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BDNF/TRK/KCC2 PATHWAY IN NICOTINE WITHDRAWAL-INDUCED HYPERALGESIA

Abstract

Purpose: To investigate the effect of brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase (Trk) on potassium chloride cotransporter 2 (KCC2) in rats following nicotine withdrawal and the roles played by BDNF/Trk/KCC2 pathway in nicotine withdrawal-induced hyperalgesia. Methods: Seventy-eight rats were randomly assigned to five groups: control group (n = 12) without any treatment, normal saline group (NS group, n = 12) and nicotine withdrawal group (NW group, n = 30) receiving a subcutaneous injection of saline or nicotine for 7 days, respectively. The NW + dimethyl sulfoxide (DMSO) (n = 12) and NW+ Trk antagonist K252a groups (n = 12) received an intrathecal injection of DMSO (10 μl) and K252a (10 μg/10 μl) for 3 days after nicotine withdrawal, respectively. Nicotine withdrawal was precipitated by subcutaneous injection of nonselective and noncompetitive antagonist of nicotinic acetylcholine receptors mecamylamine. Pain was tested using thermal withdrawal latency (TWL). A Western blot was used to examine the expression of BDNF and KCC2. Results: The TWL was significantly decreased in NW group relative to control and NS groups ($P < 0.01$). Compared with the NW group, the NW+K252a group manifested a significantly higher latency ($P < 0.01$). The BDNF expression was increased and KCC2 was decreased in NW group compared with the control group ($P < 0.01$). K252a reduced KCC2 downregulation. Conclusion: BDNF/Trk signaling may contribute to nicotine withdrawal-induced hyperalgesia via downregulation of KCC2.

Keywords

• Nicotine withdrawal • Brain-derived neurotrophic factor (BDNF) • Potassium chloride cotransporter 2 (KCC2)

Introduction

Several clinical studies have indicated that smokers experience greater postoperative pain and require more opioid analgesics than non-smokers [1-3]. It has been reported that the postoperative dosage of opioids correlates with the degree of nicotine dependence in patients undergoing anterior cervical corpectomy [4]. Therefore, current smokers scheduled for surgery are required to stop smoking before surgery. However, smokers deprived of nicotine require more opioid analgesics [5]. We have previously found that nicotine withdrawal induces hyperalgesia in rats [6]. The mechanisms underlying the effect of nicotine on pain are not yet fully understood.

It is well known that tissue injury-induced hypersensitivity is caused peripherally by the sensitization of primary afferent nerve fibers and centrally via modulation of nociceptive transmission at the spinal cord level [7, 8]. Enhanced excitability of spinal cord neurons contributes to central pain sensitivity [7]. The release of brain-derived neurotrophic factor (BDNF) from microglia has been found to control neuronal excitability by causing disinhibition [9]. It has been reported that nicotine receptors contribute to microglial activation, which enhances release of BDNF [9, 10]. Gamma-aminobutyric acid (GABA) is known to mediate neuronal inhibition, which depends on intracellular chloride (Cl^-) concentrations [11]. Cation-chloride cotransporters (CCC) play an important role in the maintenance of intracellular Cl^- concentrations. Potassium chloride cotransporter 2 (KCC2) is the main subtype of CCC in neurons of the central nervous system, and plays an important role in the maintenance of low intracellular Cl^- concentration, which is important for GABA-induced neuronal hyperpolarization [12]. It has been reported that downregulation of KCC2 reduces GABA-induced hyperpolarization, and thus increases neuronal excitability and subsequently reduces the injury-induced pain threshold [13, 14]. However, it remains unclear whether KCC2 is involved in nicotine withdrawal-induced hyperalgesia.

The expression and function of KCC2 is regulated by many signaling pathways such as BDNF signaling [15]. BDNF is released from microglia [16] and acts on the tropomyosin receptor kinase (Trk) in neurons [17]. Intrathecal injection of antibodies against Trk upregulates the expression of KCC2 in the spinal cord [18], suggesting that Trk signaling activation inhibits KCC2 expression. In animal models of inflammation-induced pain [18], neuropathic pain [19], and opiate-induced hyperalgesia [20], BDNF has been found to downregulate KCC2 expression via activation of Trk receptors. However, the involvement of BDNF/Trk/KCC2 signaling in nicotine withdrawal-induced hyperalgesia remains unclear.

