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Nicotinic acetylcholine receptors containing the α6 subunit contribute to ethanol activation of ventral tegmental area dopaminergic neurons

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ABSTRACT

Nicotine and alcohol are often co-abused suggesting a common mechanism of action may underlie their reinforcing properties. Both drugs acutely increase activity of ventral tegmental area (VTA) dopaminergic (DAergic) neurons, a phenomenon associated with reward behavior. Recent evidence indicates that nicotinic acetylcholine receptors (nAChRs), ligand-gated cation channels activated by ACh and nicotine, may contribute to ethanol-mediated activation of VTA DAergic neurons although the nAChR subtype(s) involved has not been fully elucidated. Here we show that expression and activation of nAChRs containing the α6 subunit contribute to ethanol-induced activation of VTA DAergic neurons. In wild-type (WT) mouse midbrain sections that contain the VTA, ethanol (50 or 100 mM) significantly increased firing frequency of DAergic neurons. In contrast, ethanol did not significantly increase activity of VTA DAergic neurons in mice that do not express CHRNA6, the gene encoding the α6 nAChR subunit (α6 knock-out (KO) mice). Ethanol-induced activity in WT slices was also reduced by pre-application of the α6 subtype-selective nAChR antagonist, α-conotoxin MII[E11A]. When co-applied, ethanol potentiated the response to ACh in WT DAergic neurons; whereas co-application of ACh and ethanol failed to significantly increase activity of DAergic neurons in α6 KO slices. Finally, pre-application of α-conotoxin MII[E11A] in WT slices reduced ethanol potentiation of ACh responses. Together our data indicate that α6-subunit containing nAChRs may contribute to ethanol activation of VTA DAergic neurons. These receptors are predominantly expressed in DAergic neurons and known to be critical for nicotine reinforcement, providing a potential common therapeutic molecular target to reduce nicotine and alcohol co-abuse

1. Introduction

Nicotine and alcohol are the predominant co-abused drugs in the world. A significant proportion of alcoholics smoke and the majority of smokers (~60%) binge drink or consume significant amounts of alcohol [1,2] suggesting a shared mechanism of action between ethanol and nicotine, the addictive component of cigarette smoke. In the mesocorticolimbic "reward" circuitry of the brain, both drugs stimulate dopaminergic (DAergic) neurons in the ventral tegmental area (VTA), to elicit drug reinforcement [3–6].

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that are activated by the endogenous neurotransmitter, ACh [7], as well as the tertiary alkaloid, nicotine. Twelve vertebrate genes encoding neuronal nAChR subunits have been identified (α2–α10, β2–β4) with five subunits coassembling to form a functional receptor [7]. High affinity nAChRs are heteromeric and consist of two or three α subunits coassembled with two or three β subunits. Low affinity receptors are mostly homomeric, predominantly consisting of α7 subunits. Several nAChR sub-types contribute to nicotine-mediated activation of DAergic neurons and reward/reinforcement including α4β2* (* indicates other unidentified subunits may coassemble with the indicated subunits), α6β2*, and α4α6β2* [8–13]. Of the critical VTA nAChR subunits, α6 is predominantly expressed in DAergic neurons [14,15].

While ethanol is not a direct agonist of nAChRs, ethanol may induce an increase in ACh release from cholinergic neuron inputs into the VTA, thereby driving an increase in DAergic neuron activity *via* cholinergic signaling through nAChRs [16]. Interestingly, VTA infusion of α-conotoxin MII, a nAChR antagonist selective for α6, α3, or β3* nAChRs reduces ethanol induced NAc DA release, ethanol consumption and reinforcement in rodents

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[17,18]. In addition, VTA DAergic neurons activated by acute ethanol express greater α6 nAChR subunit transcripts compared to DAergic neurons not activated by ethanol [19]. Interestingly, polymorphisms in CHRNA6, the gene encoding the α6 subunit has been associated with alcohol consumption in a representative sample of patients in the United States [20]. More recently, it has been shown that BAC transgenic mice expressing mutant α6 nAChR subunits that render α6* nAChRs hypersensitive to ACh consume more ethanol and are more sensitive to ethanol reward [21]. While these data implicate a role for α6* nAChRs in ethanol consumption and reinforcement, a direct involvement of this nAChR subtype in ethanol-induced activation of VTA DAergic neurons has not been demonstrated.

We sought to test the hypothesis that α6* nAChRs may be involved ethanol-induced activation of VTA DAergic neurons. Using a combination of pharmacology, genetics, and electrophysiology in a mouse midbrain slice preparation, we demonstrate that nAChRs containing the α6 subunit contribute to ethanol-mediated activation of VTA DAergic neurons.

