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Dual Effects of Iptakalim on Nicotine-induced Rat Behavioral Sensitization

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Abstract: Nicotine addiction is considered to drive tobacco product use and thus to be responsible for a variety of diseases. The most abundant form of nicotinic acetylcholine receptors (nAChRs) in the brain is α4β2-nAChR, which responds to nicotine at levels found in the plasma of smokers. Selective blockade of α4β2-nAChRs is likely a key approach for facilitating smoking cessation. We previous found that iptakalim hydrochloride (Ipt) is a potent, α4β2-nAChR-selective antagonist, suggesting a potential impact of this novel compound for smoking cessation. Rat Locomotor activity was measured using a VersaMax animal activity monitor. Nicotine was repetitively injected (0.1 mg day^{-1}, i.p.) to induce behavioral sensitization. To test the effects of Ipt on glutamatergic synaptic plasticity, the patch-clamp whole-cell recording was applied in middle brain slices containing the VTA. Here, we report that repetitive injection of nicotine (free-base, 0.1 mg/kg/day, i.p. for 7 days) induced a significant increase in rat locomotor activity. Pre-injection of Ipt (1 or 10 mg kg^{-1}, i.p.) prevented this nicotinic sensitization. Interestingly, Ipt alone significantly enhanced locomotor activity, suggesting a possibility that Ipt itself increases dopaminergic (DA) signaling from Ventral Tegmental Area (VTA) to Nucleus Accumbens (NAcc). To test this possibility, we examined effects of Ipt on glutamatergic synaptic plasticity in VTA DA neurons under slice patch-clamp recording conditions. The results demonstrated that systemic injection of Ipt (10 mg kg^{-1}, i.p.) significantly increased the ratio of AMPA and NMDA-receptor mediated currents (AMPA/NMDA ratio) after 24 h. Ipt exhibits a dual action on rat locomotor activity: it prevents nicotine- induced behavioral sensitization but Ipt itself promotes rat locomotor activity and VTA glutamatergic synaptic plasticity. These features of Ipt may confer a unique therapeutic potential of this compound for smoking cessation.

Key words: Iptakalim, nicotine, behavioral sensitization, locomotor activity, synaptic plasticity

INTRODUCTION

Nicotine is the addictive substance thought to promote the use of tobacco products and consequent diseases with substantial personal and economic costs (Wu, 2009). It is well known that the interaction of nicotine with nicotinic acetylcholine receptors (nAChRs) in the brain reward-associated regions and circuits contributes to nicotine reinforcement and dependence (Placzek and Dani, 2009; Wu, 2009). The most abundant form of nAChRs in the central nervous system contains α4 and β2 subunits (α4β2-nAChR) (Gopalakrishnan *et al*., 1996; Lindstrom *et al*., 1996; Albuquerque *et al*., 2009). Nicotine binds to and activates α4β2-nAChRs with high affinity at the levels found in the plasma of smokers (Benowitz *et al*., 1989; Lindstrom *et al*., 1996; Fenster *et al*., 1997; Albuquerque *et al*., 2009). α4β2-nAChRs also have been implicated in nicotine self-administration and in diseases such as Alzheimer's disease, Parkinson's disease and epilepsy (Picciotto *et al*., 1995; Lindstrom *et al*., 1996; Cordero-Erausquin *et al*., 2000; Nakamura *et al*., 2001; O'Neill *et al*., 2002; Quik, 2004). Studies using nAChR α4 or β2 subunit knockout mice indicate that these mice fail to show nicotinic agonist-induced increases in striatal dopamine release or discharge frequency of dopaminergic (DA) neurons within the midbrain dopamine system and rapidly cease self-administration of nicotine but not cocaine (Picciotto *et al*., 1998; Marubio *et al*., 2003). In addition, selective enhancement of α4-containing nAChRs is sufficient for nicotine-induced reward, tolerance and sensitization (Tapper *et al*., 2004). Therefore, brain α4β2-nAChRs appear to play pivotal roles in mediation of nicotinic reinforcement and dependence and selective blockade of α4β2-nAChRs is likely an effective strategy for preventing nicotine dependence and promoting smoking cessation.

