Electrophysiological, pharmacological and molecular evidence for α7-nicotinic acetylcholine receptors in rat midbrain dopamine neurons

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Abbreviations: ACh, acetylcholine; Bgt, α-bungarotoxin; BSA, bovine serum albumin; DA, dopamine or dopaminergic; $I_{ACh}$, current response induced by acetylcholine; $I_{choline}$, current response induced by choline; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor(s); PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; SNc, substantia nigra pars compacta; $V_H$, holding potential; VTA, ventral tegmental area.

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ABSTRACT

Dopamine (DA) neurons located in the mammalian midbrain have been generally implicated in reward and drug reinforcement and more specifically in nicotine dependence. However, roles played by nicotinic acetylcholine receptors, including those composed of α7-subunits (α7-nAChR), in modulation of DA signaling and in nicotine dependence are not clearly understood. Although midbrain slice recording has been used previously to identify functional α7-nAChR, these preparations are not optimally designed for extremely rapid and reproducible drug application, and rapidly desensitized, α7-nAChR-mediated currents may have been underestimated or not detected. Here, we use patch-clamp, whole-cell current recordings from single neurons acutely dissociated from midbrain nuclei and having features of DA neurons to characterize acetylcholine-induced, inward currents that rapidly activate and desensitize, are mimicked by the α7-nAChR-selective agonist, choline, blocked by the α7-nAChR-selective antagonists, methyllycaconitine and α-bungarotoxin, and are similar to those of heterologously expressed, human α7-nAChR. We also use reverse transcriptase-polymerase chain reaction, in situ hybridization and immunocytochemical staining to demonstrate nAChR α7 subunit gene expression as message and protein in the rat substantia nigra pars compacta and ventral tegmental area. Expression of α7 subunit message and of α7-nAChR-mediated responses is developmentally regulated, with both being absent in samples taken from rats at postnatal-day-7 (P7), but later becoming present and increasing over the next two weeks. Collectively, this electrophysiological, pharmacological and molecular evidence indicates that nAChR α7 subunits and functional α7-nAChR are expressed somatodendritically by midbrain DA neurons, where they may play important physiological roles and contribute to nicotine reinforcement and dependence.
INTRODUCTION

Dopaminergic (DA) neurons located in the midbrain reward center, including the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), play pivotal roles in drug reinforcement, motility and associative motor learning (Dani and Heinemann, 1996; Berke and Hyman, 2000). An increase in the release of DA from VTA neurons onto their targets in the nucleus accumbens is thought to contribute to drug reinforcement. Moreover, accumulating evidence supports the idea that the midbrain DA system plays a key role in nicotine dependence (Di Chiara, 1999; Dani and De Biasi, 2001). For example, in vivo studies have demonstrated that nicotine self-administration is significantly reduced by blockade of midbrain DA receptors or by lesions of DA neurons (Corrigall and Coen, 1989; Corrigall et al., 1994).

There are indications that nicotinic acetylcholine receptors (nAChR) play important roles in midbrain DA function that may be of particular relevance to nicotine dependence. For example, a high density of nAChR subunit expression is observed in the VTA and SNc (Wada et al., 1989; Azam et al., 2002), where they could assemble as diverse nAChR subtypes to serve as targets of nicotine and become involved in mediation of nicotine self-administration, and perhaps in reinforcement of other biologically rewarding events (Dani & Heinemann, 1996; Stolerman & Shoaib, 1991). Nicotine, acting on post- or pre-synaptic nAChR, can modulate DA release (Jones et al., 2001; Wonnacott et al., 2000; Zhou et al., 2001). Furthermore, DA neuronal activity in the VTA or SNc is also sensitive to the effects of nicotine in stimulating or desensitizing postsynaptic nAChR (Pidoplichko et al., 1997; Wooltorton et al., 2003).

nAChR containing α7 subunits (α7-nAChR) are prominent in both the vertebrate central and autonomic nervous systems. The presence of α7-nAChR identified by the
existence of \(\alpha\)-bungarotoxin (Bgt)-binding sites has been known for many years (Morley et al., 1979; Schmidt, 1988; Clarke, 1992; Sargent, 1993). These Bgt-binding sites have been known 1) to exhibit many of the biochemical and pharmacological features characteristic of true \(\alpha_7\)-nAChR, 2) to have brain distributions sub- or peri-synaptic to cholinergic terminals, and 3) to have levels of expression sensitive to chronic nicotine exposure and/or modification of cholinergic inputs (Lukas and Bencherif, 1992).

Subsequent study of the function of natural, Bgt-binding nAChR uncovered short-lived, nicotine-gated, toxin-sensitive, inward currents and/or elevations of intracellular Ca\(^{2+}\) in chick autonomic neurons (Vijayaraghavan et al., 1992; Zhang et al., 1996), in human ganglionic neuron-like clonal cells (Puchacz et al., 1994), and in rat CNS immature neurons maintained in primary cell culture (Alkondon et al., 1996; Bonfante-Cabarcas et al., 1996; Zorumski et al., 1992; McGee et al., 1995). Many cell types in the CNS that naturally express Bgt-binding sites have been shown to express \(\alpha_7\) subunit genes (Lukas et al., 1993). Knockout of the \(\alpha_7\) subunit gene leads to the absence of Bgt-binding nAChR in cell lines or in mice, and these animals also lack rapid kinetic responses to nicotinic agonists (Puchacz et al., 1994; Orr-Urtreger et al., 1997).

nAChR \(\alpha_7\) subunit mRNA has been detected in about 50% of the neurons tested from the SNc and VTA (Elliott et al., 1998; Klink et al., 2001), suggesting involvement of \(\alpha_7\)-nAChR in midbrain DA neuronal function. Previous patch-clamp recordings from neurons in midbrain slice preparations have demonstrated that high concentrations of acetylcholine (ACh) or choline induce whole-cell current responses with fast kinetics sensitive to blockade by Bgt or the \(\alpha_7\)-nAChR-selective antagonist, methyllycaconitine (MLA; Pidoplichko et al., 1997; Klink et al., 2001; Wooltorton et al., 2003; Champtiaux et al., 2003). However, perfusion of brain-slice preparations is slow, complicating the
ability to rapidly, and reproducibly, apply and remove drugs, as is required to detect and characterize rapidly desensitized $\alpha_7$-nAChR-mediated currents (Gray et al., 1996; Pidoplichko et al., 1997). Therefore, there may be deficiencies in our understanding of the physiology, pharmacology and channel properties of functional nAChR, including $\alpha_7$-nAChR, in midbrain DA neurons.

