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Evidence for the exclusive expression of functional homomeric $\alpha 7$ nAChRs in hypothalamic histaminergic tuberomammillary neurons in rats

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Abstract

Hypothalamic histaminergic tuberomammillary (TM) neurons in rats express high densities of nicotinic acetylcholine receptors (nAChRs) whose Ca^{2+} permeability, kinetic and pharmacological properties are similar to those of heterologous homomeric $\alpha 7$ nAChRs. However, native $\alpha 7$ nAChR subunits can co-assemble with β or $\alpha 5$ nAChR subunits to form functional heteromeric $\alpha 7$ -containing $\alpha 7\beta$ or $\alpha 7\alpha 5$ nAChRs with kinetics and pharmacology similar to those of $\alpha 7$ homomers. Therefore, although TM nAChRs have been used as an *ex vivo* model of functional $\alpha 7$ homomers, the molecular makeup of TM nAChRs has not been determined and the expression of functional $\alpha 7$ -containing heteromers in TM neurons has not been excluded. To determine the profile of TM nAChR subunit transcripts, we have conducted single-cell qRT-PCR experiments using acutely dissociated TM neurons in rats. TM neurons were found to express transcripts of only principal $\alpha 3$, $\alpha 6$ and $\alpha 7$ nAChR subunits. Transcripts of other known mammalian neuronal subunits ($\alpha 2$, $\alpha 4$ -5, $\alpha 9$ -10, $\beta 2$ -4) were not detected. In the absence of β and $\alpha 5$ subunits, the expression of functional $\alpha 7$ -containing heteromers in TM neurons is highly unlikely because principal $\alpha 3$, $\alpha 6$ and $\alpha 7$ nAChR subunits alone are not known to form functional heteromeric nAChRs. These results support the exclusive expression of native functional $\alpha 7$ homomers in rat TM neurons and introduce these neurons as a unique reliable source of native functional homomeric $\alpha 7$ nAChRs suitable for *ex vivo* and *in vitro* pharmacological assays in developing selective $\alpha 7$ nAChR agents.

Keywords

alpha7 nAChR; alpha3 nAChR; alpha6 nAChR; nicotinic; tuberomammillary; homomeric

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are cation-selective channel complexes. The highest permeability ratio of Ca^{2+} over Na^{+} ions ($P_{\text{Ca}}/P_{\text{Na}}$) is attributed to homomeric $\alpha 7$

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nAChRs [3, 7, 17, 29]. The high Ca^{2+} permeability of $\alpha 7$ nAChRs suggests important roles for these receptors in modulation of neurotransmitter release, gene expression, neuroprotection and neurotoxicity [5, 16, 22].

Twelve genes encoding for the neuronal nicotinic acetylcholine receptor (nAChR) subunits have been identified to date [11]. Three of these genes encode for subunits ($\alpha 7$, $\alpha 8$ and $\alpha 9$) that may form functional homomeric nAChRs when expressed alone in an ectopic system. Functional heteromeric nAChRs are more diverse: receptors of this family are required to contain two principal α subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 6$) and two or three complementary β subunits ($\beta 2$ or $\beta 4$). Structural subunits ($\alpha 5$ or $\beta 3$) may also be present [4]. Although $\alpha 7$ subunits can form functional homomeric nAChRs, native $\alpha 7$ subunits have been reported to co-assemble with β or $\alpha 5$ subunits to form functional heteromeric $\alpha 7\beta 2$ -, $\alpha 7\beta 3$ - and $\alpha 7\alpha 5$ -containing nAChRs with kinetic and pharmacological properties similar to those of heterologous homomeric $\alpha 7$ nAChRs (these will be referred to as $\alpha 7\beta 2^*$, $\alpha 7\beta 3^*$, $\alpha 7\alpha 5^*$, etc.) [1, 6, 12, 18, 37, 38]. Although $\alpha 7^*$ nAChRs are some of the most common in the mammalian central and peripheral nervous systems, individual neurons typically express a complicated mix of native nAChR subunits [23, 24] and to the best of our knowledge, a clearly defined brain nucleus with a stable homogenous and exclusive expression of functional $\alpha 7$ homomers across its neuronal population has not yet been identified.