In this study, using intrathecal injection of the Trk antagonist K252a, we investigated the effect of BDNF/Trk signaling on the expression of KCC2 in a rat model of nicotine withdrawal. The purpose of this investigation was to study the effect of BDNF in nicotine-induced hyperalgesia, and to explore the role of the BDNF/Trk/KCC2 signaling pathway in nicotine withdrawal-induced hyperalgesia.

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Materials and methods

Animals

The experimental protocols were approved by the Institutional Animal Ethics Committee of Xuzhou Medical College (China). Adult Sprague Dawley rats (male, weighing 180-250 g) were used in this study. The animals were obtained from the Animal Care Center of Xuzhou Medical School. Animals were housed at room temperature (24 ± 4°C), a relative humidity of 50%, and a 12 h light/dark cycle. Animals were fed standard rat chow and water *ad libitum*.

The animals were randomly assigned to five groups: control group (n = 12) receiving no treatment, normal saline group (NS group, n = 12) receiving subcutaneous injection of saline for 7 days, nicotine withdrawal group (NW group, n = 30) receiving subcutaneous injection of nicotine for 7 days, NW + DMSO group (n = 12) receiving intrathecal injection of dimethyl sulfoxide (DMSO, 10 μl) for 3 days after nicotine withdrawal, and NW+K252a group (n = 12) receiving intrathecal injection of K252a (Sigma, St. Louis, MO, USA, 10 μg in 10 μl of saline) for three days after nicotine withdrawal.

Animal model of nicotine withdrawal

A rat model of nicotine withdrawal was created as previously reported with a slight modification. Briefly, rats received subcutaneous injection of nicotine (9 mg/kg/d) three times a day (at 7:00 hrs, 15:00 hrs, and 23:00 hrs) for 7 days. Nicotine withdrawal was precipitated by subcutaneous injection of nonselective and noncompetitive antagonist of nicotinic acetylcholine receptors mecamylamine (1 mg/kg, Sigma, St. Louis, MO, USA) 60 min after the last injection of nicotine. For the NS group, rats received subcutaneous injection of the same volume of saline instead of nicotine. Rats in the control group were subcutaneously injected with mecamylamine (1 mg/kg) on the day 7.

Intrathecal injection

Rats in the NW+DMSO and NW+K252a groups were injected intrathecally with DMSO and K252a, respectively, as previously described [21]. The rats were anesthetized by inhalation of ethyl ether. The rats were held securely in one hand by the pelvic girdle followed by percutaneous injection into the L4-L5 intervertebral space in the midline toward the head, using a 25-μl microsyringe. The injection into the subarachnoid space was verified by the appearance of side tail swing. The injection of K252a or DMSO was completed in 30 s, and the needle was held for 15 s before withdrawal.

Thermal withdrawal latency (TWL) tests

Six rats from each group underwent thermal withdrawal latency (TWL) starting at 8:00 a.m. until 12:00 p.m. daily for 7 days, one day after the last injection of nicotine. TWL tests on rats in the NW+DMSO and NW+K252a groups were performed 1 h after intrathecal injection. TWL tests were performed by a researcher blinded to the experimental conditions. A thermal stimulator (IITC Life Science Inc., Victory Blvd Woodland Hills, CA, USA) was used to measure the sensitivity of the paw to thermal stimuli. Rats were placed on the surface of a 3-mm thick glass plate that was covered with the Plexiglas chamber (26 × 20 × 14 cm). After adaptation for 30 min, thermal stimuli were directed at the exposure site on the hind paw. TWL was defined as the time (in seconds) between the delivery of thermal stimulus and paw withdrawal. A cutoff time of 30 s was defined to avoid tissue damage. The latency was measured from the delivery of five thermal stimuli at 5-min intervals. The latencies from the delivery of three thermal stimuli that had the closest values were used to calculate the mean latency.

