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Localization of nAChR subunit mRNAs in the brain of *Macaca mulatta*

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Abstract

We present here a systematic mapping of nAChR subunit mRNAs in *Macaca mulatta* brain. A fragment, from the transmembrane segments MIII to MIV of *Macaca* neuronal nAChR subunits was cloned, and shown to exhibit high identity (around 95%) to the corresponding human subunits. Then, specific oligodeoxynucleotides were synthesized for *in situ* hybridization experiments. Both α4 and β2 mRNA signals were widely distributed in the brain, being stronger in the thalamus and in the dopaminergic cells of the mesencephalon. Most brain nuclei displayed both α4 and β2 signals with the exception of some basal ganglia regions and the reticular thalamic nucleus which were devoid of α4 signal. α6 and β3 mRNA signals were selectively concentrated in the substantia nigra and the medial habenula. The strongest signals for α3 or β4 mRNAs were found in the epithalamus (medial habenula and pineal gland), whereas there were no specific α3 or β4 signals in mesencephalic dopaminergic nuclei. α5 and α7 mRNA signals were found in several brain areas, including cerebral cortex, thalamus and substantia nigra, although at a lower level than α4 and β2. The distribution of α3, α4, α5, α6, α7, β2, β3 and β4 subunit mRNAs in the monkey is substantially similar to that observed in rodent brain. Surprisingly, α2 mRNA signal was largely distributed in the *Macaca* brain, at levels comparable with those of α4 and β2. This observation represents the main difference between rodent and *Macaca* subunit mRNA distribution and suggests that, besides α4β2*, α2β2* nAChRs constitute a main nAChR isoform in primate brain.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are thought to be involved in several brain functions including control of locomotor activity (Clarke *et al*., 1988), analgesia (Marubio *et al*., 1999), modulation of sensory inputs, memory processes (Picciotto *et al*., 1995; Levin & Simon, 1998) and reward mechanisms (Picciotto *et al*., 1998). They may also contribute to neuronal survival and maintenance of cognitive performance during ageing (Zoli *et al*., 1999). Moreover, mutations in nAChR subunit α4 cause nocturnal autosomal dominant frontal lobe epilepsy (Steinlein *et al*., 1995) and alterations of α7 subunit gene expression have been related to forms of schizophrenia (Leonard *et al*., 1996; Freedman *et al*., 1997). nAChRs may also be involved in the pathophysiology of Parkinson's disease, Alzheimer's disease and Gilles de la Tourette's syndrome (Léna & Changeux, 1997; Lindstrom, 1997). Yet, the most relevant health problem related to nAChRs is nicotine addiction (Dani & Heinemann, 1996; Koob, 1996; Rose & Corrigall, 1997; Picciotto *et al*., 1998).

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nAChRs belong to the superfamily of ligand-gated ion channels (LGICs) (Cockcroft *et al*., 1992; Galzi & Changeux, 1994; Le Novère & Changeux, 1995; Bargmann, 1998), which mediate rapid channel opening and desensitization, features which make them adequate for short-term neuronal signalling. nAChRs are transmembrane proteins made up of five homologous subunits arranged symmetrically around an axis perpendicular to the membrane. They form a cationic channel whose opening is controlled in an allosteric manner by the binding of agonists at sites located at the interfaces between subunits (Changeux, 1990; Unwin, 1995). Molecular cloning methods have revealed the existence of a set of homologous genes encoding nAChR subunits in the amniote nervous system, which have been named α2–α7 and β2–β4 (Sargent, 1993; Le Novère & Changeux, 1995; Role & Berg, 1996). They assemble into a number of different receptors with specific pharmacological characteristics, channel conductance, open time and allosteric properties (Mulle *et al*., 1991; Conroy *et al*., 1992; Vernallis *et al*., 1993; Conroy & Berg, 1995; Ragozzino *et al*., 1997; Vailati *et al*., 1999). The proper assembly of the subunits into receptor oligomers is crucial for their function.

The role of the different nAChR oligomers primarily depends on their anatomical location in cholinergic circuits. A basic step in the understanding of those functions is therefore to investigate the distribution of the various nAChR subunits in the brain. While the study of nAChR subunit protein has been hampered by the availability of specific antibodies (Hill *et al*., 1993; Goldner *et al*., 1997; Rogers *et al*., 1998; Sorenson *et al*., 1998), extensive anatomical studies of neuronal nAChR subunit mRNAs have been performed in adult (Deneris *et al*., 1989; Wada *et al*., 1989, 1990; Dineley-Miller & Patrick, 1992; Séguéla *et al*., 1993; Rust *et al*., 1994; Le Novère *et al*., 1996) and the embryonic (Hoover &

TABLE 1. PCR primers used to clone the genomic fragments

Subunit	PCR primers	Restriction enzymes
α2	For 5′-GGGAATTCATTCGGCGAGTACCTGCTCTTCACCATGAT-3′	
	Rev5′-GGGGATCCAGAGTCAGCATCCCTCAGACCTCAGACGGG-3′	EcoRI and BamHI
α3	For 5′-GGGAATTCATTCGGGAGTACCTCCTCTTCACTATGAT-3′	
	Rev5′-GGGGATCTTTCATGTTTTCGGCAATGTACTTCACACT-3′	EcoRI and BamHI
α4	For 5′-GGGAATTCATTCGGCGAGTACCTGCTCTTCACCATGAT-3′	
	Rev5′-GGGGATCCGAAGTCAGTGTCTTCTGCCTTGAGGTGG-3′	EcoRI and BamHI
α5	For 5′-CCTAGCATCGATAGCTGTTGCTGGTATCCGTATG-3′	
	Rev5′-TTCCAGGACTAGTCTCACGGACATCATTTTCCTTC-3′	SpeI and ClaI
α6	For 5′-CCTAGCATCGATGCCGATGTTTTACACGATTAATC-3′	
	Rev5′-TTCAGGACTAGTCTCCTTGGTTTCATTGTGGCTCT-3	SpeI and ClaI
α7	For 5′-CAGCGAAAGCGGTTGGCGATGTAGCGGACCT3′	
	Rev5′-CTCCACGAAGTTGGGAGCCGACATCAGGATG-3′	SpeI and ClaI
β2	For 5′-GGGAATTCAGCGTGCTCAACGTGCACCACCGCTCG-3′	
	Rev 5′-CCTGGTCATCGTCCTCGCTCCGCATGTGGTCTGCGA-3′	EcoRI and BamHI
β3	For 5′-AATTCACGGTTTTTGTAATTAATGGTCCACCACAGATC-3′	
	Rev5′-GATCCGCTGACGAAGTGTTCCTTTTTCACATGCCTCGA-3′	EcoRI and BamHI
β4	For 5′-CCTAGCATCGATATCATCAAGCGCAAGCCTCT-3′	
	Rev5′-TTCAGGACTAGTCATGTGCTGGGCGATGAAGCT-3′	SpeI and ClaI

Goldman, 1992; Bina *et al*., 1995; Zoli *et al*., 1995) rodent nervous system. However, only partial analysis of the distribution of nAChR subunits has been performed in the primate brain (Cimino *et al*., 1992; Rubboli *et al*., 1994; Breese *et al*., 1997). We report here an extensive survey of the distribution of nAChR subunit mRNAs in the central nervous system (CNS) of the Rhesus monkey by *in situ* hybridization histochemistry using oligodeoxynucleotide probes. The highly variable cytoplasmic region of all nAChR neuronal subunits has been cloned and sequenced from Rhesus monkey genomic DNA, and used to design the probes for morphological analysis. Part of the results of this paper has been already published in abstract form (Han *et al*., 1999).

