Research article

Nicotine enhances GABAergic inhibition of oxytocin mRNA-expressing neuron in the hypothalamic paraventricular nucleus in vitro in rats

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HIGHLIGHTS

• Nicotine inhibited PVN OT-mRNA expressing neuron in a dose-dependent manner.
• GABA A blocker abolished the nicotine-induced inhibition of OT neuron.
• Nicotine increased sIPSPs frequency of OT-mRNA expressing neurons.
• Nicotine modulated PVN OT secretion via enhancement of presynaptic GABA release.

ARTICLE INFO

Article history:
Received 4 November 2016
Received in revised form 22 November 2016
Accepted 2 December 2016
Available online 5 December 2016

Keywords:
Nicotine
Paraventricular nucleus (PVN)
OT mRNA-expressing neuron
Spontaneous spike firing activity
GABA A receptor

ABSTRACT

We recently found that extracellular administration of nicotine indirectly excited hypothalamic paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) mRNA-expressing neurons. In this study, we studied the effect of nicotine on PVN oxytocin (OT) mRNA-expressing neuron in vitro in rats, by whole-cell patch-clamp recording technique, immunohistochemistry methods and single-cell reverse-transcription multiplex polymerase chain reaction (SC-RT-mPCR) methods. Our results showed that 79.3% (73/92) of the 92 PVN putative magnocellular neurons co-expressed GAPDH mRNA and OT mRNA. Under current-clamp recording conditions, local micro-application of nicotine (1–300 μM) induced a decrease in spontaneous firing rate accompanied with a hyperpolarization of memran potential in 76.7% (56/73) of PVN OT mRNA-expressing magnocellular neurons. The nicotine induced inhibition in spontaneous activity of PVN OT mRNA-expressing magnocellular neurons was dose-dependent. The half-inhibitory concentration (IC 50 ) is 2.9 μM. The nicotine induced hyperpolarization of PVN OT mRNA-expressing magnocellular neurons was sensitive to GABA A receptor antagonist, SR95531 (10 μM) and tetrodotoxin (TTX, 1 μM). In addition, local micro-application of nicotine induced a significant increase in frequency of spontaneous inhibitory postsynaptic potentials (sIPSPs), but without changes in the sIPSPs amplitude of the OT-mRNA expressing neurons. Biocytin staining confirmed that the nicotine-sensitive OT-mRNA expressing neurons were the PVN magnocellular neurons. These results demonstrated that nicotine enhances the GABAergic inhibition, resulting in a decrease in spontaneous firing rate of the PVN OT-mRNA expressing neurons. These findings suggested that nicotine modulated PVN OT secretion via enhancement of both presynaptic action potential drive and quantal GABA release.

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Abbreviations: ACSF, artificial cerebrospinal fluid; GABA, gamma-aminobutyric acid; PVN, paraventricular nucleus; CRH, corticotropin-releasing hormone; OT, oxytocin; sIPSPs, spontaneous inhibitory postsynaptic potentials.
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http://dx.doi.org/10.1016/j.neulet.2016.12.005
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1. Introduction

Hypothalamic paraventricular nucleus (PVN) magnocellular endocrine neurons synthesize vasopressin (VP) and oxytocin (OT) that are released into the bloodstream in response to a variety of physiological stimuli [1]. OTergic neurons are located in the PVN magnocellular division, and their axons project to several brain regions to control the drug-seeking behavior, emotional regulation via activation of oxytocin receptors [2].