Western blot

After TWL tests, three rats from each group were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg/kg). The spinal cord between the L4 and L5 segments was rapidly removed, and stored at -80°C. Tissues were thawed, and homogenized on ice in lysis buffer. Cell lysates were centrifuged at 16,000 rpm for 30 min. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride membranes by electroblotting. Membranes were incubated with primary antibodies against BDNF (polyclonal rabbit anti-rat BDNF antibodies, dilution 1:500, Millipore, Bedford, MA, USA), KCC2 (polyclonal rabbit anti-rat KCC2 antibodies, dilution 1:500, Abcam, Cambridge, MA, USA), and β-actin (polyclonal rabbit anti-rat β-actin, dilution 1:1000, Boster Company, Beijing, China) at 4°C overnight. Membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (dilution 1:5000, Millipore, Bedford, MA, USA) at room temperature for one hour. Bands were visualized using a chemiluminescence detection system, and analyzed using the ImageJ program (public domain open source software, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All values are presented as mean + standard deviation. Time courses measured for TWL were analyzed by two-way ANOVA, followed by Bonferroni *post-hoc* test. After Western blot, the groups were compared by one-way ANOVA, followed by Dunnett's multiple comparison test. Probability values less than 0.05 were considered statistically significant.

Results

K252a reduced nicotine withdrawal-induced hyperalgesia in rats

There were no significant differences in the TWL between control and NS groups. Compared with the control group, the TWL significantly decreased from 1 to 7 days after nicotine withdrawal in the NW group (P < 0.01, Fig. 1), suggesting that nicotine withdrawal induced thermal hyperalgesia in rats. Nicotine withdrawal-induced hyperalgesia increased from day 1, peaked on day 4, and gradually recovered on day 7 after nicotine withdrawal. Compared with the NW group, the NW+K252a group showed a significantly higher TWL on days 2 through 7, suggesting that the intrathecal injection of K252a reduced nicotine withdrawal-induced hyperalgesia. No significant differences were observed in TWL from days 1 through 7 after nicotine withdrawal in the NW and NW+DMSO groups.

Nicotine withdrawal upregulated BDNF expression in the spinal cord

The Western blot showed that BDNF expression in the spinal cord did not differ significantly between the control and NS groups (P > 0.05, Fig. 2). Compared with the control group, BDNF expression in NW group was increased on day 1 (P < 0.01), peaked on day 4, and gradually declined to less than the control level on day 7 after nicotine withdrawal (P < 0.01, Fig. 2).

Nicotine withdrawal downregulated KCC2 expression in the spinal cord

The expression of KCC2 in the spinal cord did not differ significantly between the control and NS groups (P > 0.05, Fig. 3). Compared with the control group, KCC2 expression in NW group was decreased on day 1 (P < 0.01), peaked on day 4, and eventually declined to less than the control level on day 7 after nicotine withdrawal (P < 0.01, Fig. 3).

Intrathecal injection of K252a reduced nicotine withdrawal-induced downregulation of KCC2 expression

Compared with the control group, KCC2 expression in the spinal cord was decreased on day 4 after nicotine withdrawal in the NW group (P < 0.01, Fig. 4). Intrathecal injection of DMSO did not significantly reduce nicotine withdrawal-induced downregulation of KCC2 in the spinal cord. Compared with the NW group, KCC2 expression in the spinal cord was significantly increased in the NW+ K252a group (P < 0.01). The KCC2 expression after intrathecal injection of K252a reached the control level (Fig. 4).