Materials and methods

Molecular cloning and sequencing of Macaca α2–α7, β2–β4 subunit fragments

For each subunit two primers were synthesized, based on the conserved regions between rat and human subunits. Restriction enzyme linkers were included at the 5′-end of primers to facilitate the subcloning of the amplified DNA into the vector. PCR were conducted on genomic DNA extracted from frozen *Macaca* forebrain by a standard phenol/chloroform/isoamyl-alcohol method followed by dialysis in Tris-EDTA buffer (pH 7.5). The fragments corresponding with the cytoplasmic domain were isolated after electrophoresis in low-welt 1.2% agarose, double digested and subcloned in pBluescript II SK. The nucleotide sequence of all fragments was determined by the chain termination method as provided by the manufacturer of the automatic sequencer (ALFexpress Autoread Sequencing Kit, Pharmacia Biotech, France). Primers (Eurogentec, Belgium) used for PCR and restriction enzymes (Promega, WI, USA) are reported in Table 1. The Wisconsin package of the genetic computer group was used for data analysis (Genetic Computer Group, WI, USA).

Oligodeoxynucleotide synthesis and labelling

Following analysis for mRNA secondary structure using the routine Stemloop of the Wisconsin package, probe sequences were chosen in unique regions of the mRNA without putative secondary structure, containing 50–60% G and C, as described in Table 2. Oligodeoxynucleotides were purchased from Genset (France) and labelled at the 3′ end using [α^{33}P]dATP (NEN, MA, USA), and terminal-deoxynucleotidyl transferase (Roche, Switzerland) following the specifications of the manufacturer, to a specific activity of 200–600 kBq/pmol. Unincorporated [α^{33}P]dATP was extracted by precipitation in ethanol, filtration on ProbeQuanTM G-50 Microcolumns (Amersham Pharmacia Biotech, NJ, USA), and a second precipitation in ethanol. Probes were finally resuspended in distilled water.

Animals

Two adult rhesus monkeys (*Macaca mulatta*) were used. The animals were tranquillised with ketamine (0.1 mL/kg), and deeply anaesthetized with sodium phenobarbital (40 mg/kg, with a solution at 50 mg/mL), prior to transcardiac perfusion with 500 mL of cold saline solution. Maintenance of animals and the procedures for euthanasia were performed according to the recommendations of the Centre National de la Recherche Scientifique ethical committee for animal care and manipulation. The brain was then extracted, dissected into several pieces, frozen in dry-ice powder and kept at –80 °C until use.

In situ *hybridization procedure*

The *in situ* hybridization method used is described and discussed in Wisden & Morris (1994). Frozen tissues were cut with a cryostat (14 µm-thick sections), thaw mounted on superfrost slides and stored at –80 °C (for < 2 weeks). The procedure was carried out according to Young *et al*. (1986), modified as described in Zoli *et al*. (1995) and Le Novère *et al*. (1996). Briefly, sections were fixed with 4% paraformaldehyde, acetylated and stored in 80% ethanol at 4 °C until the hybridization. Sections were delipidated in ethanol and chloroform, prehybridized for 2–4 h at 37 °C in a moist chamber and hybridized for 20 h at 37 °C under parafilm coverslips. The composition of the prehybridization and hybridization mixtures is described in Le Novère *et al*. (1996). Probes were added in the hybridization mixture at a concentration of 0.55 nM (corresponding to 15 fmol per section or 7500–62 500 Bq/75 µL per section according to the labelling). In order to assess the level of nonspecific staining, one in every four consecutive section was hybridized in the presence of a 50X excess of unlabelled probe. Sections were rinsed twice for 5 min in 2X standard saline citrate (SSC) solution (0.3 M NaCl, 0.03 M sodium citrate) at room temperature (RT), three times for 15 min in 1X SSC at RT, once for 15 min in 1X SSC at melting

TABLE 2. Oligodeoxynucleotide probes used in the *in situ* hybridization

Subunit	Probe code	Sequence of the oligodeoxynucleotide	GC (%)	Tm (°C)
α2	200	5′-CTCCTCCTCAACAACAACCTCCCTCTCCTCGGCATCCACCTTGGT-3′	58	76
α2	207	5′-CCTTCTGCATGCGGGGTGACAGCAGCAGCTCACCTTCCTGCAGCA-3′	62	78
α3	169	5′-GCTGCGGTGGTGGCAGTAAACACACATCCTGTCCTGGCAGGGGTA-3′	61	76
α4	195	5′-TCTGGGGGCGGGAGCTCAGCCGAGTGGGTCTTGCGAGA-3′	68	75
α5	193	5′-GAAGACAGTCAAAGACACAAGTACTGAAGTGCAGAGACAAATCTTT-3′	40	56
α5	194	5′-GGCATTATGTGTTGAGGAAGAACGATGATGAATGTTGATAGCGAAGACGG-3′	44	59
α6	197	5′-AAGTTCATTTGATTTGTGATAGTGGAAGCATTATTTAAGATGTCTGGGTTC-3′	33	55
α6	198	5′-GCTATGAATTGACACTGTTAATCACCTCTTCAACTTCAGGCGAGTGCTCCC-3′	47	66
α7	204	5′-CTCCACCGAAGTTGGGAGGCGACATCAGGATGCCGATGGTGCAGAT-3′	60	71
α7	205	5′-CAGCGAAAGCGGTTGGCGATGTAGCGGACCTCCTCCAGGATCTTG-3′	60	70
β2	192	5′-ATGAAGCGCACGCCGTCCACCGCCTCCCGGAGGCCACAG-3′	71	76
β3	199	5′-AACCTCGTTAACCAGGGGGCCATGGGGTGGTATGTGGAAGAA-3′	55	68
β4	203	5′-TCACAAAGTACATGGAGTTCCCATAGAGTTTGGAGGGGCTGGTGGAG-3′	51	67

The melting temperature is calculated by the method of nearest-neighbour with the programme MELTING (www.pasteur.fr/recherche/unites/neubiomol/meltinghome.html).

TABLE 3. Accession numbers of the public sequence databases for the monkey genomic fragments and interspecies sequence identities

Subunit	DDBJ/EMBL/GenBank accession number	%ID DNA human	%ID DNA rat	Length (bp)
α2	AJ245971	94.1	73.8	423
α3	AJ245972	97.1	85.3	373
α4	AJ245973	93.5	74.1	780
α5	AJ245974	97.6	83.1	505
α6	AJ245975	95.6	78.4	591
α7	AJ245976	94.6	84.3	472
β2	AJ245977	94.8	82.1	330
β3	AJ245978	95.3	72.8	235
β4	AJ245979	95.5	83.5	577

%ID, percentage identity.

temperature minus 20 °C, and once for 15 min in 1X SSC at room temperature. After a fast dip in ice-cold water, the slides were subjected to an alcohol gradient, dried at RT, exposed to [^{3}H]-Hyperfilm (Amersham, UK) for 7–14 days and then dipped into a photographic emulsion (NTB2, Kodak, NY, USA) for 3–4 months.

