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Nicotine decreases nerve regeneration and pain behaviors via PTEN and downstream inflammation-related pathway in two rat nerve injury models

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Data availability
There are no data, software, databases, or application/tools available apart from those reported in the present study. All data are provided in the manuscript.

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Conflict of interest
The authors claim that there are no conflicts of interest.

Author contributions
Yehong Fang, Tingkai Zhang conducted experiments and analyzed the data. Jinsong Tang and Yanhui Liao designed the study. All authors participated in writing the manuscript and gave approval before submission.

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Abstract

Neuropathic pain is stubborn and associated with peripheral nerve regeneration process. Nicotine has been found to reduce pain but whether it is involved in the regulation of nerve regeneration and the underlying mechanism are unknown. In this study, we examined the mechanical allodynia, thermal hyperalgesia together with the peripheral nerve regeneration after nicotine exposure in two rat neuropathic pain models. In the spinal nerve ligation (SNL) model, in which anatomical nerve regeneration can be easily observed, nicotine reduced anatomical measures of regeneration as well as expression of regeneration marker growth associated protein 43 (GAP43). In the tibial nerve crush model, nicotine treatment significantly suppressed GAP43 expression and functional reinnervation as measured by myelinated action potential and electromyography of gastrocnemius. In both models, nicotine treatment reduced macrophage density in the sensory ganglia and peripheral nerve. These effects of nicotine were reversed by the selective α7 nicotinic acetylcholine receptor (nAChR) blocker, methyllycaconitine. In addition, nicotine significantly elevated expression of PTEN (the phosphatase and tensin homolog deleted on chromosome 10), a key player in both regeneration and pain. Pharmacological interference of PTEN could regulate GAP43 expression, pain related behaviors and macrophage infiltration in nicotine-treated nerve crush model. Our results reveal that nicotine and its α7-nAChR regulate both peripheral nerve regeneration process and pain though PTEN and downstream inflammation-related pathway.

Keywords: Nicotine; Nerve regeneration; Neuropathic pain; α7-nAChR; PTEN
**Significance Statement**

Active peripheral nerve regeneration process is closely related to neuropathic pain. Nicotine has demonstrated pain relief properties, but whether nicotine is involved in peripheral nerve regeneration and whether this is related to its analgesic effect are unknown. In two rat nerve injury models, nicotine exposure not only reduced pain behaviors but also inhibited nerve regeneration process in an α7 nicotinic acetylcholine receptor (nAChR)-dependent manner. In addition, nicotine significantly elevated expression of PTEN, a phosphatase and tensin homolog deleted on chromosome 10, and pharmacological interfering with PTEN reversed the inhibitory effect of nicotine on nerve regeneration. These findings provide a novel mechanistic understanding of nicotine’s peripheral effect on both peripheral nerve regeneration and pain.
Introduction

In clinic, tobacco smoking and pain frequently co-occur due to the positive feedback loop between them. Indeed, smokers with pain are more likely to use E-Cigarettes and other nicotine products (Powers et al., 2020). However, accumulating researches also indicate that sustained tobacco smoking alleviates pain and in turn, smoking cessation may exacerbate pain reactivity (Baiamonte et al., 2016; Ditre et al., 2021; Nakajima and Al'Absi, 2014). Subsequent animal studies verify that exposure to nicotine or systemic administration of nicotine has consistent antinociceptive effects in different rodent models (see review (Shi et al., 2010)). Furthermore, intranasal or transdermal nicotine has been explored as an adjunctive treatment for postoperative pain (Matthews et al., 2016).

Neuronal nicotinic acetylcholine receptors (nAChRs), the molecular targets of nicotine, and nicotinic cholinergic mechanisms contribute to dysfunctions such as schizophrenia, Parkinson’s disease, autism, Alzheimer’s disease (AD), and addiction (Dani and Bertrand, 2007). Drugs targeting nAChRs or ACh have curative effects on these diseases such as acetylcholinesterase inhibitors galantamine (Reminyl) for AD, α4β2 partial agonist varenicline for smoking cessation. In addition, nAChRs also be found involved in pain relieving. Hu’s research revealed that nicotine attenuates osteoarthritis pain via the peripheral α7 nAChR (Teng et al., 2019). Another study showed antagonists of α9-containing nAChRs are analgesic in animal models of neuropathic pain (Hone et al., 2018). These evidences support nicotine and nAChRs play vital roles in relieving pain.

Peripheral nerves are capable of regeneration and, when injury, may cause neuropathic pain in the peripheral nervous system (PNS). Macrophage action, chemokine C-C motif ligand 2 (CCL2) and neuroinflammation have positive
effect in regeneration (Zigmond and Echevarria, 2019). Although numerous molecules (mainly cytokines and trophic factors) implicated in both nerve regeneration and pain, few study focus on the relationship between nerve regeneration and pain until the Xie’s report (Xie et al., 2017). They found that active nerve regeneration with failed target reinnervation may be the origin of neuropathic pain. As nicotine and nAChR ligands inhibit macrophage action and neuroinflammation (Godin et al., 2020), we wonder whether nicotine and peripheral nAChR play a role in reducing nerve regeneration and relieving of neuropathic pain.