Here, we provide studies employing both perforated patch-clamp recording and molecular biological techniques describing the properties of pharmacologically-identified, somatodendritic $\alpha_7$-nAChR on acutely-dissociated, single DA neurons from the VTA and SNc. Our studies reveal new potential mechanisms by which somatodendritic, midbrain neuronal $\alpha_7$-nAChR may contribute to physiologically-important DA neuronal activity of possible relevance to nicotine dependence.
METHODS

**Acutely-dissociated DA neurons.** Single, DA neurons were acutely dissociated from the VTA or SNc of 7-28-day-old Wistar rats as previously reported (Wu and Partridge, 1998). Briefly, rats were anaesthetized using halothane. Brain tissue was rapidly removed and immersed in cold (2-4°C) artificial cerebrospinal fluid (ACSF) containing: 124 mM NaCl, 5 mM KCl, 24 mM NaHCO₃, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 2.4 mM CaCl₂, 10 mM glucose, pH 7.4, continuously bubbled with 95% O₂ - 5% CO₂. Three 400 µm coronal slices, including the SNc and VTA, were cut using a vibrotome (Vibroslice 725M, WPI, Sarasota, FL). After cutting, the slices were continuously bubbled with 95% O₂ - 5% CO₂ at room temperature in ACSF for at least one hour. Thereafter, the slices were treated with pronase (1 mg per 6 ml) at 31°C for 30 min. After enzyme treatment, the slices were washed twice with well-oxygenated incubation solution. The VTA and SNc regions were identified using a stereomicroscope and were micro-punched out from the slices using a well-polished needle. One punched piece was then transferred to a 35 mm culture dish filled with well-oxygenated, standard extracellular solution. The punched piece was then dissociated mechanically using a fire-polished micro-Pasteur pipette under an inverted microscope (Olympus IX-70). The separated cells usually adhered to the bottom of the culture dish within 30 min. In the present study, we used only SNc or VTA neurons that maintained their original morphological features, containing polygonal, large or medium somata with 2-5 thick primary dendritic processes (Fig. 2A).

**Immunostaining.** Immunohistochemical detection of the nAChR α7 subunit was accomplished via several steps. Postnatal-day-21 (P21) brains were removed, frozen in liquid nitrogen for 15 s, and stored at −80°C for ≥ 2 days. Coronal 20 µm cryostat
sections were obtained, serially mounted onto subbed slides, and stored at –80°C with desiccant until processed (≥3 days). The slides were then allowed to reach room temperature, rinsed twice in phosphate-buffered saline (PBS, made with ddH₂O), fixed in 4% paraformaldehyde, rinsed, then treated with 3% H₂O₂ for five min to block endogenous peroxidase activity, and rinsed again before blocking nonspecific binding by incubating in rinse solution PBS made with ddH₂O containing 1% bovine serum albumin (BSA), 10% dimethyl sulfoxide, and 5% Triton X-100 containing 10% normal (horse) serum for 20 min. Primary antibody mAb 306 (Sigma) was then added overnight at 4°C, followed by 30 min sequential incubations using biotinylated secondary antibody (Vector Labs; Burlingame, CA) and horseradish-peroxidase conjugated avidin-biotin complex (Vector Labs) in 1% BSA-PBS. Antigen was detected using diaminobenzidine (Vector Labs). Between addition of primary antibody and antigen detection, slides were rinsed twice for 10 min in rinse solution between steps. Slides were then dehydrated and permanently mounted using Permount (Sigma). Sections were viewed under brightfield (Olympus IX70 microscope) and images were captured at 20x magnification using Magnafire 2.1 software (Media Cybernetics; Carlsbad, CA) (Fig. 1A right). Parallel slides where primary antibody was omitted showed no staining (data not shown).

To identify single, dissociated cells after a patch-clamp recording session, the recording pipette was filled with Lucifer yellow CH (Sigma, 1.0 mg/ml dissolved in the recording solution), and dye was ejected by a pulse (200 ms, 0.5 Hz) of hyperpolarizing current (1.0 nA) for 3 min. Cells were then fixed with 4% paraformaldehyde for 10 min, rinsed three times with PBS, treated for 20 min with 0.03% hydrogen peroxide to quench endogenous peroxidase activity, and permeabilized with blocking solution (10% horse serum in PBS) containing 0.02% Triton-X 100. Rabbit anti-tyrosine hydroxylase primary
antibody (AB152; Chemicon International) was added overnight at 4°C in blocking solution, followed by incubation (30 min) with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology; Santa Cruz, CA), with three, 10-min PBS rinses between steps. Lucifer yellow was visualized using epifluorescence (Fig. 2A left); tyrosine hydroxylase staining was detected using diaminobenzidine (Vector Labs), and images were obtained as described above (Fig. 2A right).

**In situ hybridization.** Wistar rats (P7-P21) were sacrificed, and brains were immediately removed and snap-frozen in isopentane. Samples were stored in desiccant at -80°C and subsequently sectioned (20 µm) using a cryostat. Sections were mounted on subbed (chrome alum and gelatin), poly-d-lysine-coated slides and kept under desiccant at -80°C until required. Generation of cRNA probes from known nAChR subunit templates involved utilizing Ambion’s Lig’n Scribe no-cloning promoter addition systems and MAXIscript *in vitro* transcription. Briefly, α7 subunit primers were generated in forward and reverse orientations to allow polymerase chain reaction (PCR)-based amplification of heterologous, cytoplasmic domains from human, mouse and rat α7 subunit cytoplasmic regions. PCR products were run on a 1% agarose gel to confirm size and quantity of DNA. After product purification, cytoplasmic domain templates for α7 subunits were non-directionally ligated to the T7 phage promoter adapter. Gene-specific α7 subunit primers were chosen according to the desired orientation. Concomitant with the use of Lig’n Scribe PCR adapter primers, subsequent PCR of the ligation product was performed, and transcription templates for sense and anti-sense orientations were selected for *in vitro* transcription reactions. α7 sense and anti-sense cRNAs, 232 bp in length, were transcribed incorporating biotinylated UTPs. Stored sections were thawed by
rinsing in 1X PBS and then fixed in 4% paraformaldehyde. Samples were subsequently rinsed in 1X PBS for 5 min, and sections were then acetylated, delipidated with chloroform, and serially dehydrated with ethanol (50%, 75%, 85% and 95%). Sections were then incubated in pre-hybridization solution containing final concentrations of 250 µg/ml of tRNA, 25% formamide, 10% dextran sulfate, 2.5X Denhardt's solution, 0.05 mg/ml of salmon sperm DNA, 4X SSCP, 4 mM EDTA, pH 8.0 for 2 hr at 50°C. Sections were then incubated in hybridization solution containing anti-sense or sense probes (final concentration ranging from 0.2 ng/µl - 0.7 ng/µl) for 20 hr at 50°C. After hybridization, sections were rinsed twice in 2X SSC for 5 min each, 1X SSC for 10 min, 0.5X SSC for 10 min, and 0.1X SSC for 20 min at 60°C, and then briefly rinsed in ddH2O. Slides were then dehydrated in a graded ethanol series and vacuum-dried under desiccant. Samples were conjugated using avidin-Alexa fluorophore complexes and subjected to fluorescence microscopy and image analysis using Image pro Plus v. 4.1(Silver Spring, Maryland) (Fig. 1A left and middle). Staining was only observed in samples treated with anti-sense probe, and additional control studies using cell lines expressing α7-nAChR and excess unlabeled anti-sense riboprobe also were devoid of signal, demonstrating specificity of the technique.