Histaminergic tuberomammillary (TM) neurons of the rat posterior hypothalamus express high densities of functional nAChRs whose Ca^{2+} permeability, kinetic and pharmacological properties are similar to those of heterologous homomeric $\alpha 7$ nAChRs [8, 19, 29-34]. Moreover, the kinetic and pharmacological properties of functional TM nAChRs are highly homogenous across the TM nucleus [2, 8, 29-34]. Therefore, TM nAChRs have been proposed as a useful *ex vivo* model of functional $\alpha 7$ -containing nAChRs [19], however the molecular makeup of TM nAChRs has not been determined and the expression of functional $\alpha 7$ -containing heteromers (e.g., $\alpha 7\beta^*$) in TM neurons has not been excluded. In this study, single-cell quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the profile of nAChR subunit transcripts in acutely dissociated rat TM neurons.

Materials and Methods

Animals

Sprague-Dawley rats (P22-30) were used in experiments. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD) and was approved by the Animal Care and Use Committee, SIU School of Medicine.

Dissociation of neurons

The tissue preparation was described previously [29, 32, 34]. In brief, three-four coronal TM slices (280 μm , thickness) were cut at 3°C using Vibratom-1000+ (Vibratom, St. Louis, MO). The cutting sucrose-rich solution was bubbled with carbogen (95% O_2 +5% CO_2) and contained (in mM): sucrose 250, KCl 3, NaH_2PO_4 1.26, MgCl_2 5, CaCl_2 0.5, NaHCO_3 26, glucose 10 (pH 7.4). Slices were maintained for ~30 min at room temperature in an oxygenated artificial cerebral-spinal fluid (ACSF) containing (in mM): NaCl 125, KCl 3, NaH_2PO_4 1.26, MgCl_2 1, CaCl_2 2, NaHCO_3 26, glucose 10 (pH 7.4) and then, transferred into 40 ml beaker containing papain (~0.4 mg/ml, crude form; Sigma-Aldrich) added to ACSF bubbled with carbogen. After ~40 minutes of treatment, slices were transferred into normal ACSF bubbled with carbogen and maintained at room temperature for up to 8 hours.

Cytoplasm collection

TM neurons were manually dissociated from E1-3 areas of acute slices pre-treated with papain. Polished recording pipettes were used to rupture the ventral edge of slices and to completely or partially dissociate TM neurons under visual control using a Nikon TMS microscope (Figure 1). During dissociation and cytoplasm collection, TM slices were constantly perfused at a rate of 1 ml/min with an RNase-free solution (in mM): NaCl, 150; KCl, 3; CaCl₂, 0.2; MgCl₂, 5; glucose, 10; HEPES 10 (pH 7.35). Cytoplasm was collected from individual neurons using pipettes (tip diameter, ~2-3 μ m) pulled from RNase-free, autoclaved borosilicate glass (Sutter Instruments, Novato, CA) and pre-filled with ~2 μ l reverse transcriptase reaction solution. The other end of the collection pipette was attached to a switch that allowed connection to either a 10 ml syringe or a tank filled with gaseous 100% N₂. Prior to sample collection, a slight positive pressure was applied to the collection pipette via a syringe pre-filled with 100% N₂, and the tip of the pipette was advanced toward a selected TM neuron until the neuron exhibited a slight movement. Suction was applied to the pipette and the selected neuron was drawn into the collection pipette. The pipette was then disconnected from the syringe and connected to the N₂ tank via a picospritzer. The collection pipette was inserted into an RNase-free PCR tube containing reverse transcriptase reaction solution and its tip was broken onto the tube wall. Pressure puffs (~400 ms) of 100% N₂ via a picospritzer (20 psi) were applied to empty the pipette contents into the tube.