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN), has been studied extensively in cancers and neurological and psychiatric disorders (Chang et al., 2007; Wang et al., 2015). Besides, it has reported PTEN’s role in neuropathic pain (Huang et al., 2015). Our previous study has also found that spinal astrocytic PTEN plays a beneficial role in pain by regulating cholesterol biosynthesis (Fang et al., 2022). Moreover, studies revealed concurrent activation of PTEN/mTOR and STAT3 pathways as key for sustaining long-distance axon regeneration in adult CNS (Sun et al., 2011). These results indicate PTEN as key player both in nerve regeneration and pain.

In this study, we examined the analgesic effect of nicotine in two rat neuropathic pain models. Then, we reported that nicotine administration reduced peripheral nerve functional and anatomical regeneration in a α7 nAChR dependent manner. One molecule that was downregulated by nerve crush and reversed after intraperitoneal nicotine injection was PTEN. We found that inhibiting PTEN function in the DRG could mimic many of the behavioral and biochemical effects of nicotine in the tibial crush model. The macrophage density in the sensory ganglia and peripheral nerve were also
regulated by nicotine and PTEN. This research sheds light on the dual influence of nicotine on nerve regeneration and pain, providing a novel mechanistic understanding of its peripheral effects.

**Materials and methods**

**Animals and surgery**

All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the Academy of Medical Sciences. Adult Sprague–Dawley rats (female or male, 180 - 220 g) were purchased from the SPF (Beijing) Biotechnology Co., Ltd. Rats were kept in the Laboratory Animal Center of Academy of Medical Sciences, in a temperature- and humidity-controlled environment under a controlled diurnal cycle of 12 h light/dark with free access to food and water. All animal procedures performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Academy of Medical Sciences and were conducted in accordance with the guidelines of the International Association for the Study of Pain.

**For rat spinal nerve ligation (SNL) model** (n = 56), procedures were produced according to the description by Kim and Chung (Ho Kim and Mo Chung, 1992) and our previous study (Chen et al., 2019). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Sigma Aldrich, St. Louis, MO, USA). After dissection of L5 and L4 transverse processes and isolation from the overlying muscles, the ventral ramus of the L5 spinal nerve was exposed. Then, the L5 spinal nerve was tightly ligated with 6-0 silk 2-3 mm distal to the ganglion and then cut 1 mm distal to the suture. The muscle and skin incisions were closed in layers. **For rat tibial nerve crush model** (n=70), the procedures were modified from spared nerve injury (SNI) model (Decosterd and Woolf, 2000). Briefly, under anesthesia by isoflurane, the sciatic nerve and its three terminal branches (the sural and common
peroneal and tibial nerves) were exposed by incising the right thigh skin and blunt dissecting through the biceps femoris muscle. The tibial nerve was then crushed 2 to 3 mm distal to the trifurcation for 30 seconds by a small arterial microclip with smooth protective pads formed by silicon tubing placed over the blades. At the end of this procedure, the tibial nerve was completely flattened and transparent. The incision was closed in layers. For sham operations, the surgery was performed only to expose the L5 spinal nerve or tibial nerve but without ligation and crush.

Drugs and administration
Nicotine, methyllycaconitine (MLA), a selective α7 nAChR blocker (Gowayed et al., 2019; Papke and Horenstein, 2021) and a potent PTEN protector (Lee et al., 2019) Indole-3-carbinol (I3C) were purchased from Sigma–Aldrich (St. Louis, MO). The inhibitor of PTEN, SF1670, was purchased from Selleckchem (Houston, TX, USA). SF1670 and I3C were dissolved in dimethylsulfoxide (DMSO) first and diluted in saline solution while nicotine and MLA were dissolved in saline solution directly. Rats had already received i.p. injections of nicotine at 1 mg/kg and MLA at 1 mg/kg once daily for 1 wk before surgery and continued received i.p. injections of 1 mg/kg nicotine or MLA in sterile saline once daily for 3 wk (Teng et al., 2019), whereas matched control rats received an equal volume of i.p. saline. SF1670 (0.3 mg/kg (Li et al., 2011)) and I3C (2 mg/kg (Lee et al., 2019)) was injected intraperitoneally in nicotine-treated rats simultaneous to surgery date to observe the nociceptive effect of PTEN deficiency and analgesic effects under prevention of PTEN degradation, respectively.

Behavioral testing
Rats were acclimatized to the testing environment for 3 consecutive days before baseline testing and were tested for mechanical and thermal
hyperalgesia in a blind manner to avoid potential bias. Before each test, habituation was performed for 30 minutes, as described in our previous studies (Fang et al., 2022). The region tested in the SNL model was centrally located and within the L4 dermatome, while the test site was moved somewhat more laterally to the sural nerve territory in the tibial nerve crush model. For the Hargreaves test (Hargreaves et al., 1988), a thermal stimulator (BME-410C Plantar Test Apparatus, China) was used. Rats (n = 84) were placed individually in a Plexiglas enclosure (10 × 20 × 20 cm) on top of a 2 mm-thick glass plate, and the plantar surface was exposed to a beam of radiant heat from the stimulator. A cutoff time was 20 s and the test time interval between the tests of two feet was 5 min. The average of 3 recorded test times was considered the paw withdrawal thermal latency (PWL, s). For the Von Frey test, rats (n = 84) were placed in a Plexiglas chamber (10 × 20 × 20 cm) placed on a wire grid floor, and the plantar surface of the hind paw was stimulated using an electronic von Frey Anesthesiometer (Electronic von Frey 2390, IITC Life Science, Woodland Hills, CA, USA). The maximum force (g) was automatically recorded, and the average of 3 successive force (g) readings was used as the paw withdrawal mechanical threshold (PWT, g).