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed as previously described (Kuo et al., 2002). Briefly, total RNA was isolated from tissue punches representing the SNc via dissociation through a 23 gauge needle in TRIZOL® reagent. RNA was prepared according to the manufacturer’s instructions followed by DNAse I (Invitrogen) treatment to remove any residual genomic DNA, and quantitated spectrophotometrically for reverse transcription using the Superscript II Preamplification system (Invitrogen). Typically, 1 µg of RNA was used.
for each RT reaction consisting of a cocktail of 10 rat-specific nAChR subunit anti-sense primers plus a primer for the housekeeping gene, gapdh. Each reaction was carried out at 48°C, heat-inactivated, and RNAse H–treated prior to PCR. PCR was performed with approximately 70 ng of RT template, 200 nM of each sense and anti-sense gene-specific primer pair, 200 µM dNTPs and 2.5 units of RediTaq® (Sigma). Standard PCR reactions were performed using a RoboCycler (Stratagene) for 35 cycles using the following amplification protocol: 95°C for 60 s, 55°C for 90 s, 72°C for 90 s, followed by a single-cycle, 4-min 72°C extension. One-fifth of each reaction was run on an ethidium bromide/1% agarose gel for visualization against a 100 bp sizing ladder (NEBiolabs). Representative samples without template DNA or minus reverse transcriptase were routinely run to monitor potential contamination. Whole rat brain RNA was purchased from Ambion. Rat oligonucleotide primer pairs used in this study are presented in the 5’→3’ orientation with the sense primer listed first: 

\[
\begin{align*}
\alpha_2 & : \text{cgggtgccagttgctgatga/gaggtgacagcagaatctcgctag} \\
\alpha_3 & : \text{gctgtgcttcggtggtcagcacc/gccggcgggatccaagtcacttc} \\
\alpha_4 & : \text{gaatgtcacctccatccgcatc/ccggca(a/g)ttgtc(c/t)ttgaccac} \\
\alpha_5 & : \text{cctagaggaccaagatgtcgacag/gtccagccccactctggtctc} \\
\alpha_6 & : \text{gtgtttgtgtgacata/ctacctcctt(g/t)tttgacctgct} \\
\alpha_7 & : \text{gttctatgagtgctcaagagcc/ctccacactgcccgctgctag} \\
\alpha_9 & : \text{gctcagaattgttgccagacat/cagcaggcatgccatggacc} \\
\beta_2 & : \text{ctccaactcgaatgcgtcagacatccagacgacgtgcaatggct} \\
\beta_3 & : \text{ggttcagagcatagtgatcggct} \\
\text{gapdh} & : \text{eggattgccgctatgagccgccttgccagcacttgatgc}.
\end{align*}
\]

All primer pairs except \(\alpha_2, \alpha_5, \alpha_6,\) and \(\beta_3\) span one or more exons. All nAChR primer sets, except \(\alpha_2\) and \(\alpha_5\) (Keiger and Walker, 2000), were designed to be subunit-specific, species-universal (rat/mouse/human) in our laboratory using DNASTAR software.
**Patch-clamp whole-cell recordings.** Perforated and conventional patch-clamp whole-cell recordings were employed (Wu and Partridge, 1998). The perforated patch recordings were crucial for obtaining stable ACh responses from dissociated midbrain neurons, although the precise mechanism involved is still unclear. The pipettes (3-5 MΩ) used for perforated patch recording were filled with intracellular recording solution containing nystatin. After tight seal (>2 GΩ) formation, it usually took 5-30 min to convert to perforated patch mode (Horn, 1991), which was monitored by access resistance. An access resistance of less than 60 MΩ was accepted in order to start the experiments. Series resistance was not compensated in this study. The data were filtered at 2 kHz, acquired at 5 kHz, and digitized on-line (Axon Instruments Digidata 1200 series A/D board). All experiments were performed at room temperature (22±1°C). Studies of acutely-dissociated neurons were done using cells attached to the cell culture dish, whereas studies of cells transfected to express human α7-nAChR were lifted off the dish via the recording pipette before study.

**Solutions and drug application.** The standard external solution contained 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM N-2-hydroxyethylpiperazine-N'2- ethanesulfonic (HEPES), pH 7.4 with Tris-base. The nystatin perforated patch pipette solution used for current-clamp and voltage-clamp recordings contained 150 mM potassium gluconate, 5 mM MgCl₂ and 10 mM HEPES, pH 7.2 with Tris-OH. Nystatin was dissolved in acidified methanol, and the stock solution was diluted with the internal (patch-pipette) solution to a final concentration of 200-300 µg/ml just before use. The pipette solution for conventional whole-cell recordings contained 140 mM KCl, 4 mM MgCl₂, 0.1 mM EGTA, 4 mM ATP, 10 mM HEPES, pH 7.4 with KOH. To initiate whole-cell current responses, under constant...
superfusion of the recording chamber, nicotinic agonists were rapidly delivered into the
bath medium by a computer-controlled U-tube system, in which the applied drug
completely surrounds the recorded cell in less than 30 ms (Zhao et al., 2003). The times
between drug applications (~3 min) were adjusted specifically to ensure stability of
nAChR responsiveness (absence of functional rundown). The drugs used in the present
study were (-)nicotine, ACh, choline, MLA, mecamylamine (MEC), dihydro-β-
erthyroidine (DHβE), Bgt and atropine sulfate (Sigma Chemical Co., St. Louis, MO). RJR
2403 fumarate (C_{10}H_{14}N_{2}C_{4}H_{4}O_{4}) was purchased from Tocris (Ellisville, MO). In
experiments using ACh as the agonist, 1 µM atropine sulfate was routinely added to the
standard solution in order to exclude any possible influences of muscarinic receptors,
although the drug had no clear effects on nAChR-mediated currents (Fig. 3A).