Iptakalim (Ipt) was initially designed and synthesized for hypertension (Wang *et al*., 2005). Ipt has also been reported to have neuroprotective effects in various

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in vivo and *in vitro* ischemia and Parkinson's disease models (Wang *et al*., 2004; Yang *et al*., 2004, 2005a, b, 2006a, b, 2009a, b; Wang *et al*., 2006; Zhang *et al*., 2009). Furthermore, Ipt has been shown a potential in preventing drug addiction since it inhibits cocaine challenge-induced enhancement of dopamine release in the rat Nucleus Accumbens (NAcc) (Liu *et al*., 2003). We have previously reported that Ipt is a potent antagonist that selectively blocks α4β2-nAChRs heterologously expressed in human SH-EP1 cell line (Hu *et al*., 2006b) or natively expressed in rat midbrain DA neurons (Hu *et al*., 2006a). In addition, Ipt prevents systemic nicotine-induced increase in extracellular DA levels in the NAcc (Liu *et al*., 2006). Together, these lines of evidence suggest that Ipt could be potentially developed as a novel drug for smoking cessation.

In the present study, we further evaluated effects of Ipt on systemic exposure to nicotine- induced rat behavioral sensitization. We found that Ipt prevented nicotine-induced increase in locomotor activity in both low and high doses. Interestingly, Ipt itself exhibited an enhancing effect on locomotor activity and could induce glutamatergic synaptic plasticity in VTA DA neurons. Our data suggest that Ipt is likely an ideal compound for promoting smoking cessation.

MATERIALS AND METHODS

Animals subjects: All experiments were carried out using young adult male Wistar rats (Harlan Labs) between 7 and 9 weeks of age. The rats were housed in an animal facility maintained at constant temperature (20±1°C) and humidity (18-25%). The lighting was set to a regular 12 h light (7 AM)-dark (7 PM) cycle and all experiments took place during the light cycle. Food and water were available *ad libitum*. All experimental protocols were pre-approved by the Institutional Animal Care and Use Committee of the Barrow Neurological Institute.

Measurement of animal locomotor activity: Locomotor activity was measured using a VersaMax animal activity monitor (AccuScan Instruments, Inc., Columbus, Ohio). Open field locomotor experiments were carried out in a Plexiglas box measuring 42×42 cm. A variety of movement types can be measured using activity monitors that consist of a series of horizontal and vertical sensors each consisting of 16 infrared beams located 2.5 cm. apart. Locomotion data were saved directly to a PC computer and analyzed using VersaMax software.

Nicotine sensitization studies: Our daily protocol consisted of giving tested rats an intraperitoneal (i.p.) injection of Phosphate-Buffered Saline (PBS) or test drug and placing them in the recording box for 10 min. They then received a second injection of either PBS or test drug and were monitored without disturbance for a further 60 min. The first three days of the study (accommodation days 1-3) represented an acclimatization period during which the rats received only injections of PBS. In the subsequent 7 days (Test days 1-7) tests were carried out using nicotine (0.1 mg kg^{-1} i.p.) with or without pretreatment with iptakalim.

Patch-clamp whole-cell recordings from VTA slices: Wistar rats (3-4 weeks old) were used as previously described (Gao *et al*., 2010; Jin *et al*., 2011). Briefly, rats were anesthetized (isoflurane USP) and then sacrificed by decapitation. Horizontal midbrain slices (250 μm) containing the VTA region were cut using a vibratome 1000 (Vibratome 1000 plus; Jed Pella Inc., Redding, CA) in an ice-cold artificial cerebrospinal fluid containing (ACSF, in mM): 125 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose. ACSF was continuously saturated with 95% O_2 and 5% CO_2. Conventional whole-cell recordings were made using a patch clamp amplifier (Multiclamp 700A, Axon Instruments, Foster City, CA) under infrared-DIC (differential interference contrast) microscopy as previous described (Gao *et al*., 2010; Jin *et al*., 2011). Data acquisition and analysis were performed using a digitizer (DigiData 1322A, Axon Instruments) and the analysis software pClamp 9.1 (Axon Instruments) and Mini Analysis 6 (Synaptosoft, Leonia, NJ). Signals were filtered at 2 kHz and sampled at 10 kHz. For presynaptic stimulation, a bipolar tungsten stimulation electrode (WPI, Sarasota, FL) placed ~150 μm rostral to the recording electrode was used to stimulate excitatory inputs, using a stimulation pulse of duration 40 μsec and frequency 0.1 Hz. For measurements of the ratio of AMPA and NMDA receptor-mediated currents, the DA neuron was voltage-clamped at +40 mV. Picrotoxin (100 μM) was added to the bath solution to block $GABA_A$-receptor-mediated inhibitory synaptic transmission. First, a stable baseline recording of total evoked excitatory postsynaptic currents (EPSCs) was obtained for 5 min. Then the NMDA receptor antagonist AP5 (50 μM) was applied to the bath for 10 min to obtain AMPA-receptor-mediated EPSCs. Digital subtraction of AMPA-receptor-EPSCs from the total eEPSCs from the same neuron yielded NMDA-receptor-EPSCs. An average of 15 evoked EPSCs was