**In vivo electrophysiological recording**

In vivo fiber recording method (Zhu et al., 2022) was used to estimate functional regeneration of myelinated action potential through the tibial crush site. On day 10 after tibial crush treated with saline, nicotine or nicotine plus MLA, rats (n = 18) were anesthetized with sodium pentobarbital (50 mg/kg) with additional boluses of i.v. pentobarbital as needed and put on a warm blanket to keep the body temperature close to 37°C. Both the tibial nerve around the injury site and sciatic nerve were exposed. Skin flaps were produced to form pools filled with warm light mineral oil to isolate the tibial nerve from surrounding nerves. For recording compound action potentials
(CAPs), a 1 mm diameter silver wire was inserted into the sciatic nerve transversely to serve as the recording electrode and a bipolar stimulating cuff electrode was placed distal to the crush site or proximal to the crush site, respectively as a stimulating electrode. For recording evoked electromyography (EMG), a pair of needle recording electrodes was inserted into the gastrocnemius, which is innervated by the tibial nerve. The stimulating electrodes were placed proximal or distal to the crush site. Both CAP and EMG were elicited with 0.5 and 1.0 mA, 1.0 msec stimuli and recorded by a low-noise differential amplifier and analyzed using Spike 2 software (Cambridge Electronic Design, Inc., Cambridge, UK). The fast peak (Aα and Aβ fiber) of the CAP (average value from 3 stimuli per condition) and the peak of EMG were measured and the ratio of distal to proximal amplitude was used as a measure of the fraction of fibers that failed to functionally regenerate through the crush site.

Nerve and DRG microscopy
To measure anatomical of nerve regeneration, the following two methods were used: 1) Images of regenerated spinal nerves after 21 day of SNL surgery were obtained after the dissection of perfused rats, using a ZOOM-90 6.3 megapixel camera inserted into the eyepiece of a dissecting microscope. 2) Fast DiI oil (5 mg/ml in DMSO, D3899, Invitrogen) was injected into the paw subcutaneously using 31 gauge insulin syringes after the surgery of SNL. At the end of behavior testing, sections (13 μm) of L5 dorsal root ganglions (DRGs) and longitudinal sections (40 μm) of proximal L5 spinal nerves were obtained to determine whether DiI had been transported back to the ligated L5 DRG. Co-staining of DiI and subtype of neuronal markers was then conducted (Immunohistochemistry details see below) to show a clear DiI-positive neurons.
Nerve and DRG Immunohistochemistry

Tissues for immunohistochemistry are from rats post 28 days of SNL and 4 days after tibial nerve crush. Rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde in 0.01 M PBS for 30 min. The L3-L5 DRGs in both models, L5 spinal nerve in SNL model and tibial nerve in crush model were isolated, postfixed and cut into sections for immunohistochemistry as described previously (Jiang et al., 2019; Liu et al., 2017). DRG sections were cut at 13 μm while tibial nerve longitudinal sections were cut at 40 μm. The following primary antibodies were used: GAP43 (rabbit, 1:400, Abcam, ab16053.), Iba-1 (goat, 1:500; Abcam, ab5076), NF200 (mouse, 1:400, proteintech, 60331-1-1g), NeuN (mouse, 1:400, Abcam, ab104224), CGRP (rabbit, 1:400, CST, 14959T), TRPV1 (guinea pig, 1:400, Abcam, ab10295), PTEN (rabbit, 1:400, Cayman Chemical, 10005059) and PGP9.5 (guinea pig, 1:400, Abcam, ab10410). Sections were then incubated with the proper secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Alexa Fluor 594-conjugated IB4 (1:400, Invitrogen) was added as a secondary antibody. Negative control followed the same steps without primary antibodies. The stained sections were examined using a laser confocal microscopic imaging system (FV1000 and Olympus FluoView software, Olympus, Japan). Images from 5 to 8 sections of each DRG or nerve then were analyzed with ImageJ software for quantification. The signal intensity of selected area in each channel was summed up. For GAP43 staining, both the neuronal cell bodies and areas containing predominantly axons were analyzed to get the intensity of the signal.

Western blotting

After transcardial perfusion with PBS, the DRGs, L5 spinal nerve and peripheral tibial nerve were harvested and homogenized in lysis buffer (CWBio,
Beijing, China) containing a protease inhibitor cocktail and a phosphate inhibitor cocktail (CWBio). After measuring protein concentrations using a Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), the homogenates (20 µg total protein) were separated on 12% SDS–PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skim milk in Tris buffered saline wash buffer with Tween 20 (TBST), the membrane was incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: GAP43 (rabbit, 1:1000, Abcam, ab16053), Iba-1 (goat, 1:100; Abcam, ab5076), PTEN (rabbit, 1:1000, Abcam, ab170941), GAPDH (rabbit, 1:3000, BBI Life Sciences, 110016). Followed by incubated with an HRP-conjugated secondary antibody for 30 min at room temperature, Bands were detected using an eECL Kit and scanned by the Chemidoc XRS system (Bio–Rad). The intensity of the selected bands was analyzed using ImageJ software.

