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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.

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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 membrane 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 sEPSPs 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.

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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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 addictive 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, biocytin staining, and single-cell reverse transcription-multiplex PCR techniques. Our results showed that extracellular administration of nicotine indirectly inhibited 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 $MgSO_4$, 1.4 NaH_2PO_4, 5 2-[4-(2-hydroxyethyl)-1- piperazinyl] ethanesulfonic acid (HEPES), 11 D-glucose, 2.4 $CaCl_2$ and 3.25 NaOH. The pH was 7.3, the osmolarity was 290–300 mOsm, and the fluid was bubbled with 100% O_2. 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 biocytin histochemistry

Patch pipettes were prepared from thick-wall borosilicate glass (GD-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 gluconate, 10 HEPES, 1 EGTA, 5 KCl, 3.5 $MgCl_2$, 4 NaCl, 8 biocytin, 4 Na_2ATP, and 0.2 Na_2GTP. 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× 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 gabazine (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 aspired 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′-GATGGTGAAGGTCGGTGTG-3′ (position 849), external antisense: 5′-GGGCTAAGCAGTTGGTGGT-3′ (position 1318); GAPDH internal sense: 5′-TACCAGGG CTGCCTTCTCT-3′, internal antisense: 5′-CTCGTG GTTCACACCCATC-3′ (361 bp); OT (accession No. NM_012996) external sense: 59-ACACACCAGAAGAGGGCATC (position 1814), external antisense: 59-GTCAGAGCCAGTAGGCCAAG (position 2580); OT internal sense: 5′-AGGGCCTTTGGTAGAGCAGT-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.