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Individual Variation in the Expression Profiles of Nicotinic Receptors in the Olfactory Bulb and Trigeminal Ganglion and Identification of α2, α6, α9, and β3 Transcripts

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ABSTRACT. Nicotine evokes dose-dependent and often variable chemosensory responses in animals and humans. Earlier observations that nicotine binds to some nicotinic acetylcholine receptor (nAChR) subtypes in the olfactory bulb (OB) and trigeminal ganglion (TG) led us to investigate the complete nAChR expression profile in each tissue and to determine whether inter-individual differences exist in male and female rats. Total RNA was extracted from individual samples of dissected OB and TG and analyzed by a sensitive reverse transcription–polymerase chain reaction (RT–PCR) assay to determine the messenger RNA profiles of ten transcripts encoded by the α2, α3, α4, α5, α6, α7, α9, β2, β3, and β4 nAChR genes. We found that (a) in the OB, all animals expressed α2, α3, α4, α5, α7, β2, and β4 subunit mRNAs, whereas α6, β3, and α9 transcripts were expressed in only 17, 28, and 33% of the animals, respectively, and (b) in the TG, all animals expressed α2, α3, α6, α7, β2, and β4 subunit mRNAs, whereas α9, β3, α4, and α5 transcripts were expressed in 4, 38, 88, and 92% of the animals, respectively. These results also identified new subunits that are expressed in each tissue (α2, α6, α9, and β3) and demonstrated that individual rats may have different tissue-specific expression profiles for α4, α5, α6, α9, and β3 transcripts. Such variations are likely to be reflected in the composition of functional receptor subtypes in the rat OB and TG that have different activation and desensitization characteristics to acetylcholine and nicotine. BIOCHEM PHARMACOL **59**;3:233–240, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. acetylcholine; nicotine; nicotinic receptors; chemosensory; olfactory bulb; trigeminal ganglion

Neuronal nAChRs¶ in the mammalian nervous system are composed of subunits α2–α7, α9, and β2–β4; however, it is very likely that other unidentified subunits are yet to be detected and cloned. Functional receptors from this superfamily of ligand-gated ion channels are formed by a pentameric arrangement of homogeneous (e.g. α7, α9) or heterogeneous (e.g. α4β2, α2β2, or α3β4α5) subunit combinations [1–3]. Understanding the composition and role of nAChRs in the olfactory and trigeminal systems is of interest for at least three reasons.

First, both chemosensory systems exhibit high sensitivity to *S*(-)-nicotine [4], and humans can discriminate between the *S*(-), the predominant form in tobacco [5], and the *R*(+) isomers [6, 7]. We have hypothesized [4] that, in both the olfactory and trigeminal periphery, at least one type of neuronal nAChR binds *S*(-)-nicotine as the first step in sensory transduction. A second type of neuronal nAChR is hypothesized to be sensitive to *R*(+)-nicotine.

Second, various experimental approaches have provided evidence that nAChRs are involved in OB and TG cellular functions. Alkondon and Albuquerque [8] used the whole cell patch-clamp technique to demonstrate high sensitivity to α-bungarotoxin in cultured OB neurons that were morphologically similar to periglomerular and granule cells. This and later work by Alkondon *et al.* [9] indicated that these responses were due to α7 receptors, which function presynaptically to influence information processing in the glomerular and mitral cell layers of the OB. Le Jeune *et al.* [10] used autoradiographic methods to demonstrate the presence of α4β2 nAChRs in the glomerular, mitral, and granule layers of the OB. Evoked field potential studies by Elaagouby *et al.* [11] suggested that tonic nicotinic modulation of bulbar interneuronal activity reduced the inhibitory action of granule cells. Flores *et al.* [12] reported that α3, α4, α5, β2, and β4 transcripts were expressed in the TG. Immunoprecipitation and [^{3}H]epibatidine binding experiments provided further evidence for α4β2 and α3β4

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¶*Abbreviations:* nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; OB, olfactory bulb; TG, trigeminal ganglion; RT–PCR, reverse transcription–polymerase chain reaction; AMV, Avian Myeloblastosis Virus; *Tfl*, *Thermus flavus*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and TBS, Tris-buffered saline.