**Statistical analysis**

SPSS software (version 24.0) was used for statistical analysis. Data values are presented as the mean ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) with Tukey’s post-test was used in western blotting and immunohistochemistry experiment for comparing of differences among 3 or more groups. Two-way ANOVA followed by the Bonferroni post hoc test was used to determine significant differences in behavioral tests. Two-sided tests were used throughout. A statistically significant difference was defined as P value < 0.05.
**Result**

Nicotine alleviates pain behavior in SNL rats in a α7-nAChR dependent manner

Next, we confirmed the analgesic effect of nicotine in animal models. SNL model is a commonly used neuropathic pain model in which we confirm the nicotine's analgesic effect. After SNL, pronounced mechanical allodynia and heat hyperalgesia occurs on day 3 and sustained for many weeks (Ho Kim and Mo Chung, 1992). The nicotine was intraperitoneally (i.p.) injected into naïve rats 7 days before surgery, and continued daily injection at a dose 1 mg/kg. We checked the time course of pain behavior 3, 7, 14, 21, and 28 days after surgery (as shown in the experimental timeline in Fig. 1A). The i.p. administration of nicotine significantly reduced mechanical allodynia and thermal hyperalgesia from 14 to 28 days after surgery compared with that of i.p. injection of saline ($P < 0.01$ vs. saline injection group; Fig. 1B, 1C). In addition, MLA (1mg/kg), the selective α7 nAChR inhibitor, was able to reverse the analgesic effect of nicotine in SNL model (Fig. 1B, 1C). These results indicate that nicotine alleviates neuropathic pain mainly through activating α7 nAChR.

Nicotine reduces anatomical measurement of regeneration in SNL rats

L5 spinal nerve ligation previously supposed could not regenerate or reinnervate and the evoked pain behaviors are deemed to be mediated only by the intact neurons in the L4 DRG. However, in our previous study, we also found ectopic firing of C-neurons and even spontaneous discharges in L5 DRG(Chen et al., 2019). Moreover, direct evidences given by Xie and her colleagues shown that L5 nerve can regenerate into the original sciatic nerve and reconstruct function (Xie et al., 2017). Here, similar to Xie's report, we also observed the nerve regeneration since 2 weeks after the initial SNL surgery and the newly nerve regeneration site became white more like the normal nerve after 28 days (Fig. 2A-C). Nicotine adiministration inhibited L5 spinal nerve regeneration whereas applying both MLA and nicotine seems have no
effect on nerve regeneration compared with that of i.p. injection of saline (Fig. 2D, 2E). Additionally, the neuronal tracer DiI was injected into the ipsilateral hindpaw simultaneously with the SNL surgery to determine whether L5 nerve regenerate after ligation (Fig. 2A). At the end of pain behavior testing, DiI was observed both in L5 DRG neurons and proximal L5 spinal nerves (Fig. 2F-H, L-N). The further co-staining of DiI and neuronal markers showed that DiI could be observed in both NF200-positive myelinated neurons and CGRP- and IB4-positive unmyelinated neurons (Fig. 2I-K). Nicotine administration blocked DiI transmitting to the L5 DRGs which indicated the effect of nicotine on preventing regeneration and that could be reversed by MLA injection (Fig. 2F-H, L-N).

Nicotine reduces GAP43 expression in DRG and spinal nerve after L5 nerve ligation

To examine the effect of nicotine and α7 nAChR on regeneration, the growth associated protein GAP43 expression was examined by immunofluorescence analysis and western blotting. GAP43 is protein kinase C-activated phosphoprotein and often serves as a marker in axonal plasticity and regeneration (Hung et al., 2016). The basal level of GAP43 expression was low in rats with sham surgery and markedly increased (nearly threefold change) at postoperative days 28 in SNL rats (Fig. 3A, 3B). Nicotine significantly reduced the GAP43 expression level and MLA reversed that expression (Fig. 3C, 3D and 3E for summary data). The following western blotting and quantification results further confirmed the effect of nicotine and MLA on GAP43 expression (Fig. 3F). In addition, marked upregulation was also observed in the axon just distal to the L5 DRG and proximal to the ligation site after SNL surgery. The GAP43 expression in axon after nicotine and MLA were changed similar with that in DRG as shown in both immunofluorescence and western-blot analysis in Fig. 4G-L.
Nicotine suppresses neuropathic pain and GAP43 expression after tibial nerve crush

Nerve crush model was commonly used as an experimental model in modeling nerve injury and regeneration (Bridge et al., 1994) such as optic nerve crush in CNS and sciatic nerve crush in PNS. Here, the tibial nerve crush model was used to reconfirm the effect of nicotine on nerve regeneration and neuropathic pain. In contrast to the SNL model, tibial nerve crush significantly induced thermal hyperalgesia and mechanical allodynia from 3 days and peaked at 7 days after surgery compared with sham operation (P < 0.05 or 0.01 Fig. 4A, 4B). The i.p. administration of nicotine significantly reduced mechanical allodynia and thermal hyperalgesia from 7 to 14 days after surgery compared with that of i.p. injection of saline (P < 0.01 vs. saline injection group; Fig. 4A, 4B). In addition, MLA (1mg/kg) could reverse the analgesic effect of nicotine also in nerve crush model (Fig. 4A, 4B).