collected for each type of EPSC. The experimental data were presented as means±standard errors. Statistical analysis was done using Student's t-test (unpaired values) or one-way analysis of variance (ANOVA) with Duncan's multiple comparison when comparing the data obtained from different groups. Values of p less than 0.05 were considered as significant.

All recordings were performed at 31±1°C. The putative DA neurons were identified by their pharmacological and physiological properties. Specifically, VTA DA neurons show the presence of prominent hyperpolarization-induced currents (Gao *et al*., 2010; Jin *et al*., 2011).

Drugs: APV, picrotoxin and (-) nicotine bitartrate were purchased from Sigma-Aldrich (St. Louis, MO). Nicotine was dissolved in PBS and adjusted to pH 7.2-7.4 with 0.1 M sodium hydroxide. Nicotine concentration is expressed as the free base. Iptakalim hydrochloride was a kind gift from Dr. G. Hu (Nanjing Medical University, China).

RESULTS

Systemic nicotine (0.1 or 0.5 mg kg^{-1}) induced an increase in locomotor activity: In initial experiments, we determined an appropriate dose of nicotine that could cause a behavioral sensitization of motor activity. We examined two doses of nicotine (0.1 and 0.5 mg kg^{-1}). After 3 day's accommodation (see detail in Method section), rats were injected with either nicotine or saline for 7 days. The results showed that compared to saline, repeated injections of nicotine (0.5 mg kg^{-1}, i.p.) increased locomotor activity on the first day to the asymptote level (Fig. 1a). Whereas, nicotine 0.1 mg kg^{-1} caused a typical time-dependent behavioral sensitization (Fig. 1b). Thus, we used 0.1 mg kg^{-1} nicotine for the following Ipt experiments.

Ipt prevented nicotine-induced behavioral sensitization: To test the effects of Ipt on nicotine-induced increase in rat locomotor activity, we injected different doses of Ipt 10 min before nicotine injection (0.1 mg kg^{-1}). Experiments were classified into 3 groups: saline-nicotine (Sal-Nic, 0.1 mg kg^{-1}), 1 mg kg^{-1} Ipt-Nic and 10 mg kg^{-1} Ipt-Nic. Results showed that 10 mg Ipt significantly prevented nicotine-induced behavioral sensitization (Fig. 2a). Statistic analysis showed that animal total horizontal movement (averaged total beam breaks from 7 days) were significant different among these 3 groups (one-way

