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A role for α4(non-α6)* nicotinic acetylcholine receptors in motor behavior

Lindsey G. Soll [a], Sharon R. Grady [b], Outi Salminen [c], Michael J. Marks [b], Andrew R. Tapper [a,*]

[a] Brudnick Neuropsychiatric Research Institute, Department of Psychiatry, University of Massachusetts Medical School, 303 Belmont Street, Worcester, MA 01604, USA
[b] Institute for Behavioral Genetics, University of Colorado, Boulder, CO, USA
[c] Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) containing either the α4 and/or α6 subunit are robustly expressed in dopaminergic nerve terminals in dorsal striatum where they are hypothesized to modulate dopamine (DA) release via acetylcholine (ACh) stimulation from cholinergic interneurons. However, pharmacological blockade of nAChRs or genetic deletion of individual nAChR subunits, including α4 and α6, in mice, yields little effect on motor behavior. Based on the putative role of nAChRs containing the α4 subunit in modulation of DA in dorsal striatum, we hypothesized that mice expressing a single point mutation in the α4 nAChR subunit, Leu9′Ala, that renders nAChRs hypersensitive to agonist, would exhibit exaggerated differences in motor behavior compared to WT mice. To gain insight into these differences, we challenged WT and Leu9′Ala mice with the α4β2 nAChR antagonist dihydro-β-erythroidine (DHβE). Interestingly, in Leu9′Ala mice, DHβE elicited a robust, reversible motor impairment characterized by hypolocomotion, akinesia, catalepsy, clasping, and tremor; whereas the antagonist had little effect in WT mice at all doses tested. Pre-injection of nicotine (0.1 mg/kg) blocked DHβE-induced motor impairment in Leu9′Ala mice confirming that the phenotype was mediated by antagonism of nAChRs. In addition, SKF82958 (1 mg/kg) and amphetamine (5 mg/kg) prevented the motor phenotype. DHβE significantly activated more neurons within striatum and substantia nigra pars reticulata in Leu9′Ala mice compared to WT animals, suggesting activation of the indirect motor pathway as the circuit underlying motor dysfunction. ACh evoked DA release from Leu9′Ala striatal synaptosomes revealed agonist hypersensitivity only at α4(non-α6)* nAChRs. Similarly, α6 nAChR subunit deletion in an α4 hypersensitive nAChR (Leu9′Ala/α6 KO) background had little effect on the DHβE-induced phenotype, suggesting an α4(non-α6)* nAChR-dependent mechanism. Together, these data indicate that α4(non-α6)* nAChR have an impact on motor output and may be potential molecular targets for treatment of disorders associated with motor impairment.

1. Introduction

Balanced dopamine (DA) concentrations in striatum (ST) are essential for proper functioning of the basal ganglia circuitry and voluntary movement (Rice et al., 2011). Pathologically low DA concentrations, as caused by progressive neurodegeneration of substantia nigra pars compacta DAergic neurons in Parkinson's disease, leads to motor dysfunction, including akinesia, bradykinesia, resting tremor and catalepsy (Martin et al., 2011). DA release in striatum (as well as other brain regions such as prefrontal cortex and hippocampus) is, in part, modulated by nicotinic acetylcholine receptors (nAChRs), ligand gated cation channels expressed on DAergic cell bodies and terminals, which are activated by the endogenous neurotransmitter, acetylcholine (ACh), as well as by the addictive component of tobacco smoke, nicotine (Grady et al., 2007; Albuquerque et al., 2009; Tang and Dani, 2009). Indeed, within striatum, high basal levels of ACh are achieved via tonic activity of striatal large aspiny cholinergic interneurons, suggesting activation of DAergic terminal nAChRs as key regulators of DA release (Zhou et al., 2001; Quik and McIntosh, 2006; Threlfell et al., 2012).

There are at least three major high affinity populations of nAChRs expressed in DAergic neurons in substantia nigra pars compacta: Those that contain the α4 subunit (α4* nAChR, "*" indicates other subunits coassemble with α4 within a petameric nAChR complex), those that contain the α6 subunit (α6* nAChR),

* Corresponding author. Tel.: +1 5088568164.
E-mail address: andrew.tapper@umassmed.edu (A.R. Tapper).