We also examined the effect of nerve crush on expression of GAP43. As shown in Fig. 4C-F, the GAP43-positive neurons and GAP43-positive axon coursing between the cell bodies in DRG and GAP43-positive axon proximal to the crush site were observed 4 days after nerve crush. Nicotine reduced the intensity of GAP43 expression and the further MLA administration significantly promoted the expression of GAP43 (nearly twofold change compared with the nicotine injection group, P < 0.01).

Nicotine reduces functional measurement of regeneration

The above examination of GAP43 expression in tibial nerve crush model suggested that nicotine may reduce the nerve regeneration in a α7 nAChR dependent manner. In order to determine whether functional regeneration was also reduced by nicotine, both in vivo fiber recording and electromyography (EMG) of gastrocnemius were conducted in crush plus saline, crush plus nicotine and crush plus nicotine plus MLA rats. The recording electrode was
placed on the sciatic nerve while the stimulating electrode was placed firstly on
the tibial nerve distal to the nerve crush site and then moved to the tibial nerve
proximal to the crush site. The CAP of the sciatic nerve was measured twice
and the the ratio of the peak of the A component in CAP from the distal
stimulation to that of the proximal stimulation was analyzed as an indicator of
functional regeneration (Fig. 5A and B). This ratio was significantly lower in
rats that received nicotine and reversed under nicotine plus MLA treatment
(Fig. 5A and C).

The EMG of gastrocnemius innervated by tibial nerve was then recorded in the
same three groups. This time the ratio of EMG from the proximal stimulation to
that of the distal stimulation was analyzed. The quantification results showed
that nicotine also reduced this ratio and MLA counteracted this ratio change
tendency (Fig. 5B and D).

**Tibial nerve crush-induced decreases in PTEN and increases in
microphage density are reversed by nicotine administration**

We previously have found that nerve injury induces astrocytic PTEN
downregulation in rat spinal cord and overexpression of PTEN attenuates
neuropathic pain (Fang et al., 2022), but the PTEN in DRG remains scantily
understood. Here, we measured the immunohistochemistry of PTEN in DRG
and found that PTEN expressed in sensory neurons. Compared with sham
group (34.7%), PTEN-positive neurons were significantly down-regulated on
POD28 (9.2%) for crush rats in DRG (Fig. 6A and B). Next, the effect of
nicotine and MLA on PTEN expression was examined on POD28 crush rats.
Intraperitoneal injection of nicotine upregulated PTEN expression and MLA
reversed that expression (Fig. 6C-E). The following western blotting and
quantification results further confirmed the effect of nicotine and MLA on PTEN
expression (Fig. 6F). According to the double immunofluorescent staining
results, PTEN protein was mainly distributed on sensory neurons and was
costaining with three nociceptive markers, including TRPV1, IB4 and CGRP
(Fig. 6G-I). These data suggested crush nerve injury induces reduction of PTEN expression peripherally in DRG and nicotine mediated PTEN pathway may involve in pain and regeneration.

Previously works have found that nicotine had an obvious anti-inflammatory effect peripherally (Piao et al., 2009). Nicotine and α7-nAChR suppressed M1 macrophage polarization against inflammation (Han et al., 2021). Therefore, we next examined the effect of nicotine on microphage density using the pan-macrophage marker IBA1, in both DRG and distal nerve segment 4 days after crush. As shown in Fig. 7, both immunoreactivity and western blot of IBA1 increased in the tibial nerve crush model was reduced by nicotine and reversed by α7-nAChR blocker MLA, suggesting nicotine’s effect on immune homeostasis in peripheral DRG and nerve.

**Administration of Inhibitor of PTEN SF1670 reversed nicotine’s analgesic effect, GAP43 expression and microphage density**

To determine the role of PTEN on nicotine’s analgesic effect, the PTEN inhibitor SF1670 was injected (i.p.) into the crush rats on POD 3 and continued injecting until POD7 once daily (Fig. 8A). Then, we checked the time course of pain behavior once daily. The i.p. injection of SF1670 (3 mg/kg, 100 μl) reversed nicotine’s analgesic as shown in descending mechanical and thermal thresholds compared with that of i.p. injection of DMSO (P < 0.05 or P < 0.01 vs. DMSO injection group; Fig. 8B and C). In addition, the co-antinociceptive effect of nicotine and indole-3-carbinol (I3C) was also examined in nerve crush rats. I3C is a derivative of glucobrassicin in cruciferous vegetables, which prevents PTEN loss in vivo (Lee et al., 2019). After injection of I3C (2 mg/kg, 100 μl) on day 3 after nerve crush (Fig. 8A), both mechanical allodynia and thermal hyperalgesia induced by nerve crush operation was alleviated but showed no significant compared with nicotine injection group. (P > 0.05; Fig. 8B and C).