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TABLE 1. Oligonucleotide PCR primers for rat nAChR subunits and GAPDH

Gene and GenBank accession number	5′ Sense primer (5′ → 3′)	3′ Antisense primer (5′ → 3′)	Transcript size (bp)
nAChR subunits			
α 2 L10077	CGG GTG CCC AGG TGG CTG ATG A	GAG GTG ACA GCA GAA TCT CGC TAG	292
α 3 X03440	GGC CGT GTT CTT GAA CCT GCT CC	GGG CAG AGA GGG ACA ACA CAG CG	281
α 4 L31620	CGT AGA GTC TTC CTG GAC ATC GTG	CTG GAC GCC TTC TAC TGC CCG TG	687
α 5 J05231	CCT AGA GGA CCA AGA TGT CGA CAG	CCG AGA TTT AGG TCC AGC CCC AC	565
α 6 L08227	CCA TGT TCC TTC AGG TCT TCC CCT	CGA TCA CGT CTT CAA CAT CAG GTG G	270
α 7 S53987	GAC AAG GTG CGG CCA GCT TGT CA	GCA CCG TGC ATG AGG TGC TCA TC	221
α 9 U12336	GCC AAG GTG GTC ATC CTG AAG TAC	CCC TTG GAG TTG GTG GCC TTG TG	326
α 9 U12336	CAG CTA ATG GTG GCA GAG ATC ATG C	GAT CAA GAC GGT CAT GAC AAA CAC C	576
β 2 L31622	GCA CGT CAG CGT CTG CGC TTG AG	GCC ACA GCT GCA TGG CCC CAC A	195
β 3 J04636	CCG GAT GGA AAG GAG AGT GAT ACA G	CGA TGT ATC CAC ATC TTC AAG GCT G	293
β 4 M33952	CTT GAA GTC AGC CTG GTC AGG GTC	GTC ATC GCT CTC CAG ATG CTG GG	285
GAPDH X02231	CGG ATT TGG CCG TAT CGG ACG CC	GCC TTG GCA GCA CCA GTG GAT GC	620

nAChR subtypes in the TG [12]. These data are also consistent with electrophysiological studies showing that some neurons in the TG are activated by nicotine [13–15].

Finally, since there is a decline in both olfactory function and nicotinic cholinergic integrity associated with Alzheimer's disease, continued investigations of the composition and function of nAChR subtypes in the OB may contribute to a mechanistic understanding of dementia [16–21]. Accordingly, nicotinic agonists are currently being tested for the treatment of some memory and learning disorders [22, 23].

Because of the importance of understanding the relationship between the composition of different nAChR subtypes and their distinct pharmacology in chemosensory tissues, we have undertaken a detailed study of the normal variation and expression profiles of nAChR subunits in a large sample of rats. Our results show that adult rats differ in the mRNA expression of certain nAChR subunits among individuals and between the OB and TG tissues. This information will benefit research aimed at elucidating the role and/or function of diverse nAChR subtypes in both the processing of chemosensory information and the cellular events that may lead to neurodegenerative disease. Portions of this work have been published previously in abstract form [14, 24].

MATERIALS AND METHODS

Animals and Tissue Collection

Naive adult Sprague–Dawley rats (200–400 g, Virus Antibody Free/Plus, Charles River Laboratories) were used in this study. The animals were housed individually in polycarbonate cages in a vivarium with controlled lighting (12 hr light/12 hr dark) and had *ad lib.* access to food and water. The temperature and relative humidity were maintained at 20–24° and 40–60%, respectively. Rats were anesthetized deeply with 70% CO_2 and then decapitated. After the brain was removed from the skull, the OB and TG were dissected (average wet weight of OB, 95 mg; TG, 48 mg). Tissue was placed in a sterile tube and frozen at −80°. The Institutional Animal Care and Use Committee approved all animal procedures.

