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Neuronal Nicotinic Receptor α6 Subunit mRNA is Selectively Concentrated in Catecholaminergic Nuclei of the Rat Brain

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Abstract

Although the neuronal nicotinic receptor α6 subunit was cloned several years ago, its functional significance remains to be investigated. Here we describe an *in situ* hybridization study of the mRNA for this subunit in the adult rat central nervous system using oligonucleotide probes. Specific α6 mRNA labelling was restricted to a few nuclei throughout the brain; it was particularly high in several catecholaminergic nuclei [the locus coeruleus (A6), the ventral tegmental area (A10) and the substantia nigra (A9)] at levels significantly higher than those found for any other known nicotinic receptor subunit mRNA. Labelling for α6 mRNA was also detected at lower levels in the reticular thalamic nucleus, the supramammillary nucleus and the mesencephalic V nucleus. Some cells of the medial habenula (medioventral part) and of the interpeduncular nucleus (central and lateral parts) were also labelled. The distribution of α6 mRNA was compared with the distribution of the other known nicotinic acetylcholine receptor subunit mRNAs. In several nuclei, the expression of α6 was complementary to those of other α subunits. Moreover, some of the cell groups (such as the substantia nigra, the ventral tegmental area and the locus coeruleus) previously thought to contain mainly α3 mRNA in fact were found to contain high levels of α6 mRNA. Finally, we found extensive colocalization of α6 and β3, indicating the possible existence of nicotinic receptor hetero-oligomers containing both subunits. The present results show that α6 is the major nicotinic acetylcholine receptor α subunit expressed in dopaminergic cell groups of the mesencephalon and noradrenergic cells of the locus coeruleus. This suggests the involvement of the α6 subunit in some of the major functions of central nicotinic circuits, including the modulation of locomotor behaviour and reward.

Introduction

The nicotinic acetylcholine receptor (nAChR) belongs to the superfamily of ligand-gated ion channels (LGIC) (Cockcroft *et al.*, 1992; Galzi and Changeux, 1994) that are largely responsible for fast responses to neurotransmitters. The nAChR is an allosteric protein composed of five subunits. These subunits are named α if they carry a cysteine pair that belongs to the main component of the acetylcholine binding site, and non-α if they lack this pair (Changeux, 1991). Molecular cloning methods have revealed the existence of sets of homologous genes encoding nAChR subunits in the amniote nervous system, which have been named α2–9 or β2–4 (Sargent, 1993; Elgoyhen *et al.*, 1994; Le Novère and Changeux, 1995). In neurons, two subfamilies of subunits have been identified. Subunits α7–α9 form α-bungarotoxin-sensitive receptors, whereas nAChRs containing α2–α5 and β2–β4 are not sensitive to this toxin (Boulter *et al.*, 1987; Sargent, 1993; Gerzanich *et al.*, 1994). Sequence comparison indicates that α6 belongs to the subfamily of nAChRs insensitive to α-bungarotoxin (Le Novère and Changeux, 1995). Yet to date no data exist on the pharmacology of possible α6-containing nAChRs.

Heterologous expression studies in *Xenopus* oocytes and in various cell types, as well as patch–clamp studies in brain and ganglionic preparations, suggest that multiple subunit combinations assemble to form functional nAChRs with distinct pharmacological specificities and ionic channel properties (Wada *et al.*, 1988; Listerud *et al.*, 1991; Luetje and Patrick, 1991; Mulle *et al.*, 1991; Whiting *et al.*, 1991; Ramirez-Latorre *et al.*, 1996). Accordingly, several different combinations of nAChR subunit mRNAs and/or proteins have been identified in central and peripheral nervous structures (Sargent, 1993; Conroy *et al.*, 1992; Vernallis *et al.*, 1993). These nAChRs are likely to be involved in diverse physiological regulatory processes. For instance, nicotine is known to increase the release of dopamine in the striatum (Westfall, 1974), of noradrenaline in the hippocampus and hypothalamus (Hall and Turner, 1972; Arqueros *et al.*, 1978) and of γ-aminobutyric acid in the interpeduncular nucleus (Léna *et al.*, 1993). Moreover, the inactivation of the gene coding for the nAChR β2 subunit provides mutant mice with several characteristic alterations at the molecular, cellular and behavioural levels (Picciotto *et al.*, 1995).

