that sham and SNL rats showed similar long-lasting vulnerability to relapse, consistent with self-administration experiments. However, when nicotine priming-induced reinstatement was examined, successful reinstatement was only observed in sham animals whereas SNL rats failed to show increased lever pressing at all doses examined. Notably, priming-induced reinstatement experiments suggested that SNL rats may have developed different sensitivity than controls to the effects of acute doses of nicotine.

To identify molecular substrates responsible for comorbidity, SNL and sham rats were examined one and four weeks after surgery for mRNA levels of $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR subunits in DRGs as well as in brain regions involved in reward, pain and stress responses. At 4 weeks post-surgery dramatic downregulation of all nAChR subunit mRNA levels examined was observed in L5 DRGs, which is consistent with one previous study showing marked downregulation of functional nAChRs in DRGs after SNL [17]. In that study impaired responsiveness to nAChR agonists was not seen in L4 DRGs. Here, the decrease in nAChR subunit expression in L4 DRGs did not achieve statistical significance. Disruption in normal functioning of the somatosensory system is a most common reason for neuropathic pain [12; 14]. Thus, our data strongly suggest that dysfunction of nAChRs plays a pivotal role in the pathophysiology of neuropathic pain and help explain why various nAChR subunits, such as $\alpha 7$ [3], $\alpha 5$ [5] and $\alpha 6$ [56] nAChRs, have been highly associated with prominent symptoms of neuropathic pain including allodynia. In contrast, although in excellent agreement with self-administration experiments, mRNA levels of nAChR subunits in brain areas that form nicotine reward circuitries (VTA, NAcc and MHb) were not substantially modified following pain induction. The one exception was decreased expression of the accessory $\alpha 5$ nAChR subunit in the VTA of SNL rats. This result is difficult to interpret because $\alpha 5$ subunits potentiate nAChR function and seem to be critical for VTA dopaminergic neuronal activity. Therefore, decreased $\alpha 5$ in the VTA might be expected to increase nicotine self-administration [36]. Time-dependent neuroadaptations involving nAChR mRNAs containing $\alpha 3$, $\beta 2$ and $\beta 4$ subunits were specifically observed in the amygdala and the CgCx, two relevant regions for processing of emotional components of pain as well as substrates underlying emotional aspects of drug and alcohol dependence [18; 37]. Other studies in neuropathic animals have reported minor [57] or significant nAChR changes in isolated brain regions [49].

Motivation to self-administer nicotine in chronic pain individuals could result from reduction of negative reinforcement rather than recruitment of positive reinforcement mechanisms. To behaviorally corroborate this possibility, we examined whether nicotine treatment could alleviate sensory and affective chronic pain symptoms. Anxiety is among the most relevant emotional consequences of chronic pain and is mediated through mechanisms that occur in the amygdala and the CgCx [32; 58]. Anxiety-like behavior is typically observed at least 4 weeks after pain induction in rat and mouse chronic pain models [2; 35]. Our EPM results obtained 4 weeks post-ligation showed only a trend for a difference in basal anxiety between SNL and sham groups. The possibility that anxiety parameters of SNL rats depend upon the pain state of individual animals (e.g., animals not in pain display reduced anxiety) may account for this discrepancy. Alternatively, 4 weeks after surgery could be not enough time to induce anxiogenic-like activity in neuropathic rats under our experimental conditions. Nicotine treatment produced a similar anxiogenic response in SNL and sham rats, an observation that is not consistent with the inability of nicotine prime to trigger nicotine seeking in SNL rats and does not explain the frequent pain-smoking co-occurrence. More research is needed to understand how nicotine affects anxiety in neuropathic subjects.

We also tested the hypothesis that analgesic activity of nicotine in cigarettes is used as a coping strategy to alleviate chronic pain. In these experiments, rats received nicotine after