Single-cell qRT-PCR

Medial habenula (α 3, α 4, α 5, α 6, α 7, β 2, β 3, β 4), hippocampus (α 2, α 3, α 4, α 5, α 7, β 2, β 4) and cochlea (α 9 and α 10) served as positive control tissues for transcript expression (Figure 2). Cytoplasm of individual neurons was collected within the first 6 hours after the slice preparation, thereby minimizing the possibility of post-preparation changes in subunit composition. Individual neurons in reverse transcriptase reaction solution were frozen at -80°C without reverse transcriptase enzyme. Reverse transcription proceeded according to the manufacturer's protocol (Sensiscript kit, Catalog #252011; Qiagen). The cDNA was precipitated overnight at -20°C in presence of 5 μ g linear acrylamide, 50 μ l ethanol and 2 μ l, 3 M sodium acetate (pH 5.5). Precipitated cDNA was dissolved in 20 μ l of H₂O, aliquoted into four parts (5 μ l each) and subjected to amplification. Amplicon sizes are shown in Table 1. For each primer set, two amplifications were conducted. The first amplification was conducted using standard PCR. Reactions contained 5 μ l of the cDNA aliquot, 1 $\times$ HS Taq Polymerase Mix (Catalog #BE230301; MidWest Scientific) and 2.5 μ l of primer mix. The cycling parameters were: 5 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min (in a Bio-Rad my-cycler thermocycler). The second amplification was conducted using qRT-PCR. Reaction contained 5 μ l of the first PCR reaction, 12.5 μ l of iQ SYBR Green Supermix (Catalog #170-8880; Bio-Rad Laboratories), 5 μ l water and 2.5 μ l primer mix. The PCR cycling parameters were: 5 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min in a Cepheid thermocycler for quantitative PCR. For illustration purposes, the PCR amplified bands were also visualized on a 2% agarose gel using ethidium bromide stain.

RESULTS

Acutely dissociated rat TM neurons (n=44) from all ventral areas of coronal hypothalamic slices (Figure 1) were used in qRT-PCR experiments to determine the molecular makeup of TM nAChRs. The majority of TM neurons used in this study were dissociated from E3 area (n=32). E1-2 areas are usually less populous than E3 (Figure 1C-D), but TM neurons in E1-2 areas are often the largest (Figure 1D, arrow).

Single-cell qRT-PCR experiments were conducted and the transcript expression profile of TM nAChRs was determined (Table 1). TM neurons were found to express transcripts of only principal $\alpha 3$, $\alpha 6$ and $\alpha 7$ nAChR subunits (Table 1 and Figure 3A). Transcripts of other known mammalian neuronal subunits ($\alpha 2$, $\alpha 4$ -5, $\alpha 9$ -10, $\beta 2$ -4) were not detected (Table 1 and Figure 3B) in ventral TM neurons dissociated from E1-3 areas (Figure 1B). The transcript for the $\alpha 7$ subunit was detected in 44/44 (100%) neurons tested. Transcripts for $\alpha 3$ and $\alpha 6$ were detected in 15/15 (100%) and 10/12 (83.33%) of all neurons (i.e., E1-3 areas), respectively. By contrast, $\alpha 4$, $\alpha 5$, $\beta 2$ and $\beta 4$ mRNAs were not detected in any cells (Figure 3). A band corresponding to the appropriate size for $\alpha 2$ was detected in 1/12 neurons examined (0.83%); $\alpha 2$ primers also had a tendency to form dimers that appear below the 50 bp marker in the gel. The $\alpha 2$ primers should detect a band that is 137 bp. Similarly, a 71 bp band appropriately sized for $\beta 3$ was detected in 1/13 (0.77%) neurons. In both of these cases multiple bands were detected in those lanes, indicating a potential decay of the cDNA for those samples. It is therefore likely that this single instance of $\alpha 2$ and $\beta 3$ detection represents an anomalous result (not shown) and the occurrence of $\alpha 2$ and $\beta 3$ transcripts in TM neurons is even less frequent. Tests for the presence of $\alpha 9$ and $\alpha 10$ mRNAs were conducted using relatively large ($\sim 300 \mu\text{m} \times 300 \mu\text{m}$) chunks of ventral TM E3 area containing clusters of TM neurons. Transcripts for $\alpha 9$ and $\alpha 10$ were not detected (samples from $n=4$ rats, not shown) even in large hypothalamic chunks, as expected. Thus, single-cell qPCR tests were not conducted for $\alpha 9$ -10 subunits. Therefore, qRT-PCR results demonstrate that transcripts of only three nAChR subunits ($\alpha 3$, $\alpha 6$ and $\alpha 7$) are present in the majority of ventral TM neurons (E1-3 areas), as indicated by fluorescent detection of SYBR green incorporation and melting temperatures that are appropriate for the predicted amplicons (Table 1).