Numerous *in situ* hybridization studies have been performed in order to detect the neuronal nAChR subunits present in the nervous

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TABLE 1. Oligodeoxynucleotide probes used in this study

Target	Probe code	Accession number	Probe position	Sequence of the oligodeoxynucleotide	% GC	T_m (°C)
α2	59	L10077	1425	5′-CTCCAGCATCCATGTTAGTCTCTAGCCAATGGTATGAGGGGCTGA-3′	51	75.6
α3	31	L31621	1040	5′-CCCAAGTGGGCATGGTGTGTGTGGTTGGAGTTCTATAGTGCAC-3′	53	76.2
α3	46	L31621	138	5′-GCGCCGTAGAAGGTCCTCGTCTTAGGAGTGTCCCCCTCACCACTG-3′	62	83.5
α3	109	L31621	215	5′-GGACACCTCAAACTGGATGATGACTGGATGGGACACATTAGCCAC-3′	51	75.6
α3	110	L31621	623	5′-CTCCCAGTAGTCCTTGCGGTTCATGGAGGAGCCGATGAGGACCAG-3′	58	80.6
α4	47	M15681	1389	5′-GCTGCTTCTTGGGAGCTGGGCACATGCTGGACACTCAGGGACCTG-3′	62	83.5
α4	49	M15681	1271	5′-CCTTCTCAACCTCTGATGTCTTCAAGTCAGGGACCTCAAGGGGGG-3′	55	78.5
α5	58	J05231	1076	5′-CCGAGATTTAGGTCCAGCCCCACTCTCGGCTTCTTCTCTCTGAGT-3′	56	79.2
α6	132	L08227	325	5′-TCAAAGTGCACCGTGACGGGATCAGAAACGTTTTCCACTGGCCGG-3′	56	79.2
α6	133	L08227	1575	5′-GCCCCACAGTTCCAAACACACAGACGATTATAAACACCCAGAGGA-3′	49	74.2
β2	36	L31622	1341	5′-AGCCAAGCCCTGCACTGATGCAGGGTTGACAAAGCAGGTACATGG-3′	55	78.5
β2	37	L31622	1455	5′-TCGCATGTGGTCCGCAATGAAGCGTACGCCATCCACTGCTTCCCG-3′	60	82
β2	51	L31622	1315	5′-TGACAAAGCAGGTACATGGGTCAGCCGCAGGACCTTCACCGAAGA-3′	55	78.5
β3	130	J04636	1306	5′-CAGAACTCTTTCTCCATCGCTGGCGGGAGTCTGTTTCCTTTTGCC-3′	53	77
β3	131	J04636	431	5′-ATTCTTCCGGATTCCAGCGTAATTTTTGGTCTCTGCATTCCTGCT-3′	42	69.4
β4	61	J05232	1020	5′-ACCAGGCTGACTTCAAGACCGGGACGCTTCATGAAGAGGAAGGTG-3′	55	78.5
β4	62	J05232	1259	5′-AGCTGACACCCTCTAATGCTTCCTGTAGATCTTCCCGGAACCTCC-3′	53	78.5
Tyrosine hydroxylase	73			5′-AGGGTGTGCAGCTCATCCTGGACCCCCTCCAAGGAGCGCT-3′	65	83.4

The melting temperature is calculated for the most stringent wash condition (0.01 M NaCl, 0% formamide) with equation 3 of Wahl *et al.* (1987). The sequences can be viewed and downloaded from the World Wide Web address http://www.pasteur.fr/units/neubiomol/LGIC.html or from GenBank (see accession numbers in the table).

system of the adult (Goldman *et al.*, 1986; Deneris *et al.*, 1989; Wada *et al.*, 1989, 1990; Dineley-Miller and Patrick, 1992; Séguéla *et al.*, 1993; Rust *et al.*, 1994; Cimino *et al.*, 1995) and embryonic (Zoli *et al.*, 1995) rat. Yet, the distribution of α6 mRNA has not been described in detail. Here we present a detailed study of the distribution of α6 mRNA in the adult rat central nervous system (CNS). The pattern of labelling, like the codistribution of α6 with the other subunits of the nAChR, leads us to suggest that α6-containing nAChRs contribute to several of the known pharmacological effects of nicotine.

Materials and methods

The *in situ* hybridization method used is described and discussed in great detail in Wisden and Morris (1994).

Oligodeoxynucleotide synthesis and labelling

Following analysis for mRNA secondary structure using GCG Sequence Analysis Software 7.1 (Genetic Computer Group, Madison, Wisconsin), probe sequences were chosen in unique regions of the mRNAs, without putative secondary structure, containing 50–60% GC. Oligodeoxynucleotides were synthesized in a Cyclone DNA synthesiser (Biosearch) or obtained from Genset (Paris, France). The probes are described in the Table 1. Each of the nAChR subunit sequences used can be retrieved at the World Wide Web address http://www.pasteur.fr/units/neubiomol/LGIC.html, or from GenBank (accession numbers are specified in Table 1).