The PTEN’s effect on nerve regeneration was also examined by
immunofluorescent staining of GAP43. As expected, SF1670 induced
upregulated GAP43 expression in both DRG and distal nerve segment, as
observed on day 7 after the nerve crush ($P < 0.001$, vs. DMSO group, Fig. 9D-I
and M-N). This upregulation was reduced by I3C injection on day 3 after the
nerve crush (Fig. 8D-I and M-N).

Next, the immunoreactivity of IBA1 reduced by nicotine, and was increased
with PTEN’s inhibitor SF1670. The protector of PTEN I3C injection reduced the
IBA1 signal in DRGs (Fig. 8J-L and O).

Together, these data indicate that PTEN play an essential role in nicotine
related analgesia and inhibition of nerve regeneration.
Discussion

Clinically, it seems intricate and disputable for understanding the interrelationship between nicotine intake and pain condition. Since the comorbidity of tobacco smoking and pain and the reciprocal model of positive feedback loop (Kosiba et al., 2018), a collection of evidences support nicotine may increase pain. The epidemiologic evidence also shows smoking is a risk factor for chronic pain (Shi et al., 2010). However, abundant of empirical and epidemiologic studies reveal that nicotine has analgesic properties, especially in individuals with mental disorders (Crocq, 2003; Ditre et al., 2016). Animal studies show consistent support for direct pain-inhibitory effects of nicotine but remain potential mechanism obscure. Here, we investigated the analgesic effect of nicotine and indicated that nicotine may alleviate peripheral nerve regeneration process and pain though regulating PTEN and downstream inflammation-related pathway.

Present results show that daily injection of nicotine reduced both mechanical and thermal pain intensity robustly in SNL and nerve crush models for 28 days. These data reflect the acute analgesic effect of nicotine on pain, especially neuropathic pain. It is worth noting that the analgesic effect was partial, because the von Frey threshold in two models were regained from low values (<8 g) in saline group to 10 to 13 g in nicotine injection group (as measured on day 14 after surgery), compared with baseline level of nearly 17g (Fig. 2B and Fig. 5A). This is concurred with study in osteoarthritis pain (Teng et al., 2019).

As some studies point that chronic exposure to nicotine may impair the sensitivity of nAChRs (Buisson and Bertrand, 2001), the long-term effect of nicotine is questionable and should be verified in the future. Also the analgesic effect of nicotine on other types of pain, like inflammatory pain, cancer induced pain should be investigated.

How nicotine mitigates pain intensity and experience is currently not fully understood. Nicotine exerts its pharmacological function by interacting with
nAChR family. In peripheral, the α7 and α9α10 nAChRs are found expressed in dorsal root ganglion (DRG) and are vital for pain regulation and relieving (Papke and Horenstein, 2021; Shi et al., 2010; Zhou et al., 2022). Thus, we used α7 nAChR blocker, methyllycaconitine (MLA), to confirm its function on pain and found MLA reversed the analgesic effect of nicotine. This result indicates that nicotine functions as a pain killer in a α7 nAChR dependent manner.

Nerve regeneration per se is the normal physiological process for function recovery after nerve injury but may be the origin of neuropathic pain (Cranfill and Luo, 2022; Gangadharan et al., 2022; Xie et al., 2017). The active nerve regeneration, the miswiring or failed target reinnervation may cause neuropathic pain and blocking regeneration in multiple ways relieves pain (Ritzel and Wu, 2023; Xie et al., 2017). To this end, we wonder nicotine also influence nerve regeneration process. As reinnervation in spared nerve injury (SNI) model often results in neuroma formation (Toia et al., 2015), the SNL and tibial nerve crush model were used, in which the effective nerve regeneration can be observed. To our surprise, nicotine inhibited the newly nerve regeneration in a α7 nAChR dependent manner as showed in the anatomical images and Dil tracer results. The following immunofluorescence and western-bloting analysis of GAP43 (a molecule found strongly correlated with nerve regeneration process (Mahar and Cavalli, 2018; Zhang et al., 2023)) and functional regeneration measured by in vivo fiber recording and electromyography also showed the reduction regeneration after nicotine administration. Taken together, these results indicate that nicotine disrupted nerve regeneration process and this may partly explained nicotine’s analgesic effect. These results also give a reminder that smoking cessation is necessary for smokers after nerve injury.

Neuroimmune crosstalk and macrophages have been uncovered play a role in both pain and nerve regeneration (Domoto et al., 2021; Luo et al., 2021). Both
the tissue-resident macrophages and peripheral nerve macrophages secrete a variety of mediators, such as high mobility group box 1 (HMGB1), interleukin (IL)-1β and IL-6, that regulate the excitability of primary afferents and neurons and contribute to the pathogenesis of inflammatory and neuropathic pain (Sekiguchi et al., 2018). We observed nicotine reduced microphage density in both DRG and distal nerve, which indicated nicotine’s effect on immune and inflammatory. This result is consistent with the idea that nicotine is an anti-inflammation molecule in both nerve system and immune system (Zhang et al., 2022). As M2 phenotype macrophages have been found promote the regeneration and repair (Kwon et al., 2015), the nicotine-induced reduction of regeneration is harder to explain. One possible explanation is that regeneration may require an initial period of type 1 inflammation, and the nicotine’s anti-inflammatory effect weakens the nerve regeneration.