Discussion

In the present study, we found that the TWL significantly decreased from days 1 through 7 after nicotine withdrawal. Intrathecal injection of K252a reduced nicotine-induced hyperalgesia. In addition, we found that nicotine withdrawal increased BDNF expression along with a decrease in KCC2 expression in the spinal cord. Furthermore, intrathecal injection of K252a reduced nicotine withdrawal-induced downregulation of KCC2
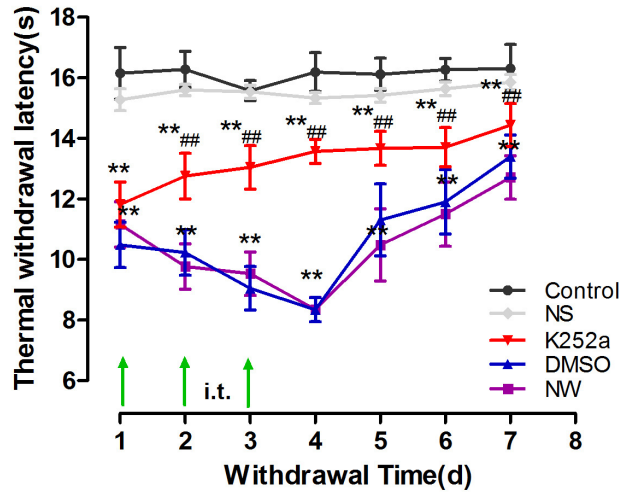

Figure 1. K252a reduced nicotine withdrawal-induced hyperalgesia in rats. The thermal withdrawal latency (TWL) was measured from 1 to 7 days after the last injection in rats in the control, NS, NW, NW+DMSO, and NW+K252a groups (*P < 0.05, **P < 0.05 vs. control; #P < 0.05 vs. NW group).
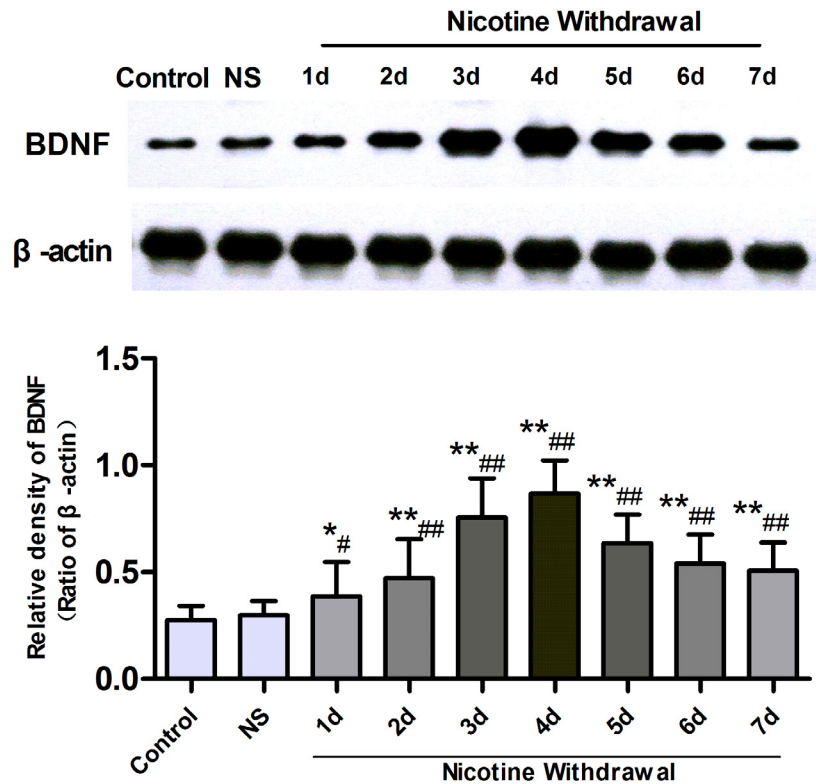