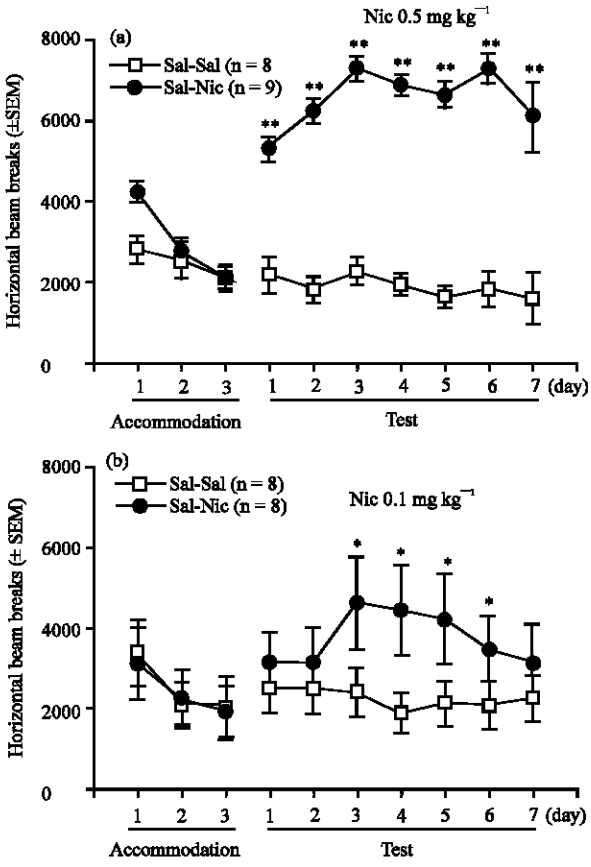

Fig. 1(a-b): Nicotine-induced increase in rat locomotor activity, (a) Rats were initially accommodated for 3 days, Then nicotine (Sal-Nic 0.5 mg/kg/day, i.p.) was administrated for 7 days and the total locomotor activity was measured for 60 min after each nicotine injection, The control rats were injected with saline (Sal-Sal), The results showed that 0.5 mg/kg/day nicotine (free-base) increased locomotor activity at first day and maintain high level for rest tested days, (b) The similar experiments but the nicotine was reduced to 0.1 mg/kg/day, Under this condition, nicotine did not induce a clear increase in locomotor activity at the first two-day but gradually increased it to the top level at the 3rd day, * means $p<0.05$, ** means $p<0.01$ (compared to Sal- Sal group)

ANOVA: F = 7.86, p = 0.0003). Figure 2b summarized the averaged total beam breaks from 3 experimental groups. These results suggest that both high (10 mg) and low (1 mg) doses of Ipt prevents nicotine-induced behavioral sensitization.

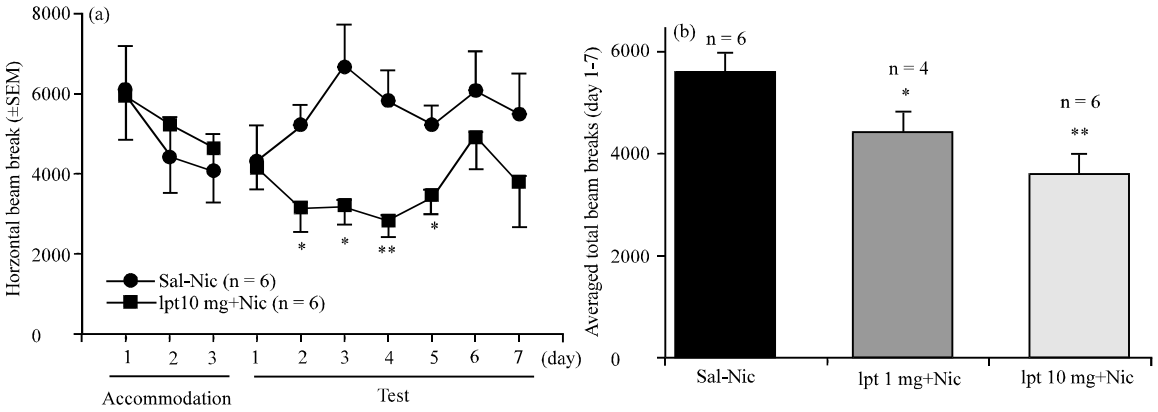

Fig. 2(a-b): Ipt prevented nicotine-induced increase in locomotor activity, (a): The effects of Ipt (10 mg/kg/day, i.p., 10 min prior to nicotine injection) on nicotine-induced behavioral sensitization were superimposed, (b): Bar graph compares the averaged total (1-7 days) locomotor activity among saline, Ipt 1 mg/kg/day and Ipt 10 mg/kg/day groups, * means $p<0.05$, ** means $p<0.01$ (compared to Sal-Nic group)