To assure that lack of detection was not due to a technical issue with primers, qPCR was performed on RNA isolated from the entire hippocampus in a dilution series with 10-, 100-, 1,000- and 10,000-fold diluted tissue samples. These tests detected $\alpha 2$, $\alpha 4$, $\beta 2$, $\beta 3$ and $\beta 4$ subunit transcripts with as little as 1 ng starting total RNA. The corresponding c.t. values were: 27.8 ($\alpha 2$), 30.1 ($\alpha 4$); 27.04 ($\beta 2$), 28.5 ($\beta 3$), 29.6 ($\beta 4$). Therefore, even at 1 ng of total RNA, which would be expected to be near or below the level of RNA in a single neuron, these transcripts were detected after two rounds of PCR. Similarly, the $\alpha 5$ transcript was detectable only at 10 ng starting total RNA: c.t.=30.6 ($\alpha 5$). Negative controls (no RNA) gave c.t. >39.0 for all subunits tested. As a positive control, a similar analysis for the $\alpha 7$ subunit gave the following numbers: 12.43 (100 ng); 17.33 (10 ng), 21.08 (1 ng), 29.11 (100 pg), 40.09 (negative control). These results suggest that $\alpha 7$ transcript is present in the hippocampus at much higher levels than other subunits tested. However, a direct quantitative comparison between different transcripts, based on c.t. values, may not be completely accurate. Nevertheless, it is quite unlikely that $\alpha 2$, $\alpha 4$, $\alpha 5$, $\beta 2$, $\beta 3$ and $\beta 4$ mRNAs are present in rat TM neurons in amounts that play any significant role in TM responsiveness to nicotinic agents.

DISCUSSION

Hypothalamic TM neurons express high densities of native functional $\alpha 7$ -containing nAChRs and no non- $\alpha 7$ functional nAChRs [8, 29, 30, 32-34]. Specifically, the pharmacology of TM nAChRs [19, 33, 34], their Ca^{2+} -permeability [29, 31] and kinetics of whole-cell [29, 32-34] and single-channel [8, 30] currents are similar to those expected from $\alpha 7$ homomers [19, 20]. Moreover, the TM nucleus consists of a dense homogeneous population of neurons that are well-defined functionally, anatomically and cytochemically [2, 9]. Its ventral group is located on the edge of the posterior hypothalamus forming several large clusters of cells that can be easily identified and dissociated using a phase-contrast microscope (Figure 1) [31, 32]. These clusters act as a convenient source of acutely dissociated cells characterized by uniform neuronal and nAChR properties similar to those

observed in *in vivo* and *ex vivo* preparations [10, 25, 27-29, 31-35]. Therefore, TM neurons have been previously proposed as a useful source of native $\alpha 7$ -containing nAChRs. However, the expression of functional $\alpha 7$ -containing heteromers (e.g., $\alpha 7\beta^*$) in TM neurons has not been excluded. The key finding of this study is that TM neurons express detectable transcripts of only three principal nAChR subunits (i.e., $\alpha 3$, $\alpha 6$ and $\alpha 7$). In the absence of β and $\alpha 5$ subunits, the expression of functional $\alpha 7$ -containing heteromers in TM neurons is highly unlikely because: 1) The absence of β nAChR subunits eliminates the possibility of expression of non- $\alpha 7$ heterooligomers (i.e., $\alpha 3\beta^*$ and $\alpha 6\beta^*$) as $\alpha 3$ and $\alpha 6$ subunits cannot form functional nAChRs without β subunits [15]; and 2) Principal $\alpha 3$, $\alpha 6$ and $\alpha 7$ subunits alone are not known to form functional heteromeric nAChRs. Therefore, this finding supports the exclusive expression of native functional $\alpha 7$ homomers in rat TM neurons.

In the context of existing literature, this study highlights an intriguing positive correlation among expressions of $\alpha 7$ and $\alpha 3/\alpha 6$ subunit transcripts: $\alpha 7$ and $\alpha 3/\alpha 6$ mRNAs were distinctly co-expressed in 100% of TM neurons that were simultaneously tested for these 3 subunits. Regulation of cell type-specific expression of nAChRs remains unknown. Promoters of all nAChRs are similar in structure; generally, they are described as TATA-less, GC-rich regions with Sp-1 binding sites that are likely responsible for basal expression [14, 36]. Factors that may direct $\alpha 7$ and $\alpha 3/\alpha 6$ to the same cell types have yet to be determined. One possibility is that the expression of $\alpha 3/\alpha 6$ in TM neurons may be due to co-activation of their promoters by factors that target $\alpha 7$.