Data acquisition and analysis. All experimental data were recorded using a 200B
amplifier (Axon Instruments Foster City, CA), typically using data filtered at 2 kHz,
acquired at 5 kHz, displayed and digitized on-line (Axon Instruments Digidata 1200 series
A/D board), and stored to hard drive. Data acquisition and analyses were done using
Pclamp8 (Axon Instruments), and results were plotted using Origin 5.0 (Microcal, North
Hampton, MA). nAChR acute desensitization was analyzed for decay time (τ), peak
current (I_p), and steady-state current (I_s) using fits to the mono-exponential expression I =
[(I_p - I_s) e^{-kt}] + I_s. There was no significant improvement if data were fit to higher-order
exponential expressions. Data usually were fit over the period between 90% and 10% of
the peak inward current. Curve fitting for sigmoid agonist and antagonist data was
performed using Origin (Microcal) or Prism (GraphPad; San Diego, CA) and the Hill
equation.
RESULTS

**nAChR \( \alpha 7 \) subunit expression in midbrain DA nuclei.** Studies were done to assess natural expression of nAChR \( \alpha 7 \) subunits in the VTA and SNC. *In situ* hybridization and immunohistochemistry revealed extensive nAChR \( \alpha 7 \) subunit expression as mRNA and protein in the SNC and VTA (Fig. 1A). Comparisons between SNC and whole-brain expression of \( \alpha 7 \) and other nAChR subunits based on RT-PCR showed that \( \alpha 7 \) subunit message could also be detected in the SNC using an alternative approach. Our findings of native expression of nAChR \( \alpha 7 \) subunit mRNA in the VTA and SNC regions are consistent with previous reports (Wada et al., 1989; Jones et al., 2001; Azam et al., 2002).

**Identification of midbrain DAergic neurons.** Cells acutely dissociated from the VTA or SNC were subjected to electrophysiological and histological characterization in order to determine whether they met standard criteria used to identify neurons expressing DA phenotypes. The structure of such a DA neuron from the VTA was revealed after it was injected with Lucifer yellow upon termination of the electrophysiological recording session (Fig. 2A). The same neuron was subsequently shown to be tyrosine hydroxylase-positive after immunostaining based on horseradish peroxidase labeling (Fig. 2A), and similar neurons also exhibited immunofluorescence staining for \( \alpha 7 \) subunits (Fig. 2B). From the same cell as shown in Fig. 2A, the representative traces shown in Fig. 2C-D were obtained using patch-clamp recordings. Current-clamp recording from this cell showed that it exhibited spontaneous activity characterized by long-duration action potentials (>2.5 ms) appearing with a frequency of ~3 Hz (Fig. 2C). Upon application of DA (10 \( \mu M \)), there was hyperpolarization of the membrane potential and cessation of spontaneous activity (Fig. 2C). Current-clamp recordings showed a ‘sag’ in membrane potential changes (Fig. 2Da), and voltage-clamp recordings showed a hyperpolarization-
activated current (H-current, Fig. 2Db). Collectively, these features show that this neuron, and others presented in the remainder of this study, exhibited electrophysiological, immunostaining and morphological characteristics of DA neurons.

**nAChR-mediated currents in midbrain DAergic neurons.** In order to evaluate expression of functional nAChR in identified DA neurons, ACh-induced currents ($I_{ACh}$) were recorded using the perforated patch whole-cell recording technique coupled with computer-controlled, U-tube-mediated, rapid drug application. At a holding potential ($V_H$) of –60 mV, 1 mM ACh-induced inward currents consisted of a rapidly activated and desensitized transient peak response succeeded by a lower in amplitude, more slowly decaying, steady-state component (Fig. 3A; representative SNc DA neuron).

Pharmacological blockade of: 1) muscarinic acetylcholine receptors using 1 µM atropine, 2) ionotropic glutamate receptors using 50 µM CNQX plus 100 µM APV, and 3) voltage-gated Na+ channels using 1 µM tetrodotoxin, had no effect on $I_{ACh}$ when samples were pretreated for 3 min with these drugs (Fig. 3A). The $I_{ACh}$ also was insensitive to blockade by 100 nM α-conotoxin MII (Fig. 3A), which actually and significantly enhanced $I_{ACh}$ ($p<0.05$, n=6). These results indicate that $I_{ACh}$ is mediated via activation of somatodendritic nAChR on SNc (or from other results on VTA) DA neurons. In addition, in current-clamp recordings, both ACh and choline modulated DA neuron spontaneous activity by transiently increasing cell firing rates soon after ACh or choline application, and also by ceasing or lowering cell firing rates after prolonged exposure to ACh or choline (Fig. 3D and E).

**Perforated whole-cell recordings are necessary for maintaining $I_{ACh}$ stability.** One of the challenges in studying α7-nAChR-mediated currents is current run-down over time, which makes it difficult to evaluate receptor function and/or pharmacology. We
assessed run-down of $I_{ACh}$ elicited by repetitive applications of ACh at 3-min intervals using either conventional or perforated patch whole-cell recordings (Fig. 4). $I_{ACh}$ run-down occurred over time when using conventional whole-cell recordings (Fig. 4A). In contrast, when employing perforated patch whole-cell recordings, $I_{ACh}$ was stably maintained for at least 20 min (Fig. 4B and C). Figure 4C summarizes peak current amplitudes for $I_{ACh}$ recorded by these two methods and shows that the perforated patch whole-cell recording method is necessary for maintaining stable $I_{ACh}$.

**Pharmacological dissection of α7-nAChR-mediated currents.** In order to distinguish between nAChR subtypes that might be contributing to $I_{ACh}$, different pharmacological probes selective or specific for distinct nAChR subtypes were employed. Fast, peak components of $I_{ACh}$ were mimicked by application of a selective α7-nAChR agonist, choline, whereas slow, steady-state components of $I_{ACh}$ were mimicked by application of a selective α4β2-nAChR agonist (Papke et al., 2000), RJR-2403 (Fig. 5A). The slow component of $I_{ACh}$ was completely blocked by a non-competitive, non-selective nAChR antagonist, mecamylamine, whereas the remaining fast component was abolished by addition of MLA, a relatively selective α7-nAChR antagonist (Fig. 5B). Both MLA (Fig. 5Ca) and Bgt (Fig. 5Cb) selectively blocked the fast component without affecting the slow component. The responses recovered faster from MLA than from Bgt block. Moreover, 10 mM choline-induced fast inward currents ($I_{choline}$) were completely abolished by 1 nM MLA (Fig. 5Da), whereas a selective α4β2-nAChR antagonist, dihydro-β-erythroidine (1 μM), blocked the RJR-2403-induced slow inward current (Fig. 5Db). From a total of 68 DA neurons dissociated from the SNc, 66 neurons (98%) responded to 1 mM ACh while 50 neurons (73%) responded to both ACh (1 mM) and choline (10 mM). Similarly, from 40 DA neurons dissociated from the VTA,
38 neurons (96%) responded to 1 mM ACh and 33 neurons (83%) responded to both
ACh (1 mM) and choline (10 mM). There are no clear differences in cholinergic (ACh or
choline) responses when comparing these two brain regions.