Analysis of histological preparations

The analysis of the labelling pattern was carried out on both film and emulsion autoradiograms. The identification of anatomical structures was carried out after counter-staining of the sections with toluidine blue. Definition of anatomical areas in the brain was based on Szabo & Cowan (1984).

Results

Cloning of the cytoplasmic regions of the nAChR subunits from Macaca mulatta

The cytoplasmic region of all known neuronal nAChR subunits of the Rhesus monkey was cloned and sequenced. This region is generally conserved across mammalian species but appears to be highly variable between different nAChR subunits (Le Novère & Changeux, 1995; Elliott *et al.*, 1996).

Each sequence was compared with the respective rat and human cytoplasmic regions by the program GAP of the Wisconsin package. The percent identity towards human sequences was uniformly high ranging from 94.1% (α2) to 97.6% (α4) (Table 3). As expected, the percentage identity towards rat sequences was also high, although always lower than that of human, ranging from 72.8% (β3) to 85.3% (α3) (Table 3). The nucleotide sequence of each fragment was submitted to the EMBL/Genbank/DDBJ databases (accession numbers AJ245971-9). Length, accession number, % identity with human and rat sequences of each fragment are reported in Table 3.

Distribution of nAChR subunits in the brain of Macaca mulatta

The *in situ* hybridization experiments were carried out in the brains of two rhesus monkeys. The resulting staining was qualitatively identical in both brains. The same structures were labelled and the ratio of intensities between different regions appeared conserved. However, in the absence of a densitometric quantification, we cannot rule out the existence of minor differences in regional labelling intensities between the two brains.

For all probes, the signal disappeared when an excess of unlabelled probe was added to the hybridization mixture. In addition, when two probes directed against two different parts of the same subunit mRNA were used, they displayed identical staining patterns. In the mapping experiments we used oligonucleotide probes 200, 194, 198 and 204 for α2, α5, α6 and α7 mRNA, respectively.

The rostro-caudal distribution of each subunit mRNA was analysed in coronal sections, taken from the monkey brain between levels corresponding with A25 and P6.6 of the *Cynomolgus* monkey brain atlas (Szabo & Cowan, 1984). A complete account of regions containing hybridization signal for nAChR subunits as judged from autoradiographs and Nissl counter-staining is reported in Table 4.

Isocortex

Specific labelling for several nAChR subunit mRNAs was present in the isocortex. Weak to strong hybridization signals for α2, α4, α5, α7 and β2 mRNAs were detected in all cortical layers. However, some subunit mRNAs showed a specific intracortical distribution pattern. α2 and β2 were found throughout all layers, but the signal for α2 was weaker than that for β2. α4 and α5 were more concentrated in layer VI, and α7 showed a weak signal in layers II–III but was barely detectable in the other cortical layers. No specific signal was detected for the subunits α3, α6, β3 and β4 in the isocortex. No obvious difference in the intensity or distribution pattern of the nAChR mRNA signals was observed between different cortical areas.

TABLE 4. Distribution of the hybridization signal of nAChR subunit mRNAs in the CNS

	α2	α3	α4	α5	α6	α7	β2	β3	β4
Telencephalon									
Isocortex									
Layer II-III	+	–	+	+	–	+	+++	–	–
IV	+	–	+	+	–	(+)	+++	–	–
V	+	–	+	+	–	(+)	+++	–	–
VI	+	–	++	++	–	(+)	++++	–	–
Subcortical forebrain									
Septum	(+)	–	(+)	–	–	–	++	–	–
Putamen	(+)	–	–	–	–	–	++	–	–
Caudate nucleus	(+)	–	–	–	–	–	++	–	–
Globus Pallidus	–	–	–	–	–	–	(+)	–	–
Claustrum	(+)	–	(+)	–	–	–	++	–	–
Hippocampal formation									
Dentate gyrus granular lay	+	+	(+)	–	–	+++	++++	(+)	(+)
CA pyramidal lay	+	+	(+)	–	–	++	++++	(+)	(+)
Diencephalon									
Thalamus									
Anterodorsal	+++	–	+++	+	–	+	++	–	–
Anteromedial	+++	–	+++	+	–	+	++	–	–
Anteroventral	+++	–	+++	+	–	+	+++	–	–
Ventral anterior	++	–	++	–	–	–	++	–	–
Ventral lateral	++	–	++	–	–	+	++	–	–
Ventral posterolateral	++	–	++	–	–	–	++	–	–
Lateral dorsal	++	–	+++	–	–	–	+	–	–
Medial dorsal	++	–	+++	–	–	–	+	–	–
Central lateral	–	–	+	–	–	–	++	–	–
Paracentral	++	–	+	–	–	–	+	–	–
Centrum medianum	–	–	++	–	–	(+)	++	–	–
Parafascicular	–	–	++	–	–	(+)	++	–	–
Reuniens	++	–	+++	–	–	–	++	–	–
Lateral posterior	++	–	+++	–	–	(+)	++	–	–
Pulvinar	++	–	+++	–	–	(+)	++	–	–
Reticular	(+)	–	–	+	–	+	++	–	–
Lateral geniculate	++++	–	++++	+	–	–	++++	–	–
Medial geniculate	+++	–	+++	+	–	–	++	–	–
Epithalamus									
Medial habenula, medial part	+	++	+	–	–	–	++	–	+++
Medial habenula, lateral part	+	++++	+	–	+++	++	++	+	++++
Pineal gland	(+)	+++	–	–	–	+	+++	–	+++
Hypothalamus									
Medial nuclei	+	–	+	–	–	+	++	–	–
Lateral nuclei	+	–	+	–	–	(+)	++	–	–
Supraoptic nucleus	(+)	–	++	–	–	+++	++	–	–
Mesencephalon									
Substantia nigra, pars compacta	+	–	+++	++	+++	+	++++	+++	–
Substantia nigra, pars reticulata	–	–	++	–	–	–	+++	–	–
Ventral tegmetal area	(+)	–	+	++	++	+	++	+	–
Interpeduncular nucleus	+	–	(+)	–	–	–	+	–	(+)
Red nucleus	++	–	–	–	–	–	+	–	–
Oculomotor III nucleus	–	–	+	–	–	–	++	–	–

Intensity of signal was rated as follows: –, no signal; (+), barely detectable; +, weak; ++, moderate; +++, strong; ++++, very strong.

Subcortical forebrain

Only α2, α4 and β2 mRNAs were detected in septal regions. The signal intensity for β2 was moderate, whereas those for α2 and α4 were very weak.

A moderate signal for β2 was detected in the septum, caudate nucleus, putamen and the claustrum (Fig. 1), and a very weak signal was detected in the globus pallidus. A signal for α2 was also noticed in these regions although at very weak level. Very weak staining for α4 mRNA was detected in the septum and claustrum.

Hippocampal formation

Strong signals for α7 and β2 mRNA were present in the hippocampal formation, particularly in the granular layer of the dentate gyrus and the pyramidal layer of the Ammon's horn. Weak positive signals for α2 and α3 were also detected in these layers. The labelling for α4, β3 and β4 was barely detectable in hippocampal formation.