the initiation of chronic pain, which is probably different than found in the majority of smokers with chronic pain, a fact that could have an effect on overall outcome. Regardless of pain state, analgesic properties of nicotine are well established, particularly in animal models, but the mechanisms responsible for nicotine antinociception are complex and may not only involve activation of nAChRs [46]. When examining antinociceptive responses in the tail flick assay, acute nicotine dose-dependently produced a short-term antinociceptive activity, which was similar in sham and SNL animals with comparable baseline tail flick latencies [30], an outcome that does not explain the prevalence of smoking in chronic pain individuals. When SNL and sham rats were challenged with nicotine to reverse mechanical allodynia, we found a dose-dependent and potent reversal of allodynia in SNL rats. Importantly, this antiallodynic effect occurred at nicotine doses lower than those used to induce antinociception in the tail flick assay. Similar to previous evidence [43], sham rats also seemed to show less sensitivity to the von Frey filaments at the 5 min time point following nicotine treatment. However, this short-term response is consistent with tail flick latency and reflects an analgesic, not antiallodynic effect. Furthermore, the nicotine-induced antiallodynic effect was surprisingly long-lasting as it was maintained for at least 3 hours, well over the normal half-life for nicotine actions. In this experiment, the use of a within-subject design, in which every subject received nicotine doses sequentially (lowest dose to highest dose), along with potential carryover effects between multiple tests may represent possible limitations of this design. The possibility that long-lasting antiallodynic activity is mediated by nicotine metabolites is unlikely. Although a major metabolite and long-lasting, cotinine has low affinity and does not substitute for nicotine to reverse nicotine withdrawal-induced anxiety [19; 51]. Alternatively, the long-lasting antiallodynic response to nicotine may result from the involvement of other neurotransmitter or neuromodulatory systems. For example, opioid and serotonergic systems are thought to modulate the analgesic action of nicotine [46]. The understanding of how reduced nAChR expression in DRGs is consistent with increased sensitivity to nicotine is critical and will be investigated in future studies.

In conclusion, neuropathic pain induces downregulation of nAChRs mRNA in tissues that modulate both somatosensory and emotional components of pain. Although changes in mRNA levels do not necessarily directly correlate with protein levels, these dysregulations do not seem to affect positive reinforcement properties of nicotine or cue-induced relapse but may contribute to alter sensitivity to the effects of nicotine that, in turn, may be responsible for changes in nicotine-induced behavioral responses. Such altered processing may lead chronic pain individuals to smoke more cigarettes due to the potent and long-lasting antiallodynic actions of nicotine, which is fully consistent with and can explain the epidemiological studies demonstrating increased cigarette consumption in chronic pain individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Outline of experimental procedures.

Figure 2. Ligature of the L5 spinal nerve produces thermal hyperalgesia and mechanical allodynia that last for over 8 weeks

(A) Mechanical allodynia following unilateral spinal nerve ligation (SNL). Values are the mean force applied in grams (g) $\pm$ SEM for SNL and sham-operated rats (N=7–8 per group).

(B) Hyperalgesic response due to radiant heat. Values are the mean of hindlimb withdrawal latency in seconds (s, average of three trials) $\pm$ SEM for SNL and sham (N=7–8 per group).

Mechanical allodynia and heat hyperalgesia were determined 1 week after surgery in naïve rats. A separate group of rats was used to examine (C) long-lasting mechanical allodynia following SNL. Values are the mean force applied in grams (g) $\pm$ SEM at time point 1, 2, 4 and 8 weeks for SNL and sham (N=8–10 per group). * p <0.05, ** p <0.01 difference from sham. For detailed statistics, see Results.

Figure 3. Expression of genes encoding for α3, α4, α5, α7, β2 and β4 nAChR subunits

Gene expression was analyzed within a network of brain structures including cingulate cortex (CgCx), nucleus accumbens (NAcc), medial habenula (MHb), amygdala, ventral tegmental area (VTA) and periaqueductal gray (PAG) in rats subjected to spinal nerve ligation (SNL) 1 and 4 weeks prior to gene expression assessment and rats receiving sham surgery. The sham group is pooled data from sham rats that control for 1-week and 4-week groups. Expression of the same genes was assessed in L4 and L5 DRGs 4 weeks following injury in a different cohort of SNL and sham rats. GAPDH expression served as normalization control. Data are reported as fold change relative to sham controls. Data are mean (N=6–8 per group) ± SEM. *p<0.05, **p<0.01, ***p<0.001 difference from sham; ## difference from the “1 week” group. ND= not detected (only reactions with appropriate amplification and melting curves were included for analysis). For detailed statistics, see Results.

Figure 4. Intravenous nicotine self-administration patterns of SNL and sham rats

(A) No significant difference in the number of reinforcements earned during the fixed ratio 1 (FR-1) acquisition phase was detected between the two groups. (B) No significant difference in the number of reinforcement earned during the FR-3 maintenance phase of nicotine (30 μ g/kg/inf) lever pressing was detected between SNL and sham rats. (C) Sham and SNL rats also exhibited similar break point measures during the progressive ratio (PR) task for (30 μ g/kg/inf) nicotine. (D) Significant separation of nicotine self-administration patterns under FR-3 was observed when nicotine was available at the dose of 60 μ g/kg/inf. (E) PR contingency to earn a 60 μ g/kg dose did not lead to significant difference between SNL and sham groups. Values are the mean $\pm$ SEM number of infusions in 2 hours daily sessions or the mean $\pm$ SEM break point. (F) SNL rats did not show altered number of lever presses as compared to sham rats during the extinction sessions. Values are the mean $\pm$ SEM number of responses in 1 hour daily session. * $p < 0.05$ difference between SNL and sham ($N = 10-11$ per group). For detailed statistics, see Results.