Nicotine is arguably the primary additive component in tobacco and its initial actions are via nicotinic acetylcholine receptors (nAChRs) [3]. Binding with nAChRs, nicotine may affect other neurotransmitter systems such as GABAergic neurons and glutamatergic neurons to exert multi-physiological functions. Acute intravenous administration of nicotine has been shown to decrease OT content in the pituitary of rats [4]. However, nicotinic agonists directly increased the excitability of magnocellular neurons under in vitro conditions [5]. We recently found that extracellular administration of nicotine indirectly excited PVN corticotropin-releasing hormone (CRH) mRNA-expressing neurons [6]. On the other hand, systemic administration of OT abolished physical somatic symptoms of nicotine withdrawal in rats [7]. Moreover, brainstem catecholaminergic neurons projecting to the PVN showed a regionally selective and dose-dependent sensitivity to nicotine [8,9], and the PVN-projecting caudal nucleus of the solitary tract (NTS) neurons responded to nicotine via activation of presynaptic nicotinic acetylcholine receptors [10]. Although nicotine modulates PVN neuronal activity has been widely studied, the effect of nicotine on PVN OT neuronal activity is currently unclear. In the present study, we studied the effects of nicotine on PVN OT mRNA-expressing neurons in vitro in rats by whole-cell patch-clamp recordings, bicucullin staining, and single-cell reverse transcription-multiplex PCR techniques. Our results showed that extracellular administration of nicotine indirectly induced PVN OT mRNA expression neuron via an activation of presynaptic GABAergic inputs.

2. Materials and methods

2.1. Hypothalamic slices preparation

Hypothalamic slices from P12–14 day old male Wistar rats. All experiments were approved by the Animal Care and Use Committee of Jilin University and were performed in accordance with the animal welfare guidelines of the National Institutes of Health (permit no. SYXK (ji) 2007-0011). The experimental procedure was as described previously [11]. In brief, the brain was immediately placed into ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 140 NaCl, 3 KCl, 1.3 MgSO4, 1.4 NaH2PO4, 5 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 11 D-glucose, 2.4 CaCl2 and 3.25 NaOH. The pH was 7.3, the osmolality was 290–300 mOsm, and the fluid was bubbled with 100% O2. Coronal hypothalamic slices (250-μm-thick) were prepared using a vibrating brain slicer (Leica VT1200S; Leica Biosystems Nussloch GmbH, Nussloch, Germany). The slices were incubated for at least 1 h in a chamber filled with equilibrated ACSF at room temperature (24–26 °C) before electrophysiological recordings were started.

2.2. Electrophysiological recording and bicucullin histochemistry

Patch pipettes were prepared from thick-wall borosilicate glass (OD-1.5; Narishige, Tokyo, Japan) using a puller (PB-10; Narishige, Tokyo, Japan). They were filled with a solution consisting of (in mM) 120 potassium glutonate, 10 HEPES, 1 EGTA, 5 KCl, 3.5 MgCl2, 4 NaCl, 8 bicucullin, 4 Na2ATP, and 0.2 Na2GTP. The pH was adjusted to 7.3 with KOH. Patch pipette resistances were 5–7 MΩ in the bath, with series resistances in the range of 10–20 MΩ. Membrane potentials and/or currents were monitored using an Axopatch 700 B amplifier (Molecular Devices, Foster City, CA, USA), filtered at 5 kHz, and acquired through a Digidata 1440 series analog-to-digital interface on a personal computer using Clampex 10.4 software (Molecular Devices, Foster City, CA, USA). We performed whole-cell patch-clamp recordings from PVN neurons in hypothalamic slices visualized through a 60 x water-immersion lens using upright microscopy (Eclipse FN1; Nikon Corp., Tokyo, Japan) at room temperature (24–26 °C). After electrophysiological recording, the slice was removed and fixed in 4% paraformaldehyde in 0.1 phosphate buffer. The slices were incubated overnight with the avidin–biotin complex (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) at room temperature. Finally, biocytin was detected using 3,3′-diaminobenzidine tetrahydrochloride histochemistry. Reagents included nicotine, kynurenic acid, TTX and gaba-zine (SR95531) which were bought from Sigma (Sigma-Aldrich, Shanghai, China), and were dissolved in ACSF. Nicotine was applied into the area, 300–500 μm apart from the recorded neuron (Fig. 1E) at 0.1 μl/s by a micro pump (KDS-210, KD Scientific, Holliston, MA, USA). The other chemicals were added to external solutions, and applied at 1 ml/min by a peristaltic pump (Gilson Minipulse 3; Villiers, Le Bel, France). For recording spontaneous inhibitory postsynaptic potentials (sIPSPs), kynurenic acid (1 mM) was routinely included in external recording solutions to block ionotropic glutamate receptor-mediated excitatory postsynaptic events.