Thalamus

Three nAChR subunit mRNA signals were detected at high levels in the thalamus, α2, α4 and β2 (Fig. 2). The highest level of signal was observed for α4 in anterodorsal, anteromedial, anteroventral, lateral dorsal, medial dorsal, reuniens, lateral posterior and pulvinar nuclei; moderate signalling was detected in ventral anterior, ventral lateral, ventral posterolateral, centrum medianum and parafascicular nuclei; while central lateral and paracentral nuclei exhibited only a weak signal. The signal for β2 mRNA was moderate through most parts of thalamus; it was similar to, but less intense than, that for α4 mRNA. A strong signal was observed in anterior parts, especially in the anteroventral nucleus; while lateral dorsal, medial dorsal and

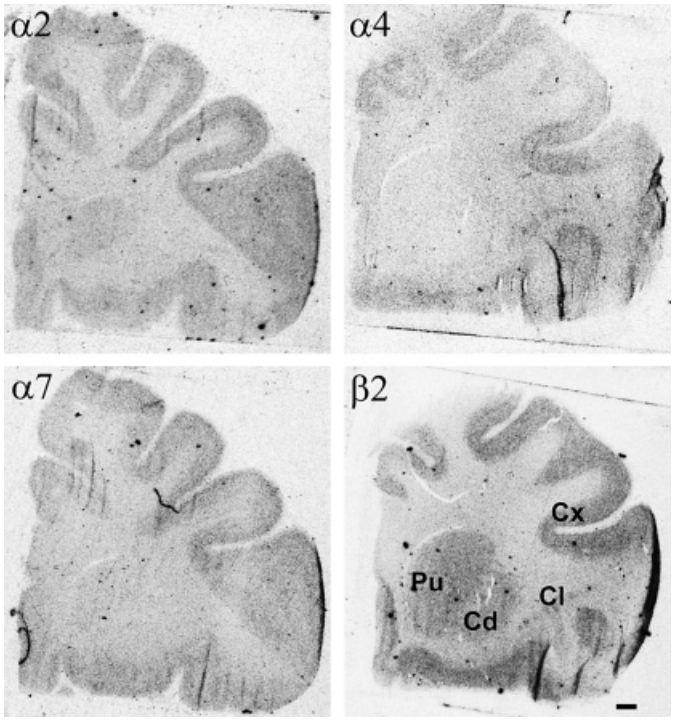

FIG. 1. Bright-field photographs of film autoradiograms showing the distribution of α2, α4, α7 and β2 nAChR subunit mRNAs in adjacent coronal sections at level A24 of Szabo & Cowan (1984). Abbreviations: Cd, caudate nucleus; Cl, claustrum; Cx, isocortex; Pu, putamen nucleus. Scale bar, 2 mm.

paracentral nuclei exhibited only weak levels of β2 mRNA signal. α2 mRNA signals exhibited an extensive distribution in thalamus, with a distribution pattern slightly different from those of α4 and β2 signals (see Figs 2 and 3). Strong signals for α2 were detected in the anterior part, moderate signals in ventral, lateral dorsal and medial dorsal parts, paracentral, reuniens, lateral posterior and pulvinar nuclei, whereas, differently from α4 (Fig. 3), the central lateral, centrum medianum and parafascicular nuclei were devoid of specific labelling.

Signals for α5 and α7 were restricted to a few nuclei throughout the thalamus. Both of them were present in the anterior part at a weak intensity, whereas a signal for α7 was also detected in ventral lateral and posterior nuclei.

The reticular thalamic nucleus exhibited signals for α2, α5, α7 and β2. While the β2 signal was moderate, signals for α5 and α7 probes were weak and for α2 the signal was very weak.

Very intense signals for α2, α4 and β2 were detected in the lateral geniculate body (Fig. 4). α2 and α4 probes also provided strong signal intensities in the medial geniculate nucleus, while the signal for β2 was moderate. A weak signal for α5 probe was found in both geniculate nuclei.

Epithalamus

Most of the nAChR subunit mRNAs were present in medial habenular nuclei (Fig. 5). Very strong signals for α3 and β4 were observed in the lateral part of medial habenula. The α3 signal was moderate in the medial part while the β4 signal was still high. β2 mRNA was present at moderate levels in both parts of medial habenula. Weak levels of α2 and α4 mRNA signals were found in medial habenula. Three subunits were detected only in the lateral part of the medial habenula, but with different intensities: α6 signal was strong, α7 signal was moderate and β3 signal was weak.

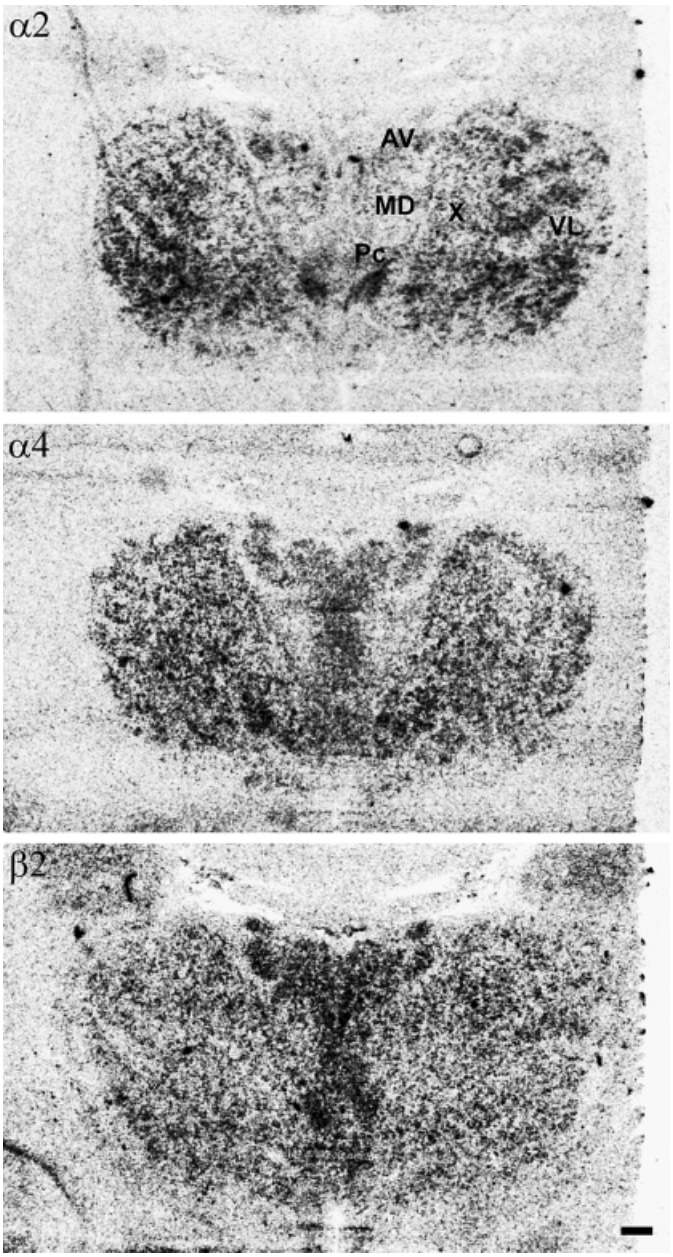

FIG. 2. Bright-field photographs of film autoradiograms showing the distribution of α2, α4 and β2 nAChR subunit mRNAs in adjacent coronal sections at level A12 of Szabo & Cowan (1984). Abbreviations: AV, antero-ventral nucleus, MD, medio-dorsal nucleus; Pc, Paracentral; VL, ventro-lateral nuclei; X, area X. Scale bar, 1 mm.