RNA Extraction and RT–PCR

Total RNA was isolated from the OB and TG of each animal using the TOTALLY RNA Kit (Ambion). In most instances, it was possible to analyze the RNA from both tissues of each animal. Prior to RT–PCR experiments, the quality of all RNAs was assessed on denaturing gels, and total RNA was quantitated by measuring absorbance at 260 nm (Beckman DU-650 spectrophotometer). The RNA samples had A_{260}/A_{280} ratios ranging from 1.8 to 2.0. All RNA samples were treated with amplification grade DNase I according to the manufacturer's instructions (Gibco BRL, Cat. No. 18068–015) to eliminate any residual DNA. For conversion of total RNA (600–800 ng) to cDNA, a 50-μL single-tube reaction mixture was prepared from a master-mix containing 10 μL of AMV/*Tfl* 5x reaction buffer, 0.2 mM dNTP mix, 1.5 mM $MgS0_4$, 0.1 unit of AMV RT, and 0.1 unit of *Tfl* DNA polymerase (Access RT–PCR System, Cat. No. A1250). Then 30 pmol of each gene-specific primer pair was added to individual tubes. Primer sequences (Table 1) were selected from the unique cytoplasmic domain region of each nAChR subunit. A search of the GenBank database revealed no significant homology of our selected primer sequences with those for other rat genes. GAPDH was used as an internal control to verify the quality of each RNA sample and its subsequent RT–PCR analysis.

The RT and PCR cycling profiles using a Thermal Cycler 9600 (Perkin Elmer) were as follows: 1 cycle at 48° for 50 min; 1 cycle at 94° for 2 min 30 sec; 38 cycles at 94° for 40 sec and 70° for 1 min 45 sec; and a final cycle at 72° for 5 min. A 12-μL aliquot of each sample was electrophoresed on a 2.4% agarose gel containing 0.8 μg/mL of ethidium

bromide, and then photographed using the Eagle Eye Imaging System (Stratagene).

The Promega Access RT–PCR System was used because this one-tube, two-enzyme system provides high sensitivity and reproducible analyses of low abundance RNAs. RT was performed at an elevated temperature 48° to minimize the effects of RNA secondary structure and to improve amplification of G + C-rich sequences. The higher annealing and extension temperature 70° also minimized nonspecific primer annealing. Negative controls in each experiment included two reaction tubes that contained different primer pairs but no AMV RT. The PCR product for each RNA transcript appeared only when RT was present. A negative control RT–PCR experiment also was performed using 5 μg of yeast tRNA (Gibco BRL) with each gene-specific primer pair; no bands were detected (data not shown). Other negative controls were reaction tubes in which sterile nuclease-free water was substituted for the tissue RNA and reaction tubes that contained all of the assay components except the primers. Following amplification by PCR, the authenticity of newly identified nAChR subunits α2, α6, α9, and β3 was verified by DNA sequencing (ABI Prism 377, Perkin Elmer). The percent identity of our α2, α6, α9, and β3 transcripts to the corresponding GenBank rat nAChR sequence was 98, 96, 99, and 98%, respectively.

Immunoblot Analyses

OB and TG total cell lysate fractions were mixed with equal volumes of 2x Laemmli buffer (Sigma) and heated for 3 min at 100°. Proteins were separated by electrophoresis on 4–20% Tris–glycine gels (Novex) and transferred onto nitrocellulose membranes (Novex) using the Trans-Blot SD semi-dry electrophoretic blotting cell (Bio-Rad). Nonspecific binding was blocked by soaking membranes overnight at 4° in 6% blocking reagent (Bio-Rad) dissolved in TBS. Monoclonal antibody 306 (Research Biochemicals International) and polyclonal antibody K-20 (Santa Cruz Biotechnology) were used to detect α7 and α2 nAChR subunit proteins, respectively. The blots were incubated in primary antibody at 2.5 μg/mL (α7) or 2 μg/mL (α2) in the blocking solution overnight at 4°, washed in TBS containing 0.05% Tween-20, and then incubated for 2.5 hr at room temperature with CruzMarker-compatible horseradish peroxidase-conjugated secondary mouse or goat antibody (Santa Cruz Biotechnology) diluted to 1:3000 in blocking solution. After further washing with TBS, blots were incubated in ECL Plus Chemiluminescence reagent (Amersham Life Science) and exposed to Hyperfilm-ECL (Amersham Life Science) for 3–5 min. Protein concentrations were determined using the *DC* Protein Assay (Bio-Rad) with bovine γ-globulin as the standard.

Antibody K-20 is an affinity-purified goat polyclonal antibody raised against a peptide corresponding to amino acids 450–469 at the carboxy terminus of the α2 subunit of the nAChR precursor of rat origin. The α7 subunit mAb 306 has been described by Dominguez del Toro *et al.* [25]. In control experiments, the primary antibody was omitted on identical blots, and no signals were detected. We were unable to obtain antibodies for α6, α9, and β3 subunits.