2. Materials and methods

Animals. α6 knockout (KO) homozygous mice and wild-type (WT) litter-mates (6–10 weeks of age) were used in all experiments. The α6 KO line has been back-crossed to the C57BL/6J strain at least nine generations. WT and KO animals were derived from heterozygous crosses. The genetic engineering of the α6 KO line has been described previously [14]. Animals were housed four/cage until the start of each experiment. Animals were kept on a standard 12-h light/dark cycle with lights on at 7:00 AM and off at 7:00 PM. Mice had access to food and water *ad libitum*. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council [22], as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

2.1. Slice preparation

Mice were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (200 mg/kg, Vortech Pharmaceuticals, Dearborn, MI) and then decapitated. Brains were quickly removed and placed in an oxygenated ice-cold high sucrose artificial cerebrospinal fluid (SACSF) containing kynurenic acid (1 mM, Sigma, St. Louis, MO). Sagittal brain slices containing VTA and lateral dorsal tegmental brain regions (~180 μm) were made using a Leica VT1200 vibratome. The brain slices were incubated in oxygenated EBSS solution supplemented with glutathione (1.5 mg/ml, Sigma), N-ω-nitro-L-arginine methyl ester hydrochloride (2.2 mg/ml, Sigma), pyruvic acid (11 mg/ml, Sigma) and kynurenic acid (1 mM, Sigma) for 45 min at 34 °C. Slices were transferred into oxygenated artificial cerebrospinal fluid (ACSF) at room temperature (24 °C) for recording. SACSF solution contains (in mM, all chemicals purchased from Sigma): 250 sucrose, 2.5 KCl, 1.2 $NaH_2PO_4{\cdot}H_2O$, 1.2 $MgCl_2{\cdot}6H_2O$, 2.4 $CaCl_2{\cdot}2H_2O$, 26 $NaHCO_3$, 11 D-glucose. ACSF solution contains (in mM): 125 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4{\cdot}H_2O$, 1.2 $MgCl_2{\cdot}6H_2O$, 2.4 $CaCl_2{\cdot}2H_2O$, 26 $NaHCO_3$, 11 D-glucose.

2.2. Electrophysiological recordings

Individual slices were transferred to a recording chamber continually superfused with oxygenated ACSF (30–32 °C) at a flow rate of ~2 ml/min. Cells were visualized using infrared differential interference contrast (IR-DIC) imaging on an Olympus BX-50WI microscope. Electrophysiological recordings were recorded using a Multiclamp 700B patch-clamp amplifier (Axon Instruments, Foster City, CA). Action potentials (APs) were obtained in the cell-attached configuration and gap-free acquisition mode in Clampex (Axon Instruments).

I_h currents in whole-cell configuration were elicited every 20 s by stepping from −60 mV to a test potential of −120 mV. APs and currents were filtered at 1 kHz using the amplifier's four-pole, low-pass Bessel filter, digitized at 10 kHz with an Axon Digidata 1440A interface and stored on a personal computer. The junction potential between the patch pipette and bath ACSF was nullified just prior to obtaining a seal on the neuronal membrane. At the end of recording, the cytoplasm was sucked into the recording pipette and the contents in the pipette were expelled into a microfuge-tube for single-cell RT-PCR experiments to verify tyrosine hydroxylase (TH) expression as previously described [13].

Pipette solution contained (in mM, all chemicals purchased from Sigma): 121 KCl, 4 $MgCl_2{\cdot}6H_2O$, 11 EGTA, 1 $CaCl_2{\cdot}2H_2O$, 10 HEPES, 0.2 GTP and 4 mM ATP. Pipette solution was made with DEPC-treated distilled water to prevent RNA degradation. ACSF was used for bath solution. Ethanol was added into ACSF. Ethanol, ACh, and α-conotoxin MII[E11A] were applied to each slice by gravity superfusion. α-conotoxin MII[E11A] was synthesized as previously described [23]. APs were recorded in the presence of a cocktail of inhibitors (Sigma) including atropine (1 μM) to block muscarinic receptors, bicuculline (20 μM) to block $GABA_A$ receptors, and CNQX (10 μM) to block AMPA receptors.

2.3. Data analysis

AP spikes were detected using a threshold detection protocol contained within pClampfit (pClamp v10.2, Axon Inst., Molecular Devices). Average fold change in AP frequency is presented as means ± standard errors of means (SEM). Paired *t*-tests were used to analyze differences between AP frequency at baseline (1 min prior to drug application) and after 10 min application of drug. Unpaired *t*-tests were used to analyze data between genotypes or drug treatment unless otherwise indicated. Results were considered significant at $p < 0.05$.

3. Results

To test the involvement of α6* nAChRs in ethanol-mediated activation of VTA DAergic neurons, we recorded from VTA DAergic neurons in midbrain slices from mice that did not express CHRNA6, the gene encoding the α6 subunit (α6 KO mice) and their wild-type (WT) littermates. Slices containing the VTA were cut in the sagittal plane (Fig. 1A). DAergic neurons typically displayed a relative slow firing rate (1–5 Hz, Fig. 1B) and a slow inward current upon hyperpolarization (Fig. 1C). DAergic neuron identity was verified after recording by detection of tyrosine hydroxylase (TH) mRNA as determine by single neuron RT-PCR (Fig. 1D). To test the effects of ethanol on DAergic neuron activity, AP frequency was monitored in cell-attached mode at baseline, during application of an intoxicating concentration of ethanol (50 or 100 mM), and after wash-out. Because the focus of our experiments was to uncover the contribution of nAChR activation in response to ethanol, recordings were made in the presence of a cocktail of inhibitors to block muscarinic receptor, AMPA receptor, and $GABA_A$ receptor activity (see methods). No significant difference in baseline firing frequency of DAergic neurons was observed between WT and α6 KO animals (5.0 ± 1.8 and 4.0 ± 1.0 Hz, respectively). In WT VTA DAergic neurons, 10 min bath application of both ethanol concentrations produced an increase in AP frequency (~33% and 35% increase from baseline, $p < 0.001$, respectively, Fig 2A and D) that was completely reversed upon wash out. In contrast, ethanol did not significantly increase VTA DAergic neuron activity above baseline in