We also show that PTEN reduced in DRG after tibial nerve crush and nicotine reversed this change. Moreover, administration of PTEN’s inhibitor SF1670 reversed nicotine’s effect on both neuropathic pain and regeneration. Studies have found simultaneous deletion of PTEN and SOCS3 enables sustained axon regeneration in CNS (Sun et al., 2011) and overexpression of astrocytic PTEN alleviates neuropathic pain (Fang et al., 2022). Combined with these findings, it is possible that PTEN is a downstream molecule involved in nicotine regulation of pain and regeneration, but the specific mechanism of how nicotine regulates PTEN needs to be further studies in the future.

In conclusion, our study provides evidence for a close association between nicotine, peripheral nerve regeneration, and neuropathic pain. Further mechanistic investigations demonstrate that nicotine inhibit peripheral nerve regeneration and pain through regulating PTEN and downstream anti-inflammatory pathways in a α7-nAChR dependent manner. Insofar as contradiction between nicotine’s effect on regeneration and pain, it is essential to understand whether it possible to block development of pain without inhibiting nerve regeneration, in the condition that such regeneration is
desirable at the early stage of nerve injury.

Reference


cross-sectional investigation between abstinent smokers and nonsmokers. Psychophysiology 51, 1015-1022.


Figure Legends

Fig. 1. Nicotine alleviates mechanical allodynia and thermal hyperalgesia in SNL rats in a α7-nAChR dependent manner. (A) The timeline shows nicotine, MLA and saline injection date, the surgery and tissue harvest date. (B and C) Intraperitoneal injection of nicotine significantly reduced mechanical allodynia (B) and thermal hyperalgesia (C) from 14 to 28 days after surgery compared with that of i.p. injection of saline and MLA (1mg/kg) reversed the analgesic effect (n=6-8/group).*p < 0.05, **p < 0.01, versus the sham group; #p < 0.05, ##p < 0.01, versus the SNL plus saline group; ^^p < 0.01, versus the SNL plus nicotine (1mg/kg) group.

Fig. 2. Nicotine inhibits L5 spinal nerve regeneration in SNL model. (A) Schematic of SNL model and the cut site of L5 spinal nerve. The red arrow shows the injection site of Dil, Dil was injected just after the L5 nerve transection. (B-E) The microscope images demonstrating the regenerated L5 nerve (arrows) distal to the injury site observed on day 14 in saline group and on day 28 in saline group, nicotine group and nicotine plus MLA group. (F-H) Representative images of L5 DRG showing Dil transported from the hindpaw in on day 28 in saline group (F), nicotine group (G) and nicotine plus MLA group(H). (I-K) Double immunofluorescence labeling of Dil with CGRP (I), IB4 (J) and NF200 (K) in the L5 dorsal horn. Scale bar, 50 μm. (L-N) Representative images of L5 spinal nerve proximal to injury site showing Dil transported from the hindpaw in on day 28 in saline group (L), nicotine group (M) and nicotine plus MLA group(N). Scale bar, 100 μm.

Fig. 3. Nicotine reduces GAP43 expression in DRG and spinal nerve after L5 nerve ligation. (A-D) Representative immunofluorescence images of L5 DRG showing GAP43 (red) and neuronal marker PGP9.5 (green) expression in sham group (A), SNL+saline group (B), SNL+nicotine group (C) and SNL+nicotine+MLA group(D). (E) Immunofluorescence intensity analysis shows that the immunoreactivity of GAP43 in the L5 DRG was reduced at
postoperative day 28 in nicotine group and reversed in nicotine plus MLA group. n = 8 sections from 3 rats per group. (F) Representative western blots and quantification of GAP43 in the above four groups. (G-J) Representative immunofluorescence images of L5 spinal nerve showing GAP43 expression in sham group (G), SNL+saline group (H), SNL+nicotine group (I) and SNL+nicotine+MLA group (J). (K) Immunofluorescence intensity analysis shows that the immunoreactivity of GAP43 in the L5 spinal was reduced at postoperative day 28 in the nicotine group and reversed in nicotine plus MLA group. n = 9 sections from 3 rats per group. (L) Representative western blots and quantification of GAP43 in L5 spinal nerve in the above four groups. n = 3. *compared with the sham group, #compared with the SNL group. Scale bar, 50 μm.

**Fig. 4. Nicotine alleviates pain behaviors and reduced GAP43 expression in nerve crush model.** (A and B) nicotine injection alleviated mechanical allodynia (A) and thermal hyperalgesia (B) in comparison with vehicle (saline solution) injection and MLA reversed this effect. n = 7/group. (C-F) DRG sections from cellular regions (C) or axonal regions (D) stained for GAP43 from crush+saline group, crush+nicotine group and crush+nicotine+MLA group. Scale bar, 100 μm. (E and F) Summary data of GAP43 intensity from cellular (E) and axonal (F) regions. **p < 0.01, versus the crush plus saline group; ##p < 0.01, versus the crush plus nicotine group; ^^p < 0.01, versus the SNL plus nicotine (1mg/kg) group.