RESULTS

Multiple Neuronal Nicotinic Receptor Subunit mRNAs Are Expressed in the OB and TG

Analysis of nAChR gene expression in the OB and TG showed individual variation and tissue-specific differences. In the OB, all animals expressed α2, α3, α4, α5, α7, β2, and β4 mRNAs in comparison with 17, 28, and 33% of the animals expressing α6, β3, and α9 mRNAs, respectively. More specifically, one male and two female rats expressed detectable levels of α6 mRNA. The same male rat also expressed a low level of α9 mRNA [see Fig. 1 (top panel) and Table 2].

Several variations in nAChR expression profiles also were observed in the TG. For example, all animals expressed α2, α3, α6, α7, β2, and β4 mRNAs, in comparison with 4, 38, 88, and 92% of the animals expressing α9, β3, α4, and α5 mRNAs, respectively. In the TG, one male rat did not express detectable α4 and α5 mRNAs and another male rat did not express detectable α5 mRNA; furthermore, two female rats did not express detectable α4 mRNA. Only one female rat in the present study expressed detectable levels of α9 mRNA in the TG. Previously, low levels of α9 mRNA were detected in the TG of a few rats using the intron–exon boundary primers [14]. An expression profile and comparison summary of nAChR subunit mRNAs in the TG are shown in Fig. 1 (bottom panel) and Table 2.

In both tissues, a subset of male and female rats expressed β3 mRNA. All animals that expressed variations in certain nAChR subunit mRNAs had similar optical density measurements of GAPDH mRNA as the other animals, indicating that the RNA samples were not degraded. Duplicate RT–PCR experiments were performed on the RNA samples from animals that showed variations in subunit expression. Duplicate experiments confirmed the initial findings.

Immunoblot Analyses

Immunoblot experiments were performed to verify the expression of the α2 nAChR protein and to visually compare the relative α2 protein levels in the OB and TG. Additionally, recent studies in chick retina [26] and transfected COS cells [27] show that the α7 protein is glycosylated and can exist as several peptides in different states of glycosylation. We, therefore, investigated whether the α7 protein was similarly expressed in the rat OB and TG. Immunoblot analyses for α2 and α7 nAChR subunits were performed with total cell lysate fractions from the OB and TG of the same animal and are shown in Fig. 2.

The α2 protein was expressed in both tissues but was more abundant in the OB. The experimentally determined molecular mass of the α2 nAChR protein is consistent with the predicted molecular mass of ~55 kDa [28]. In compar-