Strong staining for α3, β2 and β4 was found in the pineal gland. Signal for α7 was present at a weak level while α2 signal was very low. No specific signal for other subunits was detected in pineal gland.

Hypothalamus

We found α2, α4, α7 and β2 mRNA hybridization signals in the hypothalamus. Medial and lateral nuclei of the hypothalamus displayed weak signals for α2, α4 α7 mRNA and moderate signals for β2. Distinct signals for these subunits were detected in the magnocellular supraoptic nucleus: the α7 mRNA signal was strong whereas α4 and β2 mRNA signals were moderate and the α2 mRNA signal was very weak.

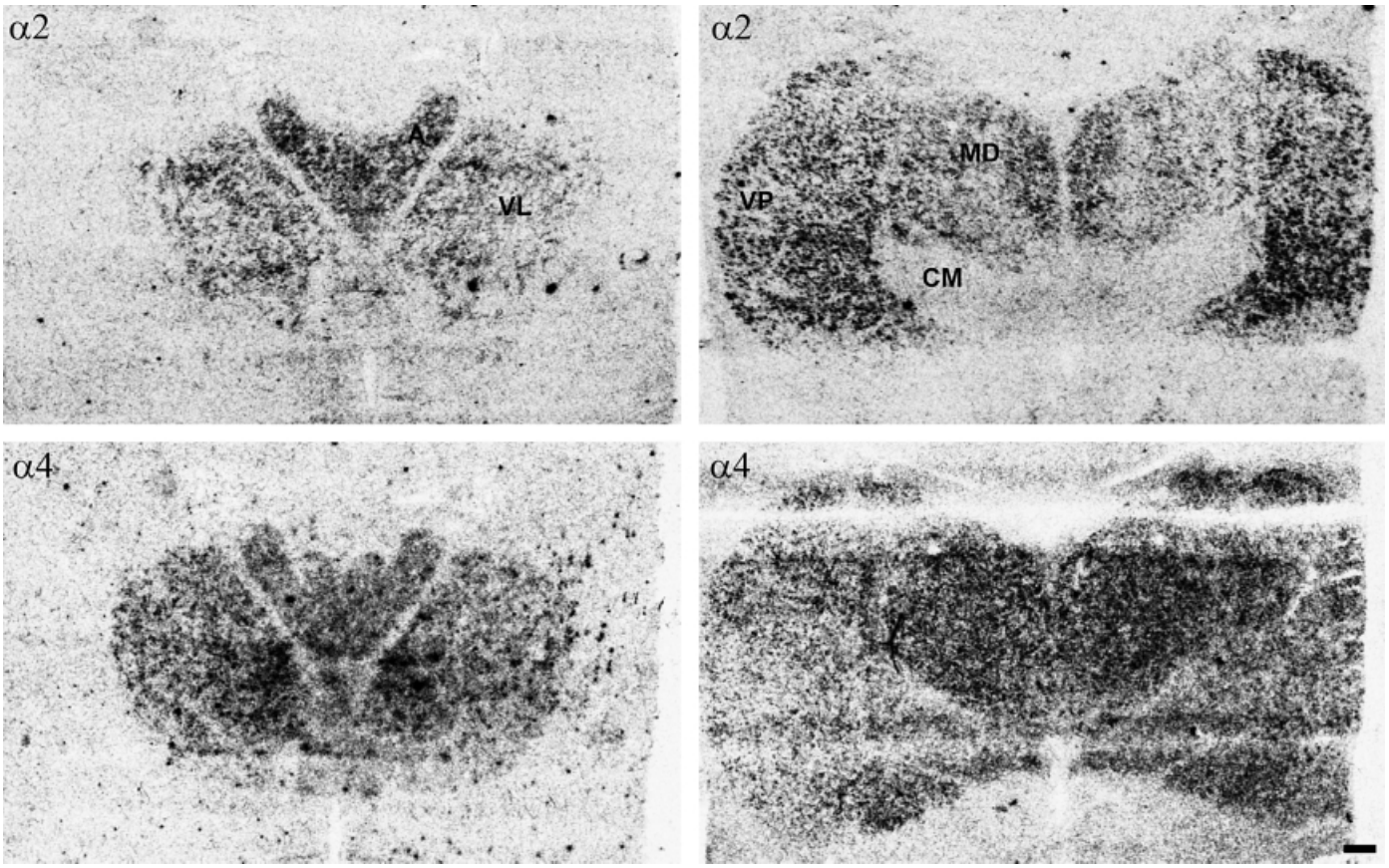

FIG. 3. Bright-field photographs of film autoradiograms showing the distribution of α2 and α4 nAChR subunit mRNAs in adjacent coronal sections at levels A14 and A8.5 of Szabo & Cowan (1984). Abbreviations: A, anterior nuclei; MD, medio-dorsal nuclei; VL, ventro-lateral nuclei; CM, centrum medianum nuclei. Scale bar, 1 mm.

Mesencephalon

The hybridization pattern in the mesencephalic dopaminergic cells was complex (Fig. 6). In order to identify the populations of dopaminergic neurons, we also used the mappings reported in other atlases (Benevento, 1975; Schofield & Everitt, 1981; Poirier *et al*., 1983; Azmitia & Gannon, 1986; Paxinos *et al*. 2000).

High levels of β2 mRNA signalling were detected in the substantia nigra pars compacta (SNc). Labelling of β2 in substantia nigra pars reticulata (SNr) was also strong whereas the signal intensity in the ventral tegmental area (VTA) was moderate. The pattern of α4 mRNA signalling was similar to that of β2 but with a bit lower signal intensity.

Signals for α6 and β3 mRNAs were strong in the SNc, while in the VTA they were moderate and weak, respectively. The intensity of α2 and α7 mRNA signals was weak in SNc. The VTA displayed weak and very weak signals for α7 and α2 mRNA, respectively. The signal for α5 mRNA was detected in the SNc and VTA at moderate levels.

Weak levels of labelling for α2 and β2 transcripts were present in the interpeduncular nucleus. Signals for α4 and β4 mRNA were also detected but at very low levels. Positive signals for α2 and β2 mRNAs were found in the red nucleus, at moderate and weak hybridization level, respectively. A moderate β2 signal was localized in the oculomotor III nucleus, whereas α4 probe displayed a weak labelling.

Discussion

We have mapped the distribution of neuronal nAChR subunit mRNAs in the forebrain and midbrain of the rhesus monkey by means of *in situ* hybridization histochemistry. In order to obtain specific oligodeoxynucleotide probes, we cloned the sequence between MIII and MIV of the nine neuronal nAChR subunits expressed in mammalian brain. The distribution of the mRNAs coding for the different nAChR subunits was subunit- and region-specific. In general, the pattern of nAChR subunit mRNA signal in the monkey brain is very similar to what has been previously observed in rodent brains (Deneris *et al*., 1989; Wada *et al*., 1989, 1990; Dineley-Miller & Patrick, 1992; Séguéla *et al*., 1993; Rust *et al*., 1994; Le Novère *et al*., 1996). A notable exception is the α2 subunit mRNA signal, which is widespread in monkey brain, but highly restricted in rodent (Wada *et al*., 1989) as well as avian (Daubas *et al*., 1990) brain.