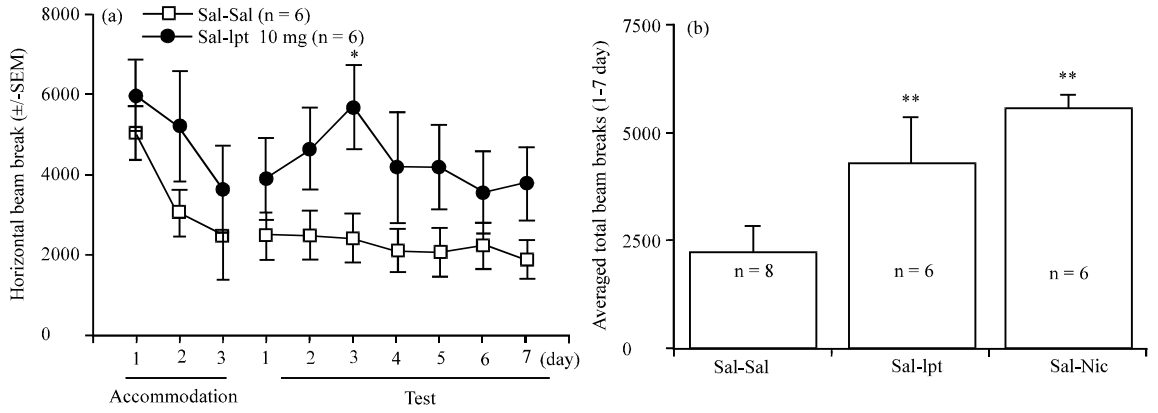

Fig. 3(a-b): Ipt itself increased rat locomotor activity, (a) Comparing to Sal-Sal group, repetitive injection of Ipt alone (10 mg/kg/day, i.p.) increased rat locomotor activity, (b) Bar graph compares the averaged total locomotor activity among Sal-Sal, Sal-Ipt and Sal-Nic groups, *Means $p<0.05$, ** means $p<0.01$ (compared to Sal-Sal group)

Ipt itself induced behavioral sensitization: To test whether Ipt itself affects rat locomotor activity, we examined the effects of Ipt alone (Ipt-Sal) on rat behavioral activity. Surprisingly, 10 mg kg^{-1} Ipt (Ipt-Sal) itself increased locomotor activity compared to Sal-Sal group (Fig. 3). Statistic analysis showed that the averaged total beam breaks of Sal-Sal and Sal-Ipt groups were 2254.6.3±571.8 (n = 8) and 4280.1±1060.9 (n = 6, $p<0.01$), respectively. Comparing to Sal-Nic group, the averaged total horizontal activity of Sal-Ipt group was not significant different ($p>0.05$) (Fig. 3b). These results suggest that Ipt itself increases locomotor activity, which might imply a possibility that Ipt increases extracellular DA level in nucleus accumbens (NAcc).

Ipt induced glutamatergic synaptic plasticity in VTA DA neurons: The results from Fig. 3 raised a question that whether Ipt, like nicotine, can elicit synaptic plasticity in excitatory synapses onto VTA DA neurons. To address this question, we measured the ratio of AMPA and NMDA receptor-mediated currents (AMPA/NMDA ratio) in DA neurons of VTA slices using patch-clamp whole-cell recording technique (Fig. 4a-c). The rats were classified into three groups: Sal-Sal (here Sal is the PBS), Sal-Nic (0.17 mg kg^{-1}, i.p.) and Sal-Ipt (10 mg kg^{-1}, i.p.). After injection of nicotine or Ipt for 24 h, the values of AMPA/NMDA ratio in these 3 groups were 0.48±0.05 (Sal-Sal, n = 6), 0.82±0.09 (Sal-Nic, n = 5, $p<0.01$) and 0.93±0.07 (Sal-Sal, n = 11, $p<0.01$), respectively (Fig. 4d). These results further suggest that Ipt exhibits stimulating effect on VTA-NAcc circuit, which may increase DA level in the NAcc and promote rat locomotor activity.