Figure 2. Nicotine withdrawal upregulated BDNF expression in the spinal cord in the control, NS, and NW groups. A. Western blot of BDNF protein expression in the spinal cord. The increase started on day 1 and peaked by day 4. The time course of change in BDNF protein was similar to that of TWL. B. The relative expression of BDNF was normalized to the expression of β-actin (n = 3, *P < 0.05, **P < 0.01 vs. control; #P < 0.05, ##P < 0.01 vs. NS).
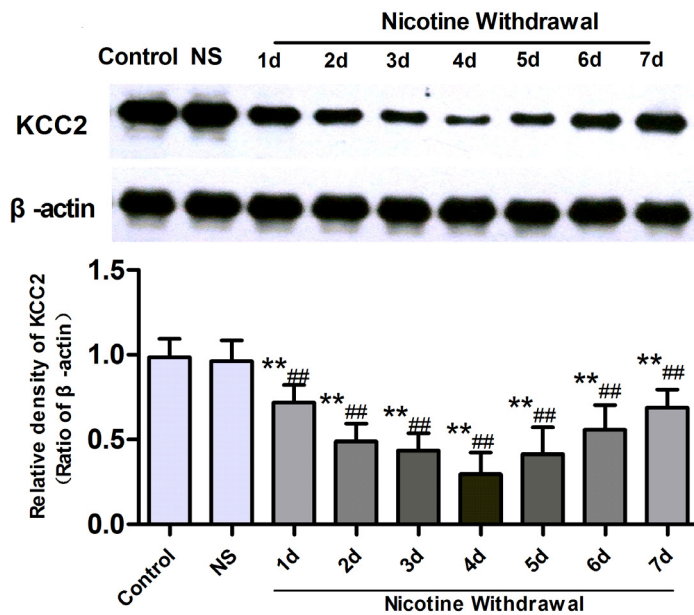
Figure 3. Nicotine withdrawal downregulated KCC2 expression in the spinal cord. A. Western blot results showing the expression of KCC2 protein in the spinal cord in the control, NS, and NW groups. The expression of KCC2 was significantly decreased in NW rats after nicotine withdrawal compared with control and saline groups, and declined to the lowest level by day 4. B. The relative expression of KCC2 was normalized to the expression of β-actin (n = 3, *P < 0.05, **P < 0.01 vs. control; #P < 0.05, ##P < 0.01 vs. NS).
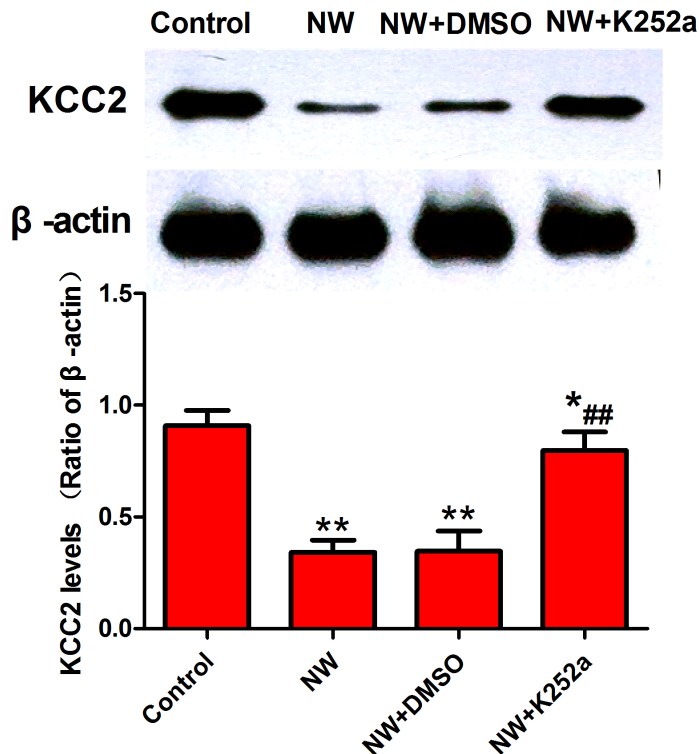

Figure 4. K252a reduced nicotine withdrawal-induced downregulation of KCC2 expression in the spinal cord. A. Western blot of KCC2 protein expression in the spinal cord of the control, NW, NW+DMSO, and NW+K252a groups. Blockade of TrkB with K252a increased the KCC2 protein level in NW rats on day 4. B. The relative expression of KCC2 was normalized to the expression of β-actin (n = 3,*P < 0.05, **P < 0.01 vs. control; #P < 0.05, ##P < 0.01 vs. NW group).

expression. Our study suggests that BDNF/Trk signaling may regulate nicotine withdrawal-induced hyperalgesia via downregulation of KCC2.