**Concentration-response relationship for α7-nAChR-mediated currents.** To
determine agonist affinity for functional α7-nAChR naturally expressed in midbrain DA
neurons, a concentration-response relationship for choline, a selective agonist of α7-
AChRs, was examined in VTA and SNc DA neurons, and was compared to
concentration-response profiles for activation of functional, human α7-nAChR
heterologously expressed in the SH-EP1-hα7 cell-line (Zhao et al., 2003; Fig. 6). Based
on summation of individual records (Fig. 6A), fits of the data to the Hill equation
indicated that EC\textsubscript{50} values for \( I_{\text{choline}} \) recorded from VTA, SNc and SH-EP1-hα7 cells
were 4.5, 2.7 and 0.9 mM, respectively, and the values of the Hill coefficient were 0.90,
0.91 and 1.10, respectively. Thus, choline showed higher functional agonist potency for
SH-EP1-hα7-nAChR than for rat SNc (3-fold) or VTA (5-fold) α7-like-nAChR.

**Kinetics of α7-nAChR-mediated currents in SNc, VTA and α7 transfected cells.**
Since α7-nAChR-mediated currents are characterized by fast activation and acute
desensitization (which is the term we apply to describe the decay from peak to steady-
state inward current levels during agonist exposure, in this case, over two seconds of drug
exposure), the kinetics of choline-induced currents were analyzed in DA neurons
dissociated from either the SNc (7 neurons) or VTA (11 neurons; using cells attached to
the dish), and also in α7-nAChR-expressing SH-EP1 cells (12 cells; using the lifted cell
technique). The results show that the required time to traverse from 10% to 90% of the
peak current response (rising time) was 20.4 ± 4.8 ms for SNc cells, 29.4 ± 5.9 ms for
VTA cells, and 13.6 ± 1.7 ms for α7-nAChR transfected cells, respectively (Fig. 7). The half times for decay from peak current to steady state levels during acute desensitization were 14.8 ± 2.6 ms (SNc), 17.5 ± 2.4 ms (VTA) and 23.0 ± 3.8 ms (α7-nAChR-expressing SH-EP1 cells), respectively (Fig. 7). Statistical analyses indicate that rising times are only statistically different (p < 0.05) when comparing activation of responses in VTA DA neurons to those in transfected SH-EP1 cells, perhaps simply reflecting faster, drug perfusion rate-limited responses obtained when using lifted, transfected cells (p<0.05). Furthermore, there is no significant difference in the rate of acute desensitization of responses from these three types of cells (Fig. 7B). These results suggest there are functional α7-nAChR-mediating fast, choline-, MLA-, and Bgt-sensitive currents located somatodendritically on single, DA neurons freshly isolated from midbrain nuclei. The slower, steady-state response, characterized by slow acute desensitization, seems to include contributions from non-α7-nAChR (likely α4β2-nAChR).

**Developmental changes in α7-nAChR expression in DA neurons.** We undertook additional studies to assess the developmental profile for nAChR α7 subunit and functional α7-nAChR expression in rat brain. From rats younger than postnatal-day-seven (P7), no detectable ACh responses were recorded (7 neurons from 3 animals). Typical traces indicated that whole-cell current responses to ACh are evident at P10, and peak current amplitudes in response to ACh continued to increase until P18-21 (Fig. 8A,C). Similarly, $I_{\text{choline}}$ could only be recorded from P10 and had amplitudes that increased through P18 (Fig. 8,C). The proportion of neurons that showed responses of greater than 25 pA magnitude to Ach that also showed responses of that same magnitude to choline increased with age and was evident in 83% of recorded VTA/SNc DA neurons.
from P18-22 (Fig. 8B). A summary of the findings taken from numerous cells (Fig. 8C) reinforces the observations illustrated as individual traces (Fig. 8A). Likewise, in situ hybridization analyses revealed a pattern of increasing nAChR α7 subunit mRNA levels with age during the first 1-3 postnatal weeks (Fig. 8D).
DISCUSSION

This study provides a detailed description of functional α7-nAChR located somatodendritically on single, midbrain DA neurons based on the following evidence: (1) in identified DA neurons, ACh-induced whole-cell currents are insensitive to blockade by atropine, APV plus CNQX, or tetrodotoxin, meaning that post-synaptic nAChR mediate the observed responses; (2) ACh-induced, fast peak current responses are mimicked by application of an α7-nAChR-selective agonist, choline, and blocked by α7-nAChR-selective antagonists, such as Bgt and MLA, also suggesting that α7-nAChR are responsible for the effects; (3) agonist concentration-response relationships showing comparatively low affinity for nicotinic agonists and the fast activation and acute desensitization of responses further confirms functional α7-nAChR-like behavior; and (4) developmentally-regulated expression of nAChR α7 subunit message and protein parallels expression of functional α7-nAChR-like responses. Thus, our approaches provide, and our findings validate, an experimental basis for understanding native α7-nAChR physiology, pharmacology and pathophysiology at the single midbrain neuronal level.

The current RT-PCR results illustrate significant expression of nAChR α7 subunit mRNA in the SNc and complement clear, in situ hybridization and immunocytochemical evidence of α7 subunit mRNA and protein expression in VTA and SNc neurons. These experimental results demonstrate the existence of building blocks for generation of α7-nAChR in the mesolimbic dopamine system (VTA and SNc), and they are consistent with previous observations (Klink et al., 2001; Azam et al., 2002; Zoli et al., 1998; Arroyo-Jimenez et al., 1999).
Other findings reported here are consistent with previous evidence showing that high concentrations of ACh induce fast inward currents sensitive to MLA or Bgt, characteristic of functional α7-nAChR, in cells taken from VTA slices (Pidoplichko et al., 1997). Our observations are also consistent with patch-clamp recording results from midbrain slices combined with single-cell RT-PCR techniques that were used to provide a rather detailed survey of molecular and physiological diversities of nAChR subtypes, including α7-nAChR, located in the VTA and SNc regions (Klink et al., 2001).