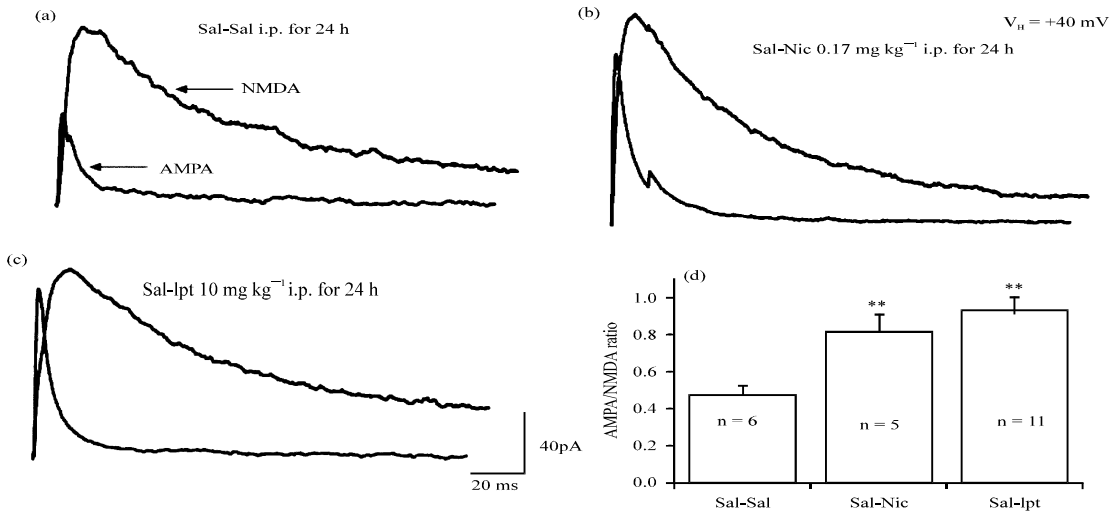

Fig. 4(a-d): Systemic injection of Ipt increased glutamatergic synaptic plasticity in VTA DA neurons, The glutamatergic synaptic plasticity was measured as AMPA/NMDA ratio, (a-c): Representative traces of AMPA and NMDA receptor-mediated currents (indicated in A), (d) Bar graph compares the AMPA/NMDA ratio among these three experimental groups, The number of inside in each column indicates the number of tested cells, ** Means $p<0.01$ (compared to Sal- Sal group)

DISCUSSION

The novel discovery of present study is that Ipt exhibits a dual action on rat locomotor activity. It prevents nicotine-induced behavioral sensitization, but increases locomotor activity on it own. This feature of Ipt on animal locomotor activity matches the concept of "double targets" drug for smoking cessation (Wu, 2010).

We previously reported that Ipt acting as a potent and selective antagonist to block human and rat α4*-nAChR function (Hu *et al*., 2006a, b). In the present experiments, we have extended our previous *in vitro* work and shown that systemic injection of Ipt significantly prevents nicotine-induced increase in rat locomotor activity. This finding is consistent with our previous reports that the Ipt is a selective α4*-nAChR antagonist (Hu *et al*., 2006a, b) and the elimination of nicotine-induced behavioral sensitization by Ipt can be interpreted as its blockade of α4*-nAChRs.

Unexpectedly, this study reveals a new feature of Ipt, it alone increases rat locomotor activity after repetitive exposures (Fig. 3). Since, increase of DA release from VTA to NAcc is the major reason to cause locomotor activity increase, systemic exposure to Ipt alone may increase extracellular DA level in the NAcc. To further confirm this possibility, we compared the effects of Ipt and nicotine on glutamatergic synaptic plasticity (AMPA/NMDA ratio) on VTA DA neurons using patch-clamp recordings. Interestingly, Ipt induced a similar increase in the AMPA/NMDA ratio after single, systemic injection, suggesting that Ipt itself enhances DA signaling in the VTA-NAcc circuit. The mechanism of how Ipt increases DA levels in the NAcc is unclear. One possible explanation is that Ipt blocks α4β2-nAChR function in VTA GABAergic neurons, in turn eliminates endogenously cholinergic modulation and leads to a disinhibition on DA neurons (Pidoplichko *et al*., 2004; Placzek and Dani, 2009). Indeed, systematic administration of the nicotinic antagonist, mecamylamine, reduces cocaine self-administration through this GABAergic mechanism in rats (Levin *et al*., 2000). Another possibility is that Ipt may alter DA transporter function. It has been reported that Ipt enhances glutamate transporter function via the opening of K_{ATP} channels (Yang *et al*., 2004, 2006a; Wang *et al*., 2006). Under our experimental conditions, Ipt seems to close KATP channels (Wu *et al*., 2006), which may impair DA transporter activity. Finally, Ipt may also block KATP channels located on DA terminals and increases DA release. Further study of these underlying mechanisms will be worth to improve our understanding of Ipt pharmacology.

It has been reported that Ipt is a promise compound as an anti-addictive agent based on its ability to inhibit

cocaine challenge-induced enhancement of dopamine levels in the NAcc (Liu *et al*., 2003). Ipt also prevents systemic nicotine-induced increase in extracellular DA level in the NAcc (Liu *et al*., 2006). The present study provides further evidence that Ipt can be developed for smoking cessation. The feature of its dual effects on animal behavioral activity is well matched the concept of double targets for smoking cessation (Wu, 2010). On one hand, Ipt selectively blocks neuronal α4β2-nAChRs that are the key targets to mediate nicotine reward, dependence and addiction (Picciotto *et al*., 1998; Marubio *et al*., 2003; Tapper *et al*., 2004). On the other hand, Ipt increases DA signaling in the VTA-NAcc circuit, which will improve the withdrawal symptoms. Combining its other properties such as water-soluble, penetrates the blood-brain- barrier and has a low side-effect profile when administered systemically, Ipt may an ideal candidate to be developed for smoking cessation.

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