2.3. Cytoplasm harvest, reverse transcription, and multiplex and nested PCR

Cytoplasm harvesting and reverse transcription were carried out as previously described [11,12]. After the whole-cell recording, the cytoplasm was aspirated into the patch pipette by applying gentle negative pressure in the pipette while maintaining a tight seal. The pipette contents (8 μl) were expelled into a 0.5 ml test tube containing the reagents for reverse transcription. First-strand cDNA was synthesized for 1 h at 42 °C. Multiplex and nested PCR were carried out as described previously [11]. PCR amplification was performed with a thermal cycler (Mastercycler, nexus gradient; Eppendorf AG, Hamburg, Germany) using a fraction (4 μl) of the single-cell cDNA as a template. First multiplex-PCR was performed as a hot start in a final volume of 30 μl containing 4 μl cDNA, 100 pmol of each primer, 0.3 mM of each dNTP, 3 μl 10 × PCR buffer, and 3.5 U HotStarTaq DNA Polymerase (Takara, Dalian, China) with the following cycling protocol: (a) 15 min at 95 °C, (b) 35 cycles of 1 min at 94 °C, 1.5 min at 57 °C, and 2 min at 72°C, (c) 10 min at 72°C, and then (d) held at 4 °C. The nested primer sequences were as follows: GAPDH (accession no. NM_017008) external sense: 5′-GATTGTTGAAGTGGTGT-GTC3′ (position 849), external antisense: 5′-GGCTAAGCTTGGATGTG-3′ (position 1318); GAPDH internal sense: 5′-TACCAGGCTGGTCTTCTT-3′; internal antisense: 5′-CTCGGTGGTACACATC-3′ (361 bp); OT (accession No. NM_012996) external sense: 59-ACACACCAGAAGGGGATC (position 1814), external antisense: 59-GTCAGGGCAGTGACCAAG (position 2580); OT internal sense: 5′-AGGGCCTTTGGTGAAGAG-3′; internal antisense: 5′-GAGCTCAAAAGGGACACAGC-3′ (416 bp). The amplified fragments were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. All individual PCR products were verified several times by direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems (ABI, Foster City, California, USA) ABI 3130xl genetic analyzer. Sequence comparison was carried out using the BLAST program.
2.4. Statistical analysis

Electrophysiological data were analyzed using Clampfit 10.4 software. Spontaneous spike firing activity and IPSPs were calculated from a train of interspike intervals recorded for 100 s before and during application of drugs, and after washout for 30 min. For normalized data, the data were divided by the mean value of baseline (100 s before application of drugs). All values are expressed as the mean ± S.E.M. and differences were evaluated using Student’s paired t-test or one-way analysis of variance using the SPSS software (SPSS Inc., Chicago, Illinois, USA). P values below 0.05 were considered to indicate a statistically significant difference between the experimental groups.

3. Results

3.1. Effects of nicotine on spontaneous spike firing rate of PVN OT-mRNA expressing neurons

A total of 92 putative PVN magnocellular neurons were sampled under whole-cell current-clamp conditions. These neurons were screened for GAPDH (positive control) and OT mRNA using
Table 1
Properties of PVN OT-mRNA expressing neurons.

<table>
<thead>
<tr>
<th></th>
<th>( V_m ) (mV)</th>
<th>( R_m ) (MΩ)</th>
<th>Frequency (Hz)</th>
<th>Inter spike interval (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine-sensitive</td>
<td>54.9 ± 0.7</td>
<td>483.2 ± 42.8</td>
<td>3.35 ± 0.45</td>
<td>298.6 ± 12.6</td>
</tr>
<tr>
<td>(n = 56)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine-insensitive</td>
<td>57.7 ± 1.1</td>
<td>553.8 ± 58.6</td>
<td>2.35 ± 0.58</td>
<td>452.6 ± 33.4</td>
</tr>
<tr>
<td>(n = 17)</td>
<td>0.008 (7.38)</td>
<td>0.02 (6.2)</td>
<td>0.027 (5.6)</td>
<td>0.001 (12.6)</td>
</tr>
<tr>
<td>P value (F Value)</td>
<td></td>
<td></td>
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</tbody>
</table>

Note: All values are expressed as the mean ± S.E.M. Differences were evaluated using one-way ANOVA.