Similarly, the results of our studies complement those obtained from alternative strategies that were employed to study α7-nAChR function in midbrain slices, e.g. employing α7-nAChR mutations (α7L250T; Revah et al., 1991) in order to slow desensitization of α7-nAChR (Ji et al., 2001), or by conducting analyses using nAChR β2-subunit knockout mice, both of which may have increased α7-nAChR function and/or the ability to detect it, since α7-nAChR-mediated currents became predominant and seemed to be more resistant to desensitization during prolonged (about 10 min) exposure to low concentrations of nicotine in these models (Wooltorton et al., 2003). However, these previous studies might have been confounded by a low expression of α7-nAChR and their rapid desensitization kinetics, which present technical difficulties for convincingly establishing the properties of functional α7-nAChR when patch-clamp recording techniques are used, especially when employing brain-slice preparations. Both slow drug application and washout, while performing slice recordings, will desensitize α7-nAChR, and even when using a pressure-injection system to increase drug application speed, it is still difficult to rapidly apply different drugs to the same neuron for pharmacological studies. Another limitation is that a brain slice is indeed a complex system, containing
both DA and non-DA neurons. Additionally, nAChR are also found post- and pre-synaptically. Bath-applied nicotinic ligands may directly or indirectly affect DA or non-DA neurons. Moreover, our current demonstration of developmentally-dependent expression of both α7 subunits and functional α7-nAChR may also have adversely impacted earlier studies.

To overcome these technical limitations, we applied perforated patch-clamp recordings to acutely-dissociated, single DA neurons. We paid special attention to neuron dissociation and recording procedures. For instance, we found that maintaining dissociated neurons in their original morphology (with relatively-long dendrites) is important in order to induce nAChR-mediated currents in VTA and SNc DA neurons. In the present study, from 108 DA neurons dissociated from the VTA and SNc, 97% responded to ACh while 78% responded to choline. The ratio of neurons responding to choline in our study is higher (using acutely-dissociated neurons) when compared to previously-published work that employed slice preparations (Wooltorton et al., 2003; Klink et al., 2001). Considering that the ratio of ACh-induced responses in the present study (97% of cells tested) and in the previous report (95% of cells tested, Klink et al., 2001) are comparable, but that the ratio of choline-induced current is higher in the present study (78% of cells tested) than in the previous report (~50% of cells tested, Klink et al., 2001), perhaps use of single, dissociated neurons has aided our ability to detect fast α7-nAChR-mediated currents. Findings may also be influenced by: 1) the animal strains/species employed, 2) postnatal ages, 3) the enzyme used for cell dissociation, or 4) the single-cell dissociation procedure. Another important finding is that perforated patch whole-cell recordings used in this study, rather than conventional whole-cell recordings (Fig. 4), resulted in relatively long-term, stable nicotinic responses (for at least 20 min),
which allowed us to analyze the pharmacological properties of α7-nAChR, such as concentration-response relationships from the same recorded neuron. Furthermore, our interesting and important finding of the developmentally-regulated increase in α7-nAChR expression and channel function allowed us to choose an optimal age range (P18-21) for the productive study of α7-nAChR function.

The electrophysiological responses to nicotinic agonists that we identified were pharmacologically distinct from those that could be mediated by other signaling molecules, such as muscarinic or ionotrophic glutamate receptors. Distinctive features of whole-cell current responses to ACh (fast peak and slower steady-state components) could be further parsed pharmacologically. The sensitivity of steady-state responses to mecamylamine, dihydro-β-erythroidine, and RJR-2403, but not to choline, suggests they are mediated by non-α7-nAChR. Because these slow responses are also insensitive to blockade by α-conotoxin MII, they are not likely to be due to somatodendritic, DA neuronal nAChR containing α6- or perhaps α3-subunits. Therefore, our interpretation is that slow, steady-state current responses to ACh are due to α4β2-nAChR. By contrast, fast, peak current responses that are sensitive to activation by choline and blockade by MLA or Bgt clearly demonstrate characteristics of α7-nAChR, and parallel studies comparing these properties to those of heterologously expressed α7-nAChR reinforce this conclusion. Thus, we demonstrate that two kinds of nAChR, the abundant α7- and α4β2-nAChR subtypes, are both expressed by most midbrain DA neurons. This means that DA neurons, as well as neurons from other brain regions (Alkondon et al., 1996), can be counted among those cells which may be used as models in future studies of the
specific roles played by diverse nAChR subtypes in the control of brain neuronal function.

Our observation that ACh induces an initial increase in DA neuronal firing rates followed by a period of membrane potential hyperpolarization and absence of cell firing upon prolonged (> 10 s) exposure is consistent with earlier observations of the complex effects of nicotinic agonist exposure on DA cell activity that probably reflects an admixture of activities across nAChR subtypes. Previous electrophysiological experiments indicated that nicotine increases VTA DA neuron firing frequency and alters firing patterns from single spikes to burst patterns, which leads to an increase of DA release in the nucleus accumbens (Calabresi et al., 1989). Our studies using ACh reveal a similar effect, but just transiently. In vivo experiments have shown that nicotine self-administration is reduced either by creating a lesion of the mesolimbic DA system or by direct micro-infusion of α7-nAChR antagonists into the VTA (Corrigall and Coen, 1989; Corrigall et al., 1994). Based on other results, it has been postulated that α7-nAChR located on pre-synaptic glutamatergic terminals in the VTA play an important role in the regulation of DA neuron activity (Jones et al., 2001; Wonnacott et al., 2000; Zhou et al., 2001), but much less is known about the roles of post-synaptic α7-nAChR in midbrain DA neuronal function. According to a recent report, VTA α7-nAChR exhibit far less desensitization when persistently exposed to a smoker’s level of nicotine than do non-α7-nAChR, which suggests an important role of α7-nAChR in nicotine addiction (Wooltorton et al., 2003). Our preliminary observation that prolonged exposure to choline also induces initial activation followed by a sustained decrease in DA neuron activity, although less dramatic when compared to exposure to ACh, is consistent with possibly lower “chronic” desensitization of α7-nAChR- compared to α4β2-nAChR-
mediated responses in the presence of agonist at lower concentrations. Thus, we now have evidence of somatodendritic – and presumably post-synaptic - α7-nAChR on midbrain DA neurons, with most of the dendrites being preserved by our acute dissociation technique, which suggests that α7-nAChR contribute to cholinergic and nicotinic control of VTA or SNc DA neuronal firing. Nevertheless, more work is needed to more completely extricate cause-effect relationships and explore the specific roles of selected nAChR subtypes in the control of DA cell activity.