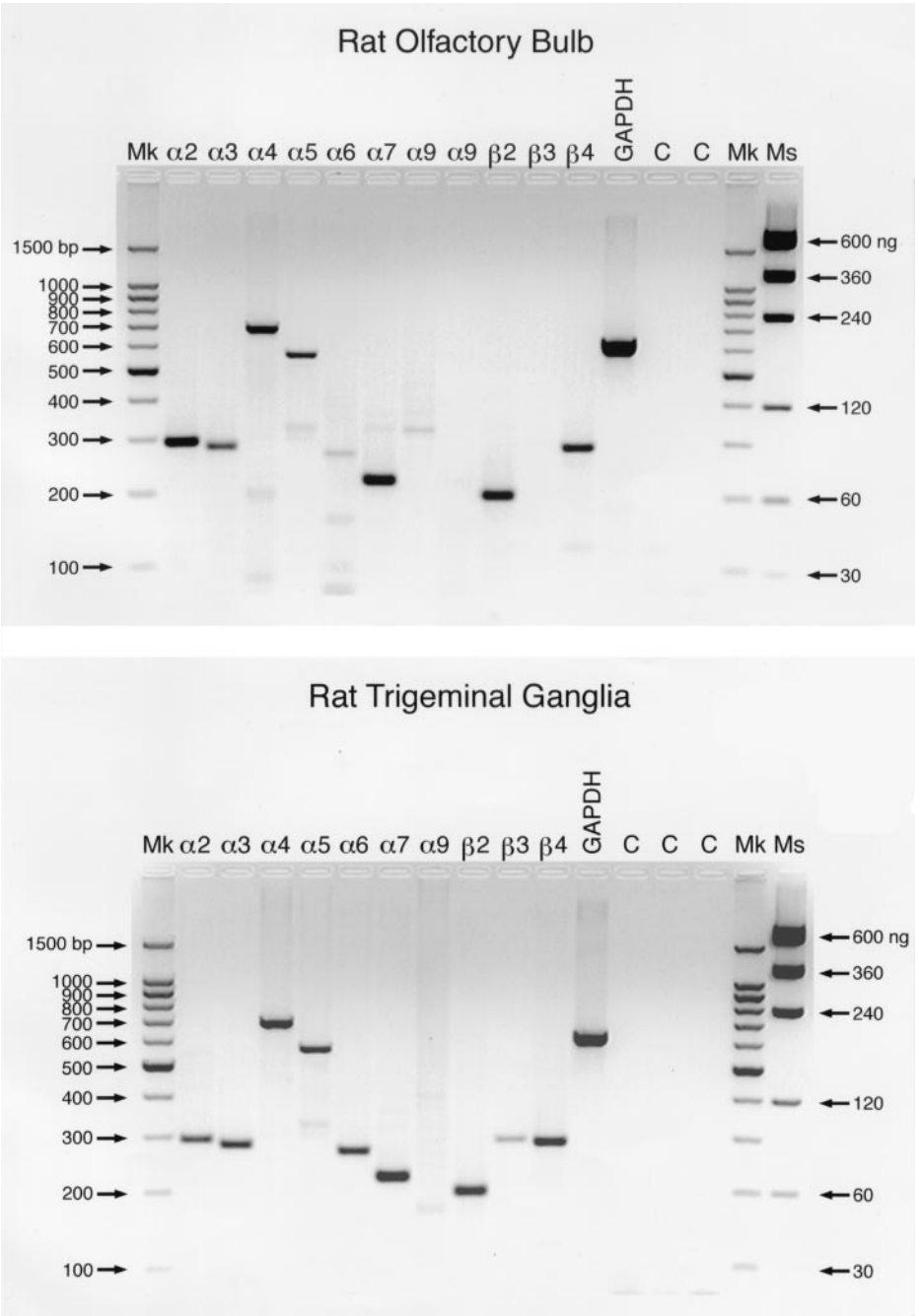

FIG. 1. Detection of multiple nAChR transcripts in the rat OB and TG. (Top panel) Inverted images of ethidium-bromide stained agarose gels demonstrating the resolution of RT–PCR amplification with rat nAChR subunit and GAPDH gene-specific primers. The mRNA profile shown for the OB is from an animal that expressed a low but detectable level of α6 mRNA. This animal is also one of six that expressed a low but detectable level of α9 mRNA as detected by the 326-bp cytoplasmic domain primer pair. No band was detected in this animal using the 576-bp intron–exon boundary primer pair. (Bottom panel) The mRNA profile shown for the TG is from one of ten rats that expressed a low but detectable level of β3 mRNA. The α6 mRNA was expressed in the TG of all animals. Transcripts for the α2 subunit were expressed in all animals in both the OB and TG. The authenticity of newly identified nAChR mRNAs (α2, α6, α9, and β3) was verified by DNA sequencing. The lanes marked 'Mk' contain DNA ladders (Promega) ranging from 100 to 1500 bp, and the lanes marked 'Ms' contain an equivalent aliquot of each amplification reaction (12 μL) of a low DNA mass ladder (Gibco BRL) ranging from 30 to 600 ng. The control reactions (lanes marked 'C') contain all of the reaction components except AMV RT.

ison, the α7 protein was expressed in both tissues but was more abundant in the TG. Differences in relative protein expression levels between structures may be due to tissue-specific proteases or more rapid protein turnover mechanisms in the OB than the TG. Four α7 peptides were detected (~62, 58, 54, and 43 kDa) in the rat OB and TG. This finding is consistent with an earlier report of the α7 protein existing in different states of glycosylation [27]. The

TABLE 2. Expression profiles of nAChR subunit mRNAs in adult rat OB and TG

	α2	α3	α4	α5	α6	α7	α9	β2	β3	β4
OB*	18/18 (100%)	18/18 (100%)	18/18 (100%)	18/18 (100%)	3/18 (17%)	18/18 (100%)	6/18 (33%)	18/18 (100%)	5/18 (28%)	18/18 (100%)
TG†	26/26 (100%)	26/26 (100%)	23/26 (88%)	24/26 (92%)	26/26 (100%)	26/26 (100%)	1/26 (4%)	26/26 (100%)	10/26 (38%)	26/26 (100%)