The functional consequences of co-expression of $\alpha 3$, $\alpha 6$ and $\alpha 7$ mRNAs in TM neurons are unclear. The concept of possible interactions among $\alpha 3$, $\alpha 6$ and $\alpha 7$ transcripts/subunits is intriguing. If indeed $\alpha 3$ and/or $\alpha 6$ transcripts/subunits interact with the expression and/or function of $\alpha 7$ transcripts/subunits, this concept may extend to other neuronal populations where the presence of β mRNAs may have made $\alpha 3$ and $\alpha 6$ subunits unexplored candidates for contributing to shaping the expression and function of $\alpha 7^*$ nAChRs.

Although it is remotely possible that $\alpha 5$ and β subunits are expressed in amounts below the level of detectability of the qRT-PCR assay, these subunits were detected in positive control samples (medial habenula and hippocampus) with as little as 1-10 ng of total RNA. Furthermore, $\beta 4$ (intracardiac ganglia, [21]), $\alpha 5$ (hippocampus, [26]), as well as $\beta 2-3$ (midbrain dopaminergic nuclei, [13]) transcripts have previously been detected using single-cell PCR analyses.

In conclusion, our results support the exclusive expression of functional $\alpha 7$ homomers in rat TM neurons and introduce these neurons as a unique reliable source of native functional homomeric $\alpha 7$ nAChRs suitable for *ex vivo* and *in vitro* pharmacological assays in developing selective $\alpha 7$ nAChR agents.

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Highlights

- Our results support the exclusive expression of native functional $\alpha 7$ homomers in rat TM neurons.
- TM neurons act as a unique reliable source of native functional $\alpha 7$ nAChRs.
- TM neurons are suitable for pharmacological assays in developing selective $\alpha 7$ nAChR agents.

Figure 1. TM neurons in E1-3 areas of coronal hypothalamic slices of rats

TM neurons completely (not shown) or partially (A, arrows) dissociated from coronal hypothalamic slices were used in this study. The majority of TM neurons used in RT-PCR experiments were acutely dissociated from E3 (A-C). E1 and E2 were found less populous than E3 (B-D), but TM neurons in E1-2 were often the largest (D, arrow). 3V indicates the third ventricle (B).

Figure 2. Positive controls for detecting known mammalian nAChR subunits in tissue chunks dissociated from the hippocampus (H), medial habenula (MH) and cochlea (C)

Agarose gel (2%) showing analysis of tissue chunks containing the hippocampus (H), medial habenula (MH) and cochlea (C) for PCR products amplified with specific primers for transcripts of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$, $\beta 4$, $\alpha 9$ and $\alpha 10$ nAChRs. All primers and experimental protocols were confirmed functional and appropriate for detection of subunit mRNAs in TM neurons.

Figure 3. Expression of $\alpha 3$, $\alpha 6$ and $\alpha 7$ nAChR subunits in individual TM neurons acutely dissociated from coronal hypothalamic slices

A) Agarose gel (2%) showing analysis of a single E3 neuron for PCR products amplified with specific primers for transcripts of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ nAChRs; **B)** Agarose gel (2%) showing analysis of a single E1-2 neuron for PCR products amplified with specific primers for $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ nAChRs. Neurons dissociated from E1 and E2 areas could not be reliably distinguished.

TABLE 1
Summary of results of single-cell qRT-PCR experiments

Primer Set	Amplicon Size	# Positive Cells	Mean ct Value
Actin	145 bp	14/15	17.8±1.9
α2	137 bp	1/12	n.d.
α3	72 bp	15/15	29.52±3.5
α4	61 bp	0/13	n.d.
α5	90 bp	0/14	n.d.
α6	73 bp	10/12	28.33±3.9
α7	90 bp	44/44	23.4±3.9
α9	107 bp	0/4 (E3 chunks)*	n.d.
α10	127 bp	0/4 (E3 chunks)*	n.d.
β2	121 bp	0/19	n.d.
β3	71 bp	1/13	n.d.
β4	73 bp	0/17	n.d.

* To detect α9 and α10 nAChR mRNAs, chunks of tissue (> 300 μm × 300 μm) near E3 were collected manually from coronal hypothalamic slices (300 μm thickness; n=4) using polished recording pipettes. The α8 nAChR subunit is strictly avian.