The fact that expression of α7 subunits and functional α7-nAChR are developmentally regulated has potential relevance to the development and activity of the midbrain DA system. First, the possible roles played by α7-nAChR in neurite extension and long-term potentiation could affect anatomic and functional substrates for reward in these cells and the systems they serve. Second, the increase in α7 subunit and α7-nAChR expression during the second and third weeks of development in the rat suggests that similar phenomena occurring at corresponding times in human development could influence not only the structure of entities comprising the reward system, but also could modulate its function during adolescence, arguably the period of highest vulnerability to the initiation of drug use, which could cause some individuals to struggle with a life-long battle of dependence. As a result, the finding that naturally expressed α7-nAChR are abundantly positioned somatodendritically on single DA neurons located in the brain reward center gives new perspectives to mechanisms involved in nicotinic mediation of DA neuronal firing and signaling, and provides a new model system with which roles of α7-nAChR (and other nAChR subtypes) can be explored in regards to their relevance in nicotine dependence and the molecular mechanisms of pleasure and reward.
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FIGURE LEGENDS

Figure 1. Native expression of α7-nicotinic receptors in mesolimbic dopaminergic nuclei. **A**: Left and Middle panels: *In situ* hybridization showing expression of nAChR α7 subunit transcripts in the SNc and VTA areas from a 21-day-old rat. Following the protocol described in Methods, VTA or SNc cells were probed with a biotinylated α7 anti-sense-orientation riboprobe before being labeled with avidin-Alexa 488 and subjected to epifluorescence microscopic analysis. Images were obtained at 30X magnification (calibration bar is 50 µm). The right panel shows α7 subunit protein expression in the SNc by immunohistochemical staining from the same 21-day-old rat (scale bar = 50 µm). Results of control studies employing no anti-sense or sense riboprobes, excess unlabeled anti-sense riboprobe, or no primary antibody as appropriate, are not shown, because they yielded no fluorescence or diaminobenzidine staining. **B**: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of products of different nAChR subunit messenger RNAs (see labels at the top of the figure and expected product sizes at the bottom of the figure) from SNc and whole rat brain. RT-PCRs were carried out using gene-specific oligonucleotide primers listed in Methods. Approximately 70 ng of the SNc or brain RT reaction was PCR amplified for each gene analyzed in 35 cycles, and one-fifth of the resulting product was resolved on a 1% agarose gel. Asterisks to the right of the partial cDNA products indicate that the correct size was obtained. nAChR β4 subunit and gapdh products were verified for whole rat brain on a separate gel, and reverse transcription-negative or null tissue controls yielded no PCR product (not shown).
Figure 2. Identification of dopaminergic neurons from the ventral tegmental area and substantia nigra pars compacta. A: The representative VTA neuron from which recordings are shown in C-D was identified by infusion with Lucifer yellow (left panel) and showed positive TH expression (right panel; horse radish peroxidase labeling). B: nAChR α7 subunit expression in single, dissociated VTA neurons after immunostaining. The top row shows 4 cells stained using both primary and secondary antibodies, and the bottom row shows that another 4 cells failed to stain when primary anti-α7 subunit antibody was omitted (scale bar = 50 µm). C: A VTA neuron (shown in A) exhibits spontaneous action potential spikes under current-clamp recording which are suppressed in the presence of 10 µM dopamine, and single action potentials exhibit long duration. D: The VTA neuron (shown in A) exhibits hyperpolarization-activated channels in current-clamp mode (Da) to show a ‘sag’ shaped membrane potential change, and in voltage-clamp mode to show an H-current (Db). Similar results were obtained in studies of SNc neurons identified as dopaminergic neurons, and the recordings shown are similar to those obtained from 6-10 cells.

Figure 3. Nicotinic receptor-mediated responses in a substantia nigra pars compacta dopaminergic neuron. A: Rapid application of 1 mM ACh induced an inward current at a holding potential of –60 mV that is not sensitive to blockade of muscarinic receptors (block by 1 µM atropine), ionotropic glutamate receptors (block by 50 µM CNQX and 100 µM APV), or voltage-gated Na+ channels (block by 1 µM tetrodotoxin, TTX), or to effects of α-conotoxin MII (Co MII; 100 nM), an α3/α6*-nAChR blocker. All five traces were recorded from the same DA neuron. B: Bar graph for records from the indicated number of cells showing average (± SE) peak current responses to 1 mM ACh in the presence of the
indicated antagonist(s) indicating insensitivity of responses to atropine, CNQX + APV, or tetrodotoxin, but potentiation by α-conotoxin MII.  

**C-E:** Representing one typical trace from studies of 6 cells, the current-clamp recording shows that both ACh (D) and choline (E) modulate DA neuron activity (identified based on sensitivity to 10 µM dopamine as shown in C) by activation (increase in cell firing rate shortly after application of ACh or choline) and desensitization (elimination or lowering of cell firing upon prolonged ligand exposure) of nAChR.

**Figure 4.** Comparison of acetylcholine-induced currents in conventional and perforated whole-cell recordings.  
**A:** A typical case of ACh-induced (1 mM, 2 s duration) currents obtained using conventional whole-cell recording. Calibration responses to 10 µM GABA at the beginning and at the end of the conventional recording session indicate stability of the patch, and numbers under each trace indicate in minutes the time (every three minutes) at which the record was obtained.  
**B:** A typical case of ACh-induced currents obtained using perforated patch whole-cell recording of responses to 2 s pulses of 1 mM ACh at three-minute intervals.  
**C:** Line graphs summarize results obtained using the two recording methods and show a clear run-down of ACh-induced currents in conventional, but not perforated patch, whole-cell recording. The vertical bars indicate the standard error and each symbol represents the mean taken from 5-7 neurons.

**Figure 5.** Pharmacologically distinguished α7-nicotinic acetylcholine receptor-mediated currents in single, midbrain DAergic neurons.  
**A:** A typical trace shows that ACh-induced (1 mM, 2 s) current consists of two components: a fast peak component and a slow steady-state component (a). The relatively selective α7-nAChR agonist, choline (10
mM), induces currents correlating with the fast component evoked by ACh (b), whereas
the relatively selective α4β2-nAChR agonist, RJR-2403 (0.1 mM), induces currents
correlating with the steady-state component of responses evoked by ACh (c). Composite
traces on the right show the current trace overlays.  
B: The non-specific nAChR antagonist, mecamylamine (MEC; 0.1 mM), selectively abolishes the steady-state component of the ACh-induced current, and further addition of a relatively selective α7-
nAChR antagonist, MLA (10 nM), dramatically diminishes the fast peak component. Responses recovered within 20 min of drug washout.  
C: The fast peak component of an ACh-induced current is sensitive to both 1 nM MLA (a) and 10 nM Bgt (b), showing faster recovery from MLA. More complete blockade by Bgt would be expected if we conducted the toxin pretreatment for a longer period or if we used a higher dose.  
D: A choline-induced current is highly sensitive to the α7-nAChR antagonist, MLA (1 nM), whereas an RJR-2403-induced current is sensitive to the α4β2-nAChR antagonist, DHβE (1 μM). Traces A-D were typical cases from 6-8 cells tested.