KCC2 is necessary for GABA-inhibitory function in the central nervous system. KCC2 has been reported to modulate pain sensation in the spinal cord [13, 14, 22]. Peripheral nerve injury reduced KCC2 expression in the spinal dorsal horn, leading to an increase in the intracellular Cl⁻ concentration, resulting in enhanced neuronal excitability via reduction of GABA inhibition [22]. Increased neuronal excitability due to KCC2 downregulation reduced the pain threshold, and thus induced pain [13, 14]. In addition, it has been reported that nicotine promoted the release of GABA via activation of nicotine receptors [23, 24]. Nicotinic receptors play an important role in the switch from excitatory to inhibitory function of GABA during early neuronal development [25]. The increased permeability of alpha-7-nicotinic acetyl cholinergic receptors (α7-nAChR) to Ca²⁺, leads to termination of GABA excitation and initiation of GABA inhibition [26]. Our findings of nicotine withdrawal-induced hyperalgesia suggest that KCC2 downregulation after nicotine withdrawal may lead to enhanced neuronal excitation by reducing GABA inhibition.

Nicotine exerts analgesic effects via nicotinic acetylcholine receptors (nAChR) centrally and peripherally [27]. The nAChR consist of five transmembrane proteins, and are permeable to cations such as Na⁺, Ca²⁺ and K⁺ [28, 29]. The α7-nAChR are expressed in the microglia of the central nervous system, and mediate the "cholinergic anti-inflammatory pathway" by inhibiting the release of proinflammatory factors[30]. In contrast, nicotine withdrawal promotes the release of proinflammatory factors via disinhibition of the cholinergic anti-inflammatory pathway [31]. Previous studies have shown that nicotine withdrawal activates microglia in rats. BDNF is released from microglia [16], and acts as an endogenous modulator of nociceptive response in the spinal cord [32]. In the present study, we found that the expression of BDNF was increased in the spinal cord after nicotine withdrawal, further suggesting that BDNF contributes to nicotine withdrawal-

induced hyperalgesia. Moreover, multiple lines of evidence have shown that BDNF expression in the spinal cord is increased in rats with peripheral nerve injury-induced neuropathic pain [33, 34]. It has been reported that BDNF upregulation promotes downregulation of KCC2 and results in hyperalgesia.

K252a, a selective Trk inhibitor [35], blocks this effect [22]. K252a inhibits Trk receptors including TrkA, TrkB, and TrkC. Neurotrophins including nerve growth factor (NGF), BDNF, neurotrophic factor 3 (NT-3), and neurotrophic factor 4 (NT-4) bind to these receptors with high affinity. It has been reported that NT-3 [36] and NT-4 [37] are not involved in the pain pathway. In contrast, NGF, which primarily binds to TrkA receptors, plays an important role in inflammatory pain in the peripheral nervous system, and BDNF, which mainly acts on the TrkB receptors, is mediating hyperalgesia in the central nervous system [38]. Both *in vivo* and *in vitro* experiments have shown that activation of the BDNF-Trk pathway [39] leads to downregulation of KCC2 expression, but NGF has no effect on KCC2 expression [40]. In the present study, we found that intrathecal injection of K252a reduced nicotine-induced hyperalgesia, accompanied by an increase in BDNF expression and a decrease in KCC2 in the spinal cord, suggesting that the BDNF/TrkB/KCC2 pathway may be involved in nicotine withdrawal-induced hyperalgesia. However, we cannot exclude the possibility that TrkC may mediate nicotine withdrawal-induced downregulation of KCC2 expression, since K252a, which is not a selective TrkB receptor, was used in the present study. Future studies with a selective TrkB inhibitor such as ANA-12 or a selective BDNF scavenger TrkB-Fc are required to confirm the specific role of the BDNF/TrkB/KCC2 pathway in nicotine withdrawal-induced hyperalgesia.

Conclusion

We found that nicotine withdrawal induced hyperalgesia, accompanied by upregulation of BNDF expression and downregulation of KCC2 expression. Intrathecal injection of K252a reduced nicotine withdrawal-induced effects. Our findings suggest that the BDNF/Trk/KCC2 pathway may contribute to nicotine withdrawal-induced hyperalgesia, and therefore, may be a therapeutic target.

Acknowledgments

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