Data are shown as the number of animals that expressed gene-specific transcripts per total number of animals studied for each issue.
* N = 18: 8 female, 10 males.
† N = 26: 18 female, 8 male.

approximate molecular mass of the α7 protein is ~58 kDa, and the calculated molecular mass of the nonglycosylated, mature α7 protein is ~54 kDa [29]. In agreement with these reports, our findings suggest that two α7 peptides (~62 and 58 kDa) may be glycosylated, and two peptides (~54 and 43 kDa) may be nonglycosylated mature α7 peptides. Alternatively, the 43-kDa band in both tissues and the low mass peptide band in the OB (~28 kDa) may be due to protein degradation.

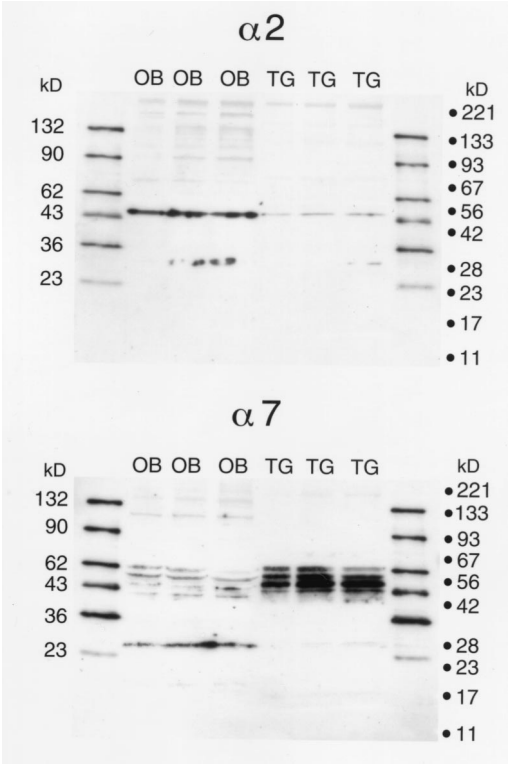

FIG. 2. Immunoblot analyses of nAChR subunits α2 and α7 in the OB and TG. Approximately 45, 90, and 135 μg of protein from the same male rat OB and TG homogenates (left to right lanes, respectively, for each tissue) were subjected to gel electrophoresis, and the blots were probed with polyclonal antibody K-20 (α2) and mAb 306 (α7). Cruz Marker molecular size standards were used on the outside lanes of each blot (132–23 kDa), and the BENCHMARK Prestained Protein Ladder (Gibco BRL) is marked on the right side of each blot (221–11 kDa). These experiments were performed in tissue preparations from three different animals with similar results.

DISCUSSION

The new information we report here shows that nAChR gene expression varies among individual rats as well as from the OB to the TG. To our knowledge, no study of this nature has been done in sensory tissue of the rat or human despite the general importance of normative tissue differences as well as the need to better understand why some individuals have a greater degree of chemosensitivity to endogenous ACh or nicotine from tobacco products. Although the range for individual chemosensory responses is variable and the OB and TG differ in function, the molecular basis for these functional variations is not known. In the rat, however, we can determine the molecular profile of the known nAChR genes and identify whether individual differences exist at the mRNA level, using sensitive subunit-specific methods.

Our mRNA data on α3, α4, α5, β2, and β4 nAChR subunits in the TG agreed with Flores *et al.* [12]; however, in contrast to their study and to work done by Wada *et al.* [30], we also detected α2 mRNA in both the OB and TG. Our detection of α2 mRNAs may be attributed to primer design from the unique cytoplasmic domain of this subunit, assay specificity, and/or sensitivity. The expression of α2 protein also was confirmed by immunoblot analyses and detection of an expected 55-kDa protein. The detection of α2 mRNA and protein in the OB and TG also raises an interesting question of whether potential α2β2 nAChRs in the rat account for and/or contribute to the high sensitivity to nicotine previously described in *Xenopus* oocytes by Luetje and Patrick [31].