Figure 5. Repeated cue-induced reinstatement sessions and reinstatement of nicotine seeking induced by various priming nicotine doses in SNL and sham-operated rats
(A) Both SNL (N=10) and sham (N=11) rats showed robust reinstatement of nicotine-seeking behavior following presentation of stimuli (cue-light, tone) previously associated with nicotine. **(B)** There was significant reinstatement induction by nicotine priming doses (0.15 and 0.3 mg/kg, sc) in sham-operated rats (N=7), whereas ligated animals (N=7) failed to show reinstatement of lever pressing at any dose examined. Values represent the mean $\pm$ SEM number of total responses on the nicotine-associated lever in 60 min for both reinstatement paradigms. +++p<0.001 difference from extinction (EXT); ***p<0.001 difference from corresponding EXT response. For detailed statistics, see Results.

Figure 6. Effect of nicotine on anxiety-like response of naïve SNL and sham-operated rats as assessed in the elevated-plus maze (EPM) test 4 weeks after surgery
(A) Nicotine (0.5 mg/kg) produced anxiogenic effect in sham as well as SNL rats as shown by decreased percent (%) time spent exploring open arms. **(B)** Open arm entries. **(C)** The elevated anxiety of nicotine-treated rats was accompanied by decreased exploration of the maze (decreased closed arm entries). Values are presented as mean percent (%) $\pm$ SEM of open arm time and mean $\pm$ SEM number of open and closed arm entries (N=30, 6–8 per group). +p<0.05 difference from vehicle-treated groups. For detailed statistics, see Results.

Figure 7. Antiallodynic responses to mechanical stimulation by von Frey filaments and antinociceptive responses to thermal stimulation in the tail flick test in sham and SNL rats following nicotine treatment

Experiments were conducted beginning 2 and 4 weeks after injury, respectively. 50% withdrawal threshold was determined before (T=0) and 5, 30 and 180 min following nicotine administration (0.0, 0.15 and 0.5 mg/kg, sc) to (A) sham (N=7) and (B) SNL (N=8) rats. Data are the mean $\pm$ SEM force (grams, g) applied. Tail flick latency was determined before (T=0) and 5, 10 and 30 min following nicotine administration (0.0, 0.5 and 1.0 mg/kg, sc) to (C) sham (N=7) and (D) SNL rats (N=7). Data are the mean $\pm$ SEM tail flick latency (seconds, s). * p <0.05 difference from sham (T=0); *** p <0.001 difference from vehicle; # p <0.05 difference from vehicle at the corresponding time point. For detailed statistics, see Results.

Table 1

Primers used to determine expression levels of genes encoding for $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nicotinic acetylcholine receptor (nAChR) subunits in rats subjected to SNL and sham-operated animals.

Rat gene	Alignment sequence	Forward (5'→3')	Reverse (5'→3')	bp
GAPDH	NM_017008.4	GGTCATCCATGACAACCTTTGG	TCAGCTCTGGGATGACCTTG	188
<i>Chrna3</i>	NM_052805.2	GTGGCTGAAGCAAATCTGGAA	CCACCTGGAAATCCCCATCA	146
<i>Chrna4</i>	NM_024354.1	GTTACAACAAGTGGTCTCGGC	TGGTTCTTCTCGTCCACGTC	103
<i>Chrna5</i>	NM_017078.2	ACGTCTGGTTGAAGCAGGAAT	CAGGGAGTCCGAAGGAACAC	98
<i>Chrna7</i>	NM_012832.3	TGCACGTGTCCCTGCAAGGC	GTACACGGTGAGCGGCTGCG	113
<i>Chrb2</i>	NM_019297.1	TGGATGGCGTACGCTTCATT	AAGATCCACAGGAACAGGCG	115
<i>Chrb4</i>	NM_052806.2	TACAACAACCTGATCCGCCC	AGTCCATTCCTGTTTCAGCCA	141

Expression of all investigated genes was normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 2

Amplification efficiency (percent, %) of each primer pair determined based on the slope of standard curves generated by 4, 1:10 serial dilutions of a cDNA sample pooled from each region examined.

Rat gene	R square	Slope	Efficiency (%)
GAPDH	0.9774	-3.277	101.91
<i>Chrna3</i>	0.9943	-3.142	108.098
<i>Chrna4</i>	0.9985	-3.197	105.491
<i>Chrna5</i>	0.9752	-3.115	109.424
<i>Chrna7</i>	0.9998	-3.22	104.437
<i>Chrb2</i>	0.9968	-3.122	109.078
<i>Chrb4</i>	0.9926	-3.12	109.176

Efficiency (%) = $-1 + 10^{(-1/\text{slope})} \times 100$.