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and those that contain both subunits (α4α6* nAChR) (Salminen et al., 2004, 2007; Grady et al., 2007; Salminen et al., 2007; Gotti et al., 2010). While the majority of data indicating an involvement of nAChRs in modulating DA release from DAergic nerve terminals stems from studies of rodent synaptosomes and striatal slices (Zhou et al., 2001; Salminen et al., 2004; Zhang et al., 2009; Exley et al., 2012; Threlfell et al., 2012), pharmacological blockade of these receptors in mice have little impact on motor behavior (Dwoskin et al., 2008; Jackson et al., 2009). In addition, mouse models that do not express the genes encoding either α4 or α6 nAChR subunits reveal few motor deficits, perhaps due to compensatory mechanisms (Ross et al., 2000; Champtiaux et al., 2002; Marubio et al., 2003). Thus, the precise impact of α4*, α6*, and α4α6* nAChRs on motor behavior is unclear.

While knock-out mice provide insight into the necessity of a targeted nAChR subunit, an alternative strategy is to study mouse models harboring "gain-of-function" mutations in a nAChR subunit (Lester et al., 2003; Drenan and Lester, 2012). To date, mice with a gain-of-function mutation in both α4 and α6 subunits have been generated (Tapper et al., 2004; Drenan et al., 2008). BAC-transgenic mice expressing α6* nAChR with a point mutation that causes agonist hypersensitivity are hyperactive in both a novel environment and in the home cage (Drenan et al., 2008). However, hyperactivity is abolished by crossing these animals with α4 KO mice, indicating that increased motor activity is a result of α4α6* nAChRs that are hypersensitive to ACh (Drenan et al., 2010). To date, motor activity of α4 gain-of-function mice has not been studied in detail. Therefore, we were interested in elucidating a role for α4* nAChRs in basal ganglia related-movement behavior by analyzing motor behavior in knock-in mice that express α4 nAChR subunits with a point mutation (a leucine mutated to an alanine, the Leu9′Ala line) in the second transmembrane pore-forming region rendering functional receptors 50-fold more sensitive to agonist including ACh (Tapper et al., 2004; Fonck et al., 2005). We hypothesized that, if endogenous ACh stimulation of α4* nAChRs were important for DA-dependent motor behavior, then blockade of these receptors in Leu9′Ala mice would have exaggerated effects helping to uncover the role of these receptors on motor output.

2. Materials and methods

2.1. Animals

Male and female (8- to 14-week-old) Leu9′Ala knock-in mice, α6 KO mice and their wild-type (WT) littermates were used for all experiments. The genetic engineering of Leu9′Ala and α6 KO mice have been described previously (Champtiaux et al., 2002; Tapper et al., 2004). These mice have been backcrossed to the C57BL/6J background for at least 9 generations. Mice, bred at University of Massachusetts Medical School or the Institute for Behavioral Genetics, University of Colorado, were housed four mice/cage, received food and water *ad libitum* and kept on a standard 12-h light–dark cycle. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996) or the guidelines for care and use of mice provided by National Institutes, as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School or the Animal Care and Utilization Committee of the University of Colorado.

2.2. Drugs

Nicotine hydrogen bitartrate, methyllycaconitine citrate salt hydrate, hexamethonium, D-amphetamine hemisulfate salt, Cloro-APB hydrobromide (SKF82958), S-(−)-eticlopride hydrochloride, nomifensine, pargyline, atropine sulfate, bovine serum albumin (BSA) and diisopropylfluorophosphophate (DFP) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Dihydro-β-erythrodine hydrobromide (DHβE) was purchased from Tocris Bioscience Bristol, UK. N-2-(hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES) and sodium salt were products of BDH Chemicals distributed by VWR (Denver, CO). [^{3}H]-dopamine ([^{3}H]-DA) (25–40 Ci/mmol) and Optiphase Supermix scintillation cocktail were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). α-Conotoxin MII (α-CtxMII) was obtained from Dr. J. Michael McIntosh (University of Utah). All drugs administered to mice were dissolved in 0.9% saline and administered via intraperitoneal (i.p.) injection at the indicated doses.