3.2. Nicotine induced inhibition of the PVN OT mRNA-expressing neuron was prevented by GABA\(_A\) receptor antagonist and TTX

Since the GABA\(_A\) receptor-mediated inhibition was expressed in the presympathetic PVN neurons and plays a key role in the modulation of PVN neuronal excitability [13], we examined the effect of local micro-application of nicotine on spike firing and membrane potential in the presence of GABA\(_A\) receptors antagonist, gabazine (10 \( \mu \)M). As shown in Fig. 3, local micro application of nicotine (10 \( \mu \)M) significantly inhibited the spike firing activity of the OT-mRNA expressing neurons. Bath application gabazine (10 \( \mu \)M) induced a weak increase in spike firing rate (112.8 ± 11.8% of baseline; \( n = 8; \ t = 1.9, P > 0.05 \)), but completely blocked the nicotine induced decrease in spike firing rate (Fig. 3B) and the hyperpolarization of membrane potential (Fig. 3C) in the OT-mRNA expressing neurons. Moreover, we examined the effect of nicotine on the membrane potential in the presence of voltage-gated sodium channel blocker, TTX (1 \( \mu \)M). Local micro application 10 \( \mu \)M nicotine reduced the nicotine-sensitive neurons spontaneous firing rate to 52.4 ± 11.9% of baseline (\( t = 18, P < 0.0001 \) vs. baseline; \( n = 6 \); Fig. 4A). Bath application of TTX abolished the spontaneous spike firing activity and prevented the nicotine induced hyperpolarization of membrane potential (\( t = 4.2, P = 0.01 \) vs. nicotine alone; \( n = 6 \); Fig. 4B). These results indicate that nicotine induced inhibition of PVN OT-mRNA expressing neurons via activation of presynaptic GABAergic components.

To determine whether local micro application of nicotine inhibited the OT-mRNA expressing neuron via increasing presynaptic GABAergic inputs, we examined the effect of local micro application nicotine on sIPSPs in the presence of ionotropic glutamate receptor antagonist, kynurenic acid (1 mM). Local micro application of nicotine did not significantly affect amplitude of sIPSPs (\( t = 1.5, P > 0.05 \)) vs control; \( n = 6 \); Fig. 5A, B), but significantly increased the frequency of sIPSPs (\( t = 26, P = 0.003 \)) vs control; \( n = 6 \); Fig. 5A, C). These results indicated that nicotine inhibited the OT-mRNA expressing neurons via increasing quantal release of GABA.

4. Discussion

In this study, we demonstrated that local micro application of nicotine induced a dose-dependent inhibition of spontaneous activity in PVN OT-mRNA expressing neurons in vitro in rats. The nicotine induced inhibition of PVN OT mRNA-expressing neurons was sensitive to GABA\(_A\) receptor antagonist. Moreover, nicotine significantly increased sIPSPs frequency of the OT-mRNA expressing neurons in the absence of ionotropic glutamate receptors activity. Our results indicated that nicotine indirectly inhibited PVN OT-mRNA expression neuron, suggesting that nicotine could modulate PVN OT secretion via a presynaptic mechanism.

The earlier studies demonstrated that nicotine stimulates the expression of cFos protein in the parvocellular neurons of PVN [14] and acetylcholine evoked a rapidly inward current in PVN magnocellular endocrine neurons in hypothalamic slices via nicotinic acetylcholine receptors (nAChRs), suggesting that nicotine could influence the magnocellular neurosecretory system by directly
increasing the excitability of magnocellular neurons [5]. However, the PVN-projecting neurons respond to nicotine via activation of presynaptic nAChRs and potentiation of synaptic release of glutamate [10].