A few animals did not express α4 and α5 mRNAs in the TG. This finding is of interest because in oocytes, channels containing the α5 subunit (α5α4β2) are activated and desensitized by nanomolar concentrations of nicotine [32]. Moreover, nAChRs containing the β2 subunit are involved in the reinforcing properties of nicotine [33], and β2 transcripts were expressed in both tissues of all animals in the present study. It is possible that genetic variation in the expression of α4 and/or α5 transcripts in the TG could affect the sensory responses of an individual to ACh and nicotine. Accordingly, Gerkovich *et al.* [34] have reported individual differences in cardiac and electromyographic activity after smoking.

The α6 transcript was expressed in the TG of all animals and in the OB of only three animals. Recently, Gerzanich

et al. [35] demonstrated that rat α6 and human β4 nAChRs have a profile in which nicotine acts as a poor partial agonist. Additionally, the α6 subunit has been co-localized in cell groups with the β3 subunit [36]. Immunoreactivity for the β6 subunit is found in adult rat dopaminergic neurons of the midbrain [37], where nicotine activates and desensitizes midbrain dopaminergic neurons [38]. Given this information, it is interesting to note that dopamine D2 receptor mRNA also is expressed in neurons of the rat TG [39].

Our RT–PCR and immunoblot data demonstrated that α7 mRNA and protein were expressed in both tissues and complement the electrophysiological findings of Alkondon *et al.* [8, 9]. Possible functional implications of an α7 nAChR in the OB and TG may be associated with neuronal plasticity [40, 41] and neuroprotective and memory-related actions [22]. The detection of four α7 peptides ranging in size from ~62 to 43 kDa suggests that the α7 protein is expressed in different states of glycosylation in the rat OB and TG. This finding is consistent with the presence of three consensus asparagine-linked glycosylation sites on the rat α7 protein and complements earlier reports of α-bungarotoxin binding sites in both tissues [42, 43].

The α9 transcript was detected in the OB of 33% of the animals and in the TG of only one animal. Elgoyhen *et al.* [44] demonstrated that the α9 homomeric receptor exhibits an unusual nicotinic–muscarinic pharmacology. Our data extend their findings in that we detected α9 mRNA in the OB and to a lesser extent in the TG, as previously reported [14]. α9 mRNA has been detected in the rat nasal epithelium of the olfactory turbinates during development [44], and we also have shown that adult rats express α9 mRNA in the olfactory epithelium, using intron–exon boundary primers [4]. The differential detection of α9 mRNA with unique primer pairs spanning different regions of the gene suggests that the α9 subunit may exist in two or more isoforms as described for the muscle-α subunit by Beeson *et al.* [45].

Transcripts for β3 mRNA were detected in the OB and TG of 28 and 38% of the animals, respectively, and these results extend the earlier findings of Hernandez *et al.* [46] and Morley *et al.* [47]. In the rat, β3 mRNA has been localized in the mesencephalic trigeminal nucleus [48] and in individual neurons of the intracardiac ganglia [49]. Recently, Forsayeth and Kobrin [50] provided evidence for a functional α4β2β3β4 receptor in the rat cerebellum. Their work underscored the possibility that certain subtypes of nAChRs may represent a very small proportion of the population of nicotinic receptors in a given tissue. Based on this report, putative α4β2β3β4 receptors may exist in the rat OB and TG; however, immunoprecipitation studies would have to be used to verify this or other nAChR arrangements in either the OB or the TG.

In summary, our results extend the molecular biology of nAChRs in the rat OB and TG and offer insight into the neural and/or cellular basis of sensory information processing by the likely existence of multiple nAChR subtypes and evidence of inter-individual expression differences. The degree to which the absence of detectable mRNA in a given case may reflect differences between the OB and TG, differences among animals, or differences over time within a given animal is not clear at the present time. However, mixed populations of nAChR subtypes whose protein subunits are translated from the varied pools of expressed mRNAs are likely to have different activation and desensitization characteristics in response to ACh and nicotine [51]. Since the pharmacology and function of a given nAChR subtype depends on its subunit composition, the inter-individual differences in subunit gene expression that we describe here could be reflected in individual chemosensory responses to endogenous ACh or nicotine from the use of tobacco products or through the therapeutic use of nicotine-containing vehicles to combat nicotine addiction or to treat Alzheimer's disease.

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