2.3. Motor characterizations

Drug Naïve mice were placed into novel cages and allowed time to habituate to the cage. At time point 0 min, mice were tested for akinesia, catalepsy, clasping and tremor (described below). Immediately after baseline testing, mice were injected with saline or DHβE and characterizations were conducted for each mouse at the indicated time points over a 180 min period. In preliminary experiments, the effects of DHβE on motor phenotypes including locomotor activity, catalepsy, tremor and akinesia was analyzed between genders in Leu9′Ala mice. Because the resulting analysis revealed no significant effect of gender (data not shown), data from male and female mice were combined.

2.4. Akinesia

Every 30 min, mice were placed into an empty cage and held by the tail so hind limbs were hovering above the floor with forelimbs in contact with the floor of the cage. The number of each forelimb steps forward was counted for 30 s. This was repeated and the two trials were averaged together.

2.5. Catalepsy

The forelimbs of mice were placed on a raised bar 5 cm from the floor. Latency to remove both forelimbs off the bar was measured for up to 2 min. Catalepsy was measured every 60 min.

2.6. Clasping and tremor

Mice were tested for clasping and tremor by raising a mouse by the tail for 30 s and giving a score to depict the degree to which the hind limbs were spread apart (clasping) or for severity of a body tremor. The scoring for clasping was as follows: 0 = hind limbs spread wide apart (normal position), 1 = hind limbs 25% closed, 2 = hind limbs 50% closed, 3 = hind limbs 75% closed with periods of hind limbs clasped, 4 = hind limbs fully clasped for 10 s. The severity of a body tremor was scored: 0 = no tremor, 1 = isolated twitches, 2 = non-continuous tremors, 3 = continuous tremor.

2.7. Locomotor activity

For all experiments measuring locomotor activity, mice were given saline injections once a day for 3 days prior to the experiment to reduce differences in locomotor activity due to stress from the injection and handling. Additionally, on the day of the experiment, mice were habituated to the room for 1 h to reduce differences in locomotor activity due to changes in environment unrelated to the novel cage. To measure locomotor activity, mice were placed into an individual cage within an infrared photobeam frame (San Diego Instruments) to freely roam for 30 min. Locomotor activity was measured by quantifying the number of beam breaks. Mice were challenged with saline or DHβE and placed into the locomotor chamber at the times indicated. Locomotor experiments were counterbalanced such that mice were exposed to either saline or drug and then one week late, drug treatments were switched. Thus, each mouse served as its own control. Additional drug treatments (MLA and nicotine) and blocking experiments were tested in separate groups of mice. On the day of the experiment, mice were pre-injected with saline, nicotine, SKF82958, eticlopride, or amphetamine followed by saline (i.p.) or DHβE 5 min after pre-injection as indicated. For the amphetamine rescue experiment, mice were injected with DHβE followed by an injection of saline or amphetamine 15 min after the first injection. Mice were placed into locomotor chambers at the times indicated post injection and locomotor activity was measured for 30 min.

2.8. Immunofluorescence

To avoid neuronal activation due to stress induced handling, all mice were injected with saline once a day for 3 days before the experiment. Separate groups of drug naïve Leu9′Ala and WT mice received either saline or DHβE and perfused 150 min later. Prior to perfusion, mice were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) and then perfused transcardially with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 10 mls ice-cold 4% (W/V) paraformaldehyde (PFA) dissolved in 0.1 M PBS (pH 7.5). The brains were harvested and post-fixed in PFA solution for 4 h and then cryoprotected in PBS containing 30% sucrose until the brain was fully fixed (>72 h) in sucrose solution. Coronal sections (20 μm thick) containing the striatum (ST) (between 1.18 and 0.38 from bregma) and the substantia nigra (SN) (between −2.92 mm and −3.8 mm from bregma) were sliced on a microtome (Leica SM 2000 R, Leica Microsystems Inc., Bannockburn IL, USA) and collected into a 24-well plate containing 1x PBS. Sections were washed for 5 min in 1× PBS, placed into 0.4% Triton X-100 PBS (PBST) for 5 min, washed again