The GABAergic neurons are located in the PVN, and a greater population of PVN-innervating GABA neurons is found in the hypothalamic zones immediately surrounding the PVN [15]. Moreover, the GABAergic input to the PVN is primarily from the peri-PVN region and the bed nucleus of stria terminals [16]. Iontropic GABAA receptors within the PVN are known to mediate a spatially and temporally restricted inhibition, which is critical for timing-based signaling [17]. The reduction in GABA release by footshock during nicotine self-administration disinhibits CRH neurons, which combined with enhanced glutamate input provides a new mechanism for HPA sensitization to stress by chronic nicotine self-administration [18]. In addition, nicotine modulates multiple regions in the limbic stress network regulating activation of hypophysiotrophic neurons in hypothalamic PVN, which decreased stress-induced c-Fos expression in prelimbic cortex and peri-PVN, but increased c-Fos expression in PVN [19].

OT is released into the PVN and peripheral blood in response to shaker stress, which is suggested that local release of OT into the PVN plays a role in the neuroendocrine stress cascade [20]. The previous study has shown that application of ACh evoked inward currents in OTergic neurons, suggesting that the OTergic neurons are sensitive to nicotine probably via their postsynaptic

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**Fig. 3.** Nicotine induced a decrease in spike firing rate of PVN OT-mRNA expressing neurons was abolished by GABAA receptors antagonist, Gabazine. (A) Under current-clamp recording conditions, representative recording traces showing the spike firing activity of a PVN OT mRNA expression neuron in ACSF, nicotine (10 μM), gabazine (10 μM) and gabazine + nicotine. (B, C) Bar graph showing the normalized spike firing rate (B) and the change in membrane potential (Vm) (C) of PVN OT-mRNA expressing neurons in ACSF, nicotine, gabazine and gabazine + nicotine. * P < 0.05 vs ACSF.

**Fig. 4.** Nicotine-induced inhibition of OT neuronal activity was abolished by TTX. (A) Under current-clamp recording conditions, local micro application of nicotine (10 μM) induced a reversible decrease in spike firing rate accompanied with a hyperpolarization of membrane potential (upper panel). Lower panel, in the presence of TTX (1 μM), re-application of nicotine (10 μM) failed to induce hyperpolarization of the neuron. (B) Summary of data showing the change in membrane potential (Vm) in the presence of nicotine, and a mixture of nicotine + TTX. * P < 0.05 vs ACSF.
nACh receptors [5]. However, our present results showed that nicotine inhibited PVN OT neuronal activity, suggesting that nicotine inhibited OT secretion in PVN via activation of presynaptic GABA<sub>A</sub> receptor. The contractile results were considered to be induced by different methods for drug application. In the previous study, Ach was applied by pulses of pressure ejection within 5–15 μm from the soma of the recorded neuron [5], whereas we here applied nicotine on the distal dendrites of the recorded OT-mRNA expressing neurons by local microinjection methods. Therefore, the local micro application of nicotine on the distal dendrites would activate neuronal presynaptic afferents, but not their postsynaptic nicotinic acetylcholine receptors. Notably, nicotine increased the frequency of sIPSPs, without changing the mean amplitude of sIPSP, which confirmed that local application of nicotine activation GABAergic inputs onto PVN OT-mRNA expressing neuron resulting in a decrease in spike firing rate accompanied by a hyperpolarization of membrane potential. Therefore, our present results indicated that nicotine indirectly modulated OTergic neuronal activity via presynaptic GABA<sub>A</sub> receptor, but not excited the OTergic neurons directly through postsynaptic nicotinic acetylcholine receptors [5]. Taken together, our results showed that local micro application of nicotine induced inhibition of PVN OT mRNA-expressing neuron via enhancing the GABAergic inputs in vitro in rats, suggesting that nicotine could be involved in stress responses via indirectly inhibiting OT secret in PVN.

**Acknowledgement**

This work was supported by the National Natural Science Foundation of China (31260245, 81160142, 31460261, 31560272 and 81260208), and Scientific and Technological Planning Project of Jilin Province, China (20150101070JC).

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