**Fig. 5. Effect of nicotine on functional measurement of regeneration.** (A) The Schematic and representative recording trace showing in vivo recording setup of compound action potentials (CAPs) ten days after tibial crush injury in saline injection, nicotine injection and nicotine plus MLA injection groups. The CAPs were measured in response to stimulation distal to the injury site (S2) and then proximal to the crush site (S1). (B) The Schematic and representative recording trace showing electromyography (EMG) recording of gastrocnemius
setup ten days after tibial crush injury in saline injection, nicotine injection and nicotine plus MLA injection groups. The EMG was measured in response to stimulation proximal to the crush site (S1) and distal to the injury site (S2). (C and D) the ratio of early component of CAPs (C) and EMG (D) evoked with distal and proximal stimuli were measured in the above three groups, respectively. A ratio value of 1 would be expected as normal nerve and 0 would be expected no regeneration of nerve. N= 6 rats/group. *p < 0.05.

Fig. 6. Nicotine increases PTEN expression in DRG nociceptive neurons
(A-D) Immunostaining shows PTEN in PGP9.5-positive neurons in Sham plus saline, crush plus saline, crush plus nicotine and crush plus nicotine plus MLA groups. Scale bar, 50 μm. (E) The percentage of PTEN positive neurons was reduced in crush plus saline group and reversed in crush plus nicotine groups. n = 3, *p < 0.05 versus the sham plus saline group; #p < 0.05, versus the crush plus saline group; ^p < 0.05, versus the crush plus nicotine group. (F) Western-blot shows nicotine’s effect on PTEN expression. n = 3, *p < 0.05 versus the sham plus saline group (G-I) Double immunofluorescence staining of PTEN with nociceptive cell markers TRPV1, IB4 and CGRP. n = 3. Scale bar, 50 μm.

Fig. 7 Nicotine administration reduces microphage density in DRG (A-D) Immunostaining of IBA1 in sham plus saline, crush plus saline, crush plus nicotine and crush plus nicotine plus MLA groups in DRG. Scale bar, 50 μm (E) The IBA1 intensity shows nicotine reduced microphage density and reversed by MLA. n = 6, ***p < 0.001 versus the sham plus saline group; ###p < 0.001, versus the crush plus saline group; ^^p < 0.01, versus the crush plus nicotine group. (F) Western-blot shows nicotine’s effect on IBA1 expression. n = 3 (G-J) Immunostaining of IBA1 in sham plus saline, crush plus saline, crush plus nicotine and crush plus nicotine plus MLA groups in nerves. Scale bar, 50 μm. (K) The IBA1 intensity analysis. n = 6, ***p < 0.001 versus the sham plus saline group; ###p < 0.001, versus the crush plus saline group; ^^^p < 0.001, versus
Fig. 8 PTEN's inhibitor SF1670 negates nicotine's analgesic effects (A)

Diagram showing nicotine, SF1670 and I3C injection date, the surgery and tissue harvest date and behavioral test timeline. (B and C) intraperitoneal injection of SF1670 significantly reduced mechanical allodynia (B) and thermal hyperalgesia (C) from 4 to 7 days after surgery compared with that of i.p. injection of DMSO (n=6-8/group). *p < 0.05, **p < 0.01, versus the nicotine plus SF1670 group. (D-I) DRG sections from cellular regions (D-F) or axonal regions (G-I) stained for GAP43 from nicotine+DMSO group, nicotine+SF1670 group and nicotine+I3C group Scale bar, 100 μm. (J-L) DRG sections from cellular regions stained for IBA1. (M and N) Summary data of GAP43 intensity from cellular (M) and axonal (N) regions. (O) Summary data of GAP43 intensity from cellular regions ***p < 0.001, versus the nicotine plus DMSO group; ##p < 0.01, versus the nicotine plus SF1670 group.
Figure A: Mechanical allodynia

Figure B: Heat hyperalgesia

Figure C: Imaging of tissue samples under Crush+Saline, Crush+Nic, and Crush+MLA conditions.

Figure D: Comparison of GAP43 intensity levels across different treatment groups.

Figure E: Histogram showing GAP43 intensity for Crush, Nic, and Nic+MLA conditions.

Figure F: Summary of GAP43 intensity across Crush, Nic, and Nic+MLA treatments.
A

Nic+DMSO | Nicotine at 1mg/kg once daily | Nic+DMSO at 0.1ml/10g
Nic+SF1670 | Nicotine at 1mg/kg once daily | Nic+SF1670 at 3mg/kg
Nic+I3C | Nicotine at 1mg/kg once daily | Nic+I3C at 2mg/kg

-7 | 0 | 3 | 4 | 5 | 6 | 7 Day

→ Nerve crush surgery  
→ Behavioral test  
→ Tissue harvest  
→ Drug injection

B

Mechanical allodynia

- Paw withdrawal threshold (g)
- Time after surgery (day)

C

Heat hyperagia

- Paw withdrawal latency (s)
- Time after surgery (day)

D-E-F

GAP43/PGP95

G-H-I

GAP43

J-K-L

IBA1

M-N-O

GAP43 intensity  
DMSO | SF1670 | EC

GAP43 intensity  
DMSO | SF1670 | EC

IBA1 intensity  
DMSO | SF1670 | EC