α4 and β2 mRNAs are almost ubiquitous

The pattern of α4 and β2 mRNA distribution in the monkey CNS closely resembles the distribution showed in a number of previous anatomical studies in rodents (Wada *et al*., 1989; Hill *et al*., 1993; Rogers *et al*., 1998). Like in rodents, α4 distribution parallels β2 distribution throughout most brain regions, with the exception of a few regions (striatum, reticular thalamus, pineal gland) where only β2 labelling was detected. In these nuclei other subunits may assemble with β2 to form functional receptors (e.g. α2, α5 in the reticular thalamus and α2, α3 in the pineal gland). Like in rodents (Hill *et al*., 1993; Wada *et al*., 1989; Zoli *et al*., 1995), β2 mRNA is expressed in all grey matter regions of the monkey brain and may be presented in every neuronal cell. It is therefore possible that in some neurons β2 is the only nAChR subunit and does not form any functional nAChR. It must be considered, however, that low levels of nAChR subunit

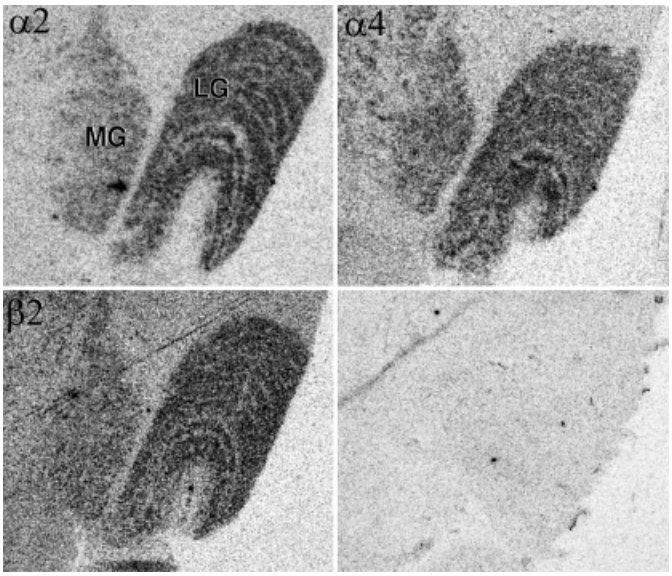

FIG. 4. Bright-field photographs of film autoradiograms showing the distribution of α2, α4 and β2 nAChR subunit mRNAs in adjacent coronal sections of level of the medial (MG) and lateral (LG) geniculate nuclei at level A7.6 of Szabo & Cowan (1984). The lower-right panel shows the displacement of the α2 mRNA signal by an excess of unlabelled oligonucleotide. Scale bar, 0.5 mm.

mRNAs only detected with highly sensitive techniques, such as single-cell PCR (Léna *et al*., 1999), may exist in these neuronal population. The very wide distribution of the β2 subunit in the brain may be related to the mechanisms of its regulation in neurons. There is, in fact, evidence that inhibition in non-neuronal cells by the activation of silencer elements is a main regulatory factor for β2 expression (Bessis *et al*., 1995). The remarkable conservation of β2 distribution between rodent and monkey brain is at odds with the reported observation of low levels of β2 in the human thalamus (Rubboli *et al*., 1994). Further studies of human brain tissue will be necessary to resolve this problem.

α2 mRNA has a distribution comparable with those of α4 and β2 mRNAs

The distribution of α2 mRNA in the monkey brain represents a remarkable exception to the general observation that nAChR subunit localization is conserved between rodent and monkey brains. In fact, α2 was found widely expressed through monkey CNS, like α4 and β2. High levels were detected in the thalamus, while positive signals were also found in pineal gland, red nucleus as well as SN, interpeduncular nucleus and hippocampus. Within these regions, α2 probes showed a specific distribution pattern when compared with α4 and β2. For example, α2 mRNA signal was undetectable in central lateral, centrum medianum and parafascicular nuclei, but high in the other thalamic nuclei. α2 mRNA distribution in the monkey markedly differs from that in the rodent (Wada *et al*., 1989; Le Novère, 1998) where α2 mRNA is detected at high levels only in the interpeduncular nucleus. Monkey distribution does not fit with that of the chick, where α2 mRNA is specifically expressed in the lateral spiriform nucleus (Daubas *et al*., 1990) and the habenula (Brussaard *et al*., 1994). On the basis of discrepancies between rat and chick α2 distributions, Le Novère & Changeux (1995) suggested that after the duplication between α4 and α2 the promoter of this latter subunit could have been unstable until the separation between the *reptilia* and the *synapsida* about 310 million years ago. The data reported here suggest that the promoter was still evolving at the time of divergence between rodent and primate lineages 110 million years ago. There is some indication that a widespread α2 mRNA distribution is not specific of the rhesus monkey but may be a common feature of primates, human included. Indeed, α2 clones were isolated from human thalamic cDNA library (Elliott *et al*., 1996).

α3, α5, α6, β3 and β4 mRNAs are highly concentrated in some brain nuclei

α3 and β4 mRNA signals were detected at high levels in the medial habenula and pineal gland. They were also present at low level in the hippocampus. In general, the regions with high expression of α3 and β4 mRNA appear conserved between rodents and monkey brain (Duvoisin *et al*., 1989; Dineley-Miller & Patrick, 1992; Zoli *et al*., 1995; Le Novère *et al*., 1996). However, especially in the case of α3 mRNA, it is not easy to reconcile the available data reported by different articles on the distribution in mammalian brain. The results of previous studies on primate brain were partially contradictory. For instance, α3 labelling in the dentate gyrus is 'very dense' in the monkey brain (Cimino *et al*., 1992) but 'very weak' in human brain (Rubboli *et al*., 1994), and in mediodorsal thalamic nucleus is 'strong signal' in human brain (Rubboli *et al*., 1994) but just 'consistently above background' in the monkey brain (Cimino *et al*., 1992). Our results fit rather well with those of Cimino *et al*. (1992), as far as hippocampus, habenula and pineal gland are concerned, i.e. regions of high expression in the present study, but not when the thalamus is considered, the main discrepancy being the lateral geniculate nucleus which is very densely labelled in Cimino *et al*. (1992) and totally devoid of labelling in our report. Important differences in α3 mRNA distribution can also be found in reports on rodent brain, the choice of the probe being in this case the main explanation of the discrepancies (see discussion in Le Novère *et al*. (1996)).

In our opinion, a strong point on α3 mRNA distribution that it is possible to make on the basis of present and previous articles is that, using the less sensitive but highly specific oligonucleotide probes, α3 mRNA distribution appears very similar in rodent and monkey brain (Picciotto *et al*., 1995; Le Novère, 1998). Weak labelling of some further regions with α3 riboprobes in rodents (Wada *et al*., 1989) and primates (Cimino *et al*., 1992; Rubboli *et al*., 1994; Terzano *et al*., 1998) may derive from the use of highly sensitive riboprobes, although it remains difficult to assess how much of this labelling is due to higher sensitivity or to lower specificity of the probes. Finally, especially in the case of human brain, where the labelling was confirmed by oligonucleotide probes (Rubboli *et al*., 1994), possible species-specific differences in α3 mRNA expression pattern should be taken into account.