Figure 6. Concentration-response relationship for choline-induced currents in single, DA neurons.  
A: Typical traces of choline-induced (1 s application) α7-type responses at different concentrations (in mM labeled above the relevant trace) in SNc (a), VTA (b) and transfected SH-EP1 cells heterologously expressing human α7-nAChR (c). All α7-type responses exhibited fast activation and desensitization kinetics.  
B: Three choline concentration-response relationship curves are superimposed for α7-nAChR responses from SNc neurons (■), VTA neurons (●) or transfected SH-EP1-cells (▲) as shown. Vertical bars indicate the standard error in normalized (to 10 mM choline) peak current measurements, and each symbol represents the results averaged from 5-8 neurons.
Figure 7. Kinetic properties of choline-induced currents in SNc, VTA and transfected SH-EP1 cells. **A:** Typical traces for choline-induced (10 mM) currents in cells obtained from SNc, VTA and SH-EP1-hα7 cells. Current decay from peak levels was fit to a single exponential as illustrated to yield half times for decay of 15.2, 15.6, and 26.4 ms, respectively. **B:** Bar graphs (n=6) compare the kinetics of α7-nAChR activation (rising time; open bars; y-axis is time in ms) and desensitization (decay time; solid bars) in these cells.

Figure 8. Developmental profile of nAChR α7 subunit gene and functional α7-nicotinic receptor expression. **A:** 1 mM ACh (black)- and 10 mM choline (red)-induced (2 s application) currents are superimposed to show a developmental age-related increase in nAChR channel function based on assessments made at P10, P14 and P18. The holding potential was –60 mV. **B:** Bar graphs illustrating numbers of cells derived from animals of the indicated ages that were tested for ACh and choline responses and having peak currents with amplitudes larger than 25 pA, with cells expressing responses to ACh only shown in black and cells responding to both ligands illustrated in red. Percentages of cells showing responses to choline are indicated by the numbers above each bar. **C:** Bar graphs compare the peak current amplitudes for ACh- and choline-induced currents at P8, P14, P18 and P21. Columns are averages from the indicated number of DA neurons dissociated from midbrain DA nuclei. **D:** *in situ* hybridization shows a developmental age-related increase in nAChR α7 subunit expression during the first three postnatal weeks. The scale bar indicates 50 µm, and there was no staining in sense riboprobe control samples.
Fig. 1.

A

SNc  VTA  SNc

\(\alpha 7\)-mRNA  \(\alpha 7\)-mRNA  \(\alpha 7\)-protein

B

Rat SNc  Rat whole brain

202  408  700  554  386  487  514  443  772  541  624 (Expected product size, bps)

* Correct product size amplified
Fig. 2.

(A) Before staining, (B) TH* (HRP), (C) DA 10 µM, (D) 300 pA, 120 mV, 50 mV.
Fig. 3.

A

ACh 1 mM  Atropine  CNQX+APV  conotoxin MII  TTX

V_h = -60 mV

50 pA

2 sec

B

Normalized peak

0  50  100  150

Atropine  CNQX + APV  α-Conotoxin MII  TTX

n = 4  n = 5  n = 6

C

DA 10 μM

D

ACh 1 mM

E

Choline 10 mM

20 mV

5 sec
Fig. 4.

**A** Conventional whole-cell

GABA 10 mM

ACH 1 mM

0 3 6 9 12 15 18 (min)

20 pA

500 pA

V_m = -60 mV

2 sec

**B** Perforated whole-cell

ACH 1 mM

0 3 6 9 12 15 18 (min)

2 sec

50 pA

**C**

Normalized peak

Time (min)

0 3 6 9 15 21 27

Perforated-WC

Conventional-WC

Normalized peak

50

0
Fig. 5.

A

\[ \text{ACh 1 mM} \quad \text{Choline 10 mM} \quad \text{RJR 0.1 mM} \quad \text{a+b} \quad \text{a+c} \]

\[ V_H = -60 \text{ mV} \]

\[ 2 \text{ sec} \]

\[ 50 \text{ pA} \]

B

\[ \text{ACh 1 mM} \quad \text{MEC 0.1 mM} \quad \text{MLA 10 nM} \quad \text{Wash 10 min} \quad \text{Wash 20 min} \]

\[ 2 \text{ sec} \]

\[ 50 \text{ pA} \]

C

\[ \text{ACh 1 mM} \quad \text{MLA 1 nM} \quad \text{wash 4 min} \quad \text{ACh 1 mM} \quad \text{α-Bgt 10 nM} \quad \text{wash 4 min} \]

\[ 2 \text{ sec} \]

\[ 50 \text{ pA} \]

D

\[ \text{Choline 10 mM} \quad \text{MLA 1 nM} \quad \text{RJR 0.1 mM} \quad \text{DHβE 1 μM} \]

\[ 1 \text{ sec} \]

\[ 20 \text{ pA} \]

\[ 2 \text{ sec} \]

\[ 50 \text{ pA} \]
Fig. 6.

A

Choline (mM) 0.3 1 3 10 30 100

SNc

V_H = -60 mV

500 ms

100 pA

VTA

Choline (mM) 0.3 1 3 10 30 100

h-α7

Choline (mM) 0.3 1 3 10 30

500 ms

200 pA

B

Normalized peak currents

Choline (mM)

EC_50 = 4.5 mM, Hill coefficient = 0.90

EC_50 = 0.9 mM

Hill coefficient = 1.10

EC_50 = 2.7 mM

Hill coefficient = 0.91

VTA

SNc

h-α7
Fig. 7

A

rat SNC
Choline 10 mM

\[ \tau_{eqv} = 15.2 \text{ ms} \]

\[ T_{\text{rise}} = 10.8 \text{ ms} \]

rat VTA
Choline 10 mM

\[ \tau_{eqv} = 15.6 \text{ ms} \]

\[ T_{\text{rise}} = 30.8 \text{ ms} \]

human \( \alpha_7 \)
Choline 10 mM

\[ \tau_{eqv} = 26.4 \text{ ms} \]

\[ T_{\text{rise}} = 14.0 \text{ ms} \]

\[ V_R = -60 \text{ mV} \]

B

\[ \text{Rising time} \]

<table>
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<th>SNC</th>
<th>VTA</th>
<th>h( \alpha_7 )</th>
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<tr>
<td>n</td>
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<td>11</td>
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\[ n=7 \]

\[ n=11 \]

\[ n=12 \]
Fig. 8.