α6 and β3 transcripts were restricted to medial habenula, SNc and VTA. The results match well with the data obtained in rodents (Deneris *et al*., 1989; Le Novère *et al*., 1996). These results suggest that also in primates, α6β3* nAChRs may be a principal isotype expressed in dopaminergic neurons of the mesencephalon (Le Novère *et al*., 1996), and strengthen the notion that these subunits are relevant for the study of nicotine reinforcement and possibly tobacco addiction. Instead, no specific labelling was detected in the monkey reticular thalamic nucleus, a region where both α6 and β3 were found in the rat (Deneris *et al*., 1989; Le Novère *et al*., 1996).

The labelling for α5 was of moderate intensity in SNc and VTA, weak in cortex and in some thalamic nuclei, including geniculate nuclei. This distribution is consistent with what has been reported in the rat (Wada *et al*., 1990), although we could not detect specific signals in the hippocampal formation.

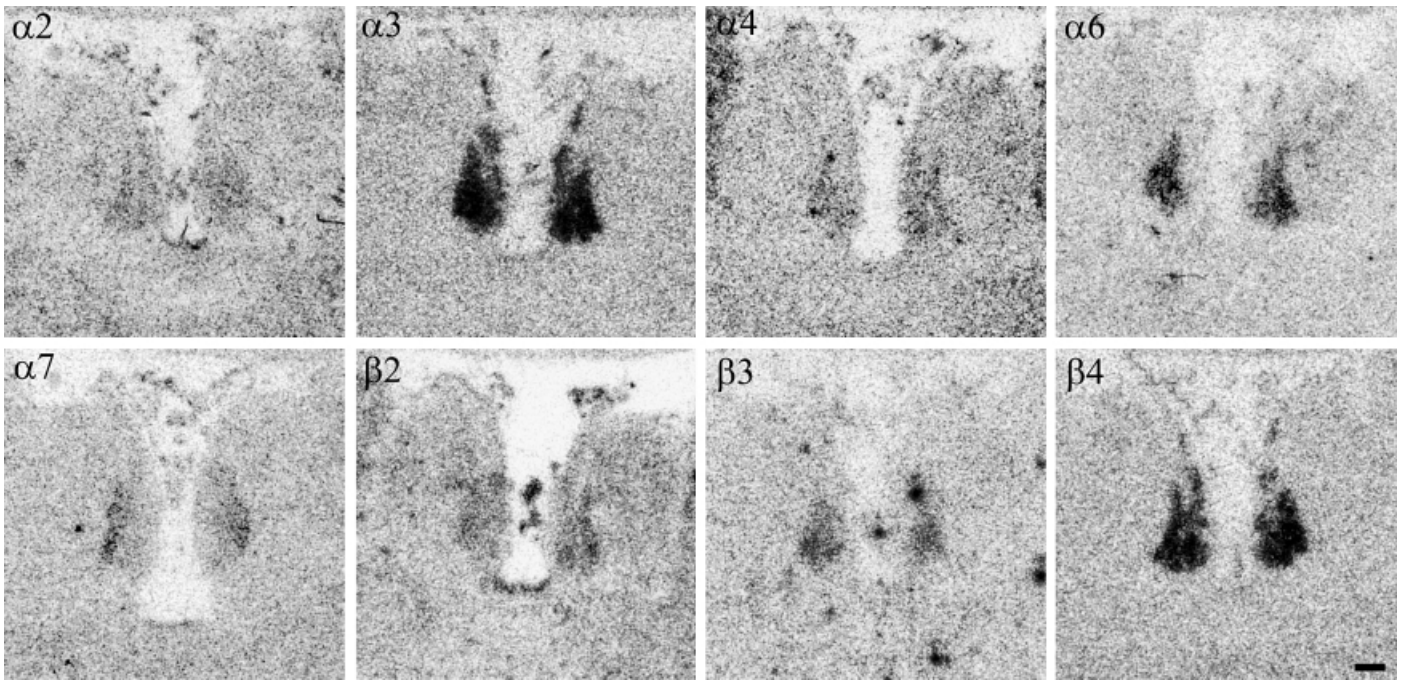

FIG. 5. Bright-field photographs of film autoradiograms showing the distribution of α2, α3, α4, α6, α7, β2, β3 and β4 nAChR subunit mRNAs in adjacent coronal sections of the habenular nuclei at level A7.6 of Szabo & Cowan (1984). Scale bar, 0.5 mm.

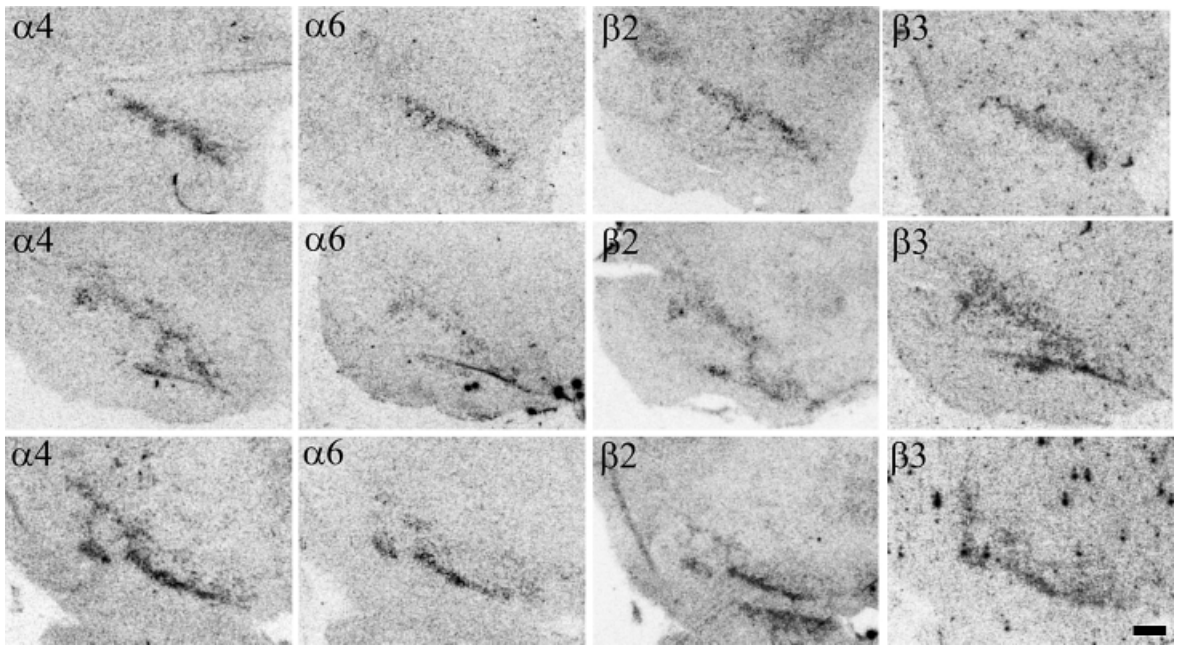

FIG. 6. Bright-field photographs of film autoradiograms showing the distribution of α4, α6, β2 and β3 nAChR subunit mRNAs in adjacent coronal sections of the substantia nigra at levels A10.8 (upper panels), A9.6 (middle panels) or A8.6 (lower panels) of Szabo & Cowan (1984). Scale bar, 1 mm.

α7 mRNA distribution is relatively wider than in rodents

The extent of α7 expression in the monkey CNS was greater than in rodents. The presence of specific signals in cortex, hippocampus, habenula and hypothalamus is consistent with the rodent distribution (Séguéla *et al*., 1993; Bina *et al*., 1995), while the positive signal in some thalamic nuclei has not been previously observed in rodents, but already detected in the human brain (Rubboli *et al*., 1994; Breese *et al*., 1997). In addition, weak but distinct labelling for α7 was found in the pineal gland (Stankov *et al*., 1993), SNc and VTA indicating that nAChRs containing α7, together with those containing α3 and β4, may be responsible for the cholinergic modulation of the pineal gland in primates. Although not clearly detected with *in situ* hybridization technique, α7 mRNA may be expressed in rodent

SNc and VTA, since putative α7* nAChRs were detected in mesencephalic dopaminergic neurons using electrophysiological techniques (Pidoplichko *et al*., 1997).

Possible distribution of defined nAChR oligomers in the monkey brain

On the basis of the distribution of nAChR subunits mRNAs in the monkey and their substantial conservation with respect to rodents, some suggestions about the distribution of nAChR oligomers in the monkey brain can be proposed. As far as heteropentameric nAChRs are concerned, an oligomer containing α4 and β2 subunits may constitute a principal isoform in monkey brain, as in rodents (Picciotto *et al*., 1995; Zoli *et al*., 1998; Marubio *et al*., 1999). The wide distribution of α2 mRNA suggests, however, that a second oligomer formed by α2β2 (and maybe mixed oligomers containing both α4 and α2) represents another principal nAChR isoform in the monkey, and perhaps human brain. These two receptor subtypes have different pharmacological and functional properties (Chavez-Noriega *et al*., 1997) and may play different roles in central neuronal circuits.

As proposed by Le Novère & Changeux (1995), and contrary to what was thought after cloning, α5 may not be an 'authentic' α subunit and may not form ACh-binding sites in combination with β subunits. Instead, α5 can form functional nAChRs when coexpressed with α3β2, α3β4 or α4β2 combinations (Ramirez-Latorre *et al*., 1996; Wang *et al*., 1996; Fucile *et al*., 1997). α5 may therefore participate to the formation of functional nAChRs with a number of other subunit combinations in the regions where it is expressed. Thus, α4β2 and/or α2β2 (with or without α5) containing nAChR oligomers may represent the major nAChR subtypes and the largest proportion of high-affinity binding for [^{3}H]-nicotine in the monkey brain (Zoli *et al*., 1998).

Again, as found in rodents, a few brain areas may contain, besides α2/4β2* nAChRs, a wide variety of other nAChRs. The brain areas particularly rich in nAChR subtypes are the medial habenula and the SNc/VTA complex. The most parsimonious interpretation of the morphological data is to suppose that the major nAChR subtypes in the medial habenula are α3β4, α4/2β2 and α6β3β2/4 oligomers, and in the SNc/VTA complex α4/2α5β2 and α6β3β2 oligomers (Le Novère *et al*., 1996). Other combinations are, however, possible and additional work is needed to elucidate this issue.

Neuronal homo-oligomeric nAChRs can be formed by α7 or α8 subunits (Séguéla *et al*., 1993; Gerzanich *et al*., 1994; Chen & Patrick, 1997; Drisdel & Green 2000). However, α8 is present in the chick but has never been detected in mammals (Schoepfer *et al*., 1990). α7 distribution in the monkey appears wider than that in rodents and may be similar to that of humans (Rubboli *et al*., 1994; Breese *et al*., 1997). These data suggest that neuronal homo-oligomeric nAChRs may contribute to a wide number of physiological functions in the primate brain. Interestingly, alterations in these nAChRs may have a pathophysiological relevance in some human diseases, such as schizophrenia (Leonard *et al*., 1996; Elmslie *et al*., 1997; Freedman *et al*., 1997).

Distribution of nAChR subunits and possible oligomers in the mesolimbic dopamine system

The neuronal systems and molecular mechanisms which mediate nicotine addiction are beginning to be understood. Among them, the meso-telencephalic dopaminergic systems appear to be main mediators of the reinforcing properties of nicotine (Imperato *et al*., 1986; Dani & Heinemann, 1996; Merlo-Pich *et al*., 1997; Picciotto *et al*., 1998).

As already mentioned, these neurons are very rich in nAChR subunits and may therefore express a wide number of different oligomers. In fact, with the exception of α3 and β4 subunits, whose levels were undetectable in this study, all the other nAChR subunits are expressed, often at high to very high levels (α4, α6, β2 and β3). This pattern of expression is substantially similar to that observed in rodents (Le Novère *et al*., 1996) (with the exception of the presence of weak levels of α2 mRNA in the monkey) and prompts an analogy between rodent and primate mechanisms. Based on what has been shown in rodents (Deneris *et al*., 1989; Wada *et al*., 1989; Hill *et al*., 1993; Le Novère *et al*., 1996; Picciotto *et al*., 1998), it can be assumed that β2* nAChRs are necessary for the sensitivity of the dopaminergic neurons to nicotine and for nicotine reinforcement in general. An isoform containing β2 and α4 and/or α2 may be the main heteropentameric nAChR expressed on dopaminergic cell bodies (Picciotto *et al*., 1998; Sorenson *et al*., 1998; Arroyo-Jiménez *et al*., 1999), while β2 coassembled with α6 and β3 may constitute the isoform mainly expressed by dopaminergic terminals (Le Novère *et al*., 1996; Booker *et al*., 1999).

In addition, both electrophysiological (Pidoplichko *et al*., 1997) and neurochemical (Schilstrom *et al*., 1998) evidence points to the relevance of α7* nAChRs for the regulation of mesencephalic dopaminergic neurons. We found weak labelling for α7 mRNA in the SNc/VTA, and high and low levels, respectively, in hippocampal and cortical areas projecting to the dopaminergic neurons. The morphological evidence is therefore consistent with the hypothesis that, besides some heteropentameric nAChR isoforms (see above), α7* nAChRs have a role in mediating nicotine reinforcing properties in the monkey.

Conclusions

The present paper reports an extensive mapping of all known neuronal nAChR subunit mRNAs expressed in the brain of *Macaca mulatta*. Overall, the distribution of nAChR subunits appears similar to that reported in rodent brains (Deneris *et al*., 1989; Wada *et al*., 1989, 1990; Dineley-Miller & Patrick, 1992; Séguéla *et al*., 1993; Le Novère *et al*., 1996), with the notable exception of α2 mRNA, which is diffusely expressed in the monkey brain but restricted to the interpeduncular nucleus in both rat and mouse brains. The complex distribution pattern of nAChR subunit mRNAs in the primate CNS illustrates the difficulties to overcome in any attempt to design pharmacological agents targeted to specific nAChR isoforms to be used in the therapy of neuropsychiatric disorders, such as schizophrenia, Alzheimer's and Parkinson's disease, in which nAChRs are thought to be involved (Perry *et al*., 1995; Gotti *et al*., 1997; Lindstrom, 1997).

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Abbreviations

CNS, central nervous system; LGIC, ligand-gated ion channel nAChR nicotinic acetylcholine receptor; SN, substantia nigra; SNc, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; SSC, saline sodium citrate buffer; VTA, ventral tegmental area.

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