The oligodeoxynucleotide probes were labelled at the 3′ end using [α^{33}P]dATP (NEN, Boston, MA) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, FRG) following the specifications of the manufacturer to a specific activity of 200–600 kBq/pmol. The labelled probes were precipitated in ethanol, separated from unincorporated [^{33}P]dATP by means of NucTrap push columns (Stratagene, La Jolla, CA), precipitated again in ethanol and resuspended in distilled water.

Animals

Six adult Sprague–Dawley rats (Iffacredo, Lyon, France), weighing 300–400 g, were used. The animals were killed by chloral hydrate injection (1 ml of 35% solution); the brain was rapidly dissected out and frozen in dry ice powder. The tissues were stored at –80°C until cut.

In situ hybridization procedure

Frozen tissues were cut with a cryostat (14 μm thick sections), thaw-mounted on poly-L-lysine-coated 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 in the following protocol. Briefly, sections were fixed with 4% paraformaldehyde for 5 min at room temperature, washed in PBS, acetylated, and stored in 80% ethanol at 4°C. Sections were then delipidated in ethanol and chloroform (5 min), prehybridized for 2–4 h at 37°C and hybridized for 20 h at 37°C under parafilm coverslips. The composition of the prehybridization and hybridization mixtures was: 50% formamide, 0.6 M NaCl, 10 mM dithiothreitol, 10% dextran sulphate, 1 mM EDTA, 1 × Denhardt's solution (50× = 1% bovine serum albumin, 1% Ficoll, 1% polyvinyl pyrrolidone), 0.1 mg/ml poly(A) (Boehringer), 0.5 mg/ml yeast tRNA (Sigma), 0.05 mg/ml herring sperm DNA (Promega, Madison, WI) in 0.02 M Tris–HCl, pH 7.5. Probes were added in the hybridization mixture at a concentration of 0.55 nM (corresponding to ~15 fmol/section or 3000–25 000 Bq/30 μl/section according to the labelling).

After removal of coverslips rinsing in 2 × standard saline citrate (SSC) solution (0.3 M NaCl, 0.03M sodium citrate) at room temperature (twice for 5 min), sections were washed three times for 15 min in 1 × SSC at room temperature, then for 15 min in 0.5 × SSC at ~55°C. The probes used in this study had the same length and approximately the same GC content. The melting temperatures of the different oligodeoxynucleotide/mRNA duplexes, calculated using equation 3 of Wahl *et al.* (1987), were similar, the same hybridization conditions being used (0.6 M NaCl, 50% formamide). The temperature of the more stringent wash was chosen to be T_m –20°C (in 0.01 M NaCl, 0% formamide; Table 1), in order to correct for the small

differences in GC content between the different probes. We have previously shown that this wash temperature gives an optimal signal-to-noise ratio, with nearly maximal specific signal and a low non-specific signal.

The sections were then rinsed for 15 min in 0.5 × SSC at room temperature. After rinsing in ice-cold water and drying through an alcohol gradient, they were exposed to [^{3}H]Hyperfilm (Amersham, Buckinghamshire, UK) and then to a photographic emulsion (NTB2, Kodak, Rochester, NY).

In order to quantitatively compare different experiments, we defined a correction factor. This factor adjusts for differences in radioactive labelling of the probes (i.e. their specific activity) and exposure time of the slides to the autoradiographic film. For instance, in a given experiment probe A may give twice the autoradiographic signal of probe B (i.e. a specific optical density two-fold higher); however, if S_A is equal to $4S_B$, the actual amount of probe B binding is twice the actual amount of probe A binding.

The total exposure factor S was calculated as follows (derived from the integration of the decay equation for ^{33}P):

$$S = 36.6 \times A_0 \times (e^{-0.00273t1} - e^{-0.00273t2})$$

where A_0 is the specific activity of the probe just after the radioactive labelling ($t0$) in KBq/pmol, $t1$ the delay between the time of probe specific activity determination and the beginning of exposure to the film, and $t2$ the delay between the time of probe specific activity determination and the film development, in days.

Specificity controls of the oligonucleotides were performed as reported in Zoli *et al.* (1995).

Analysis of histological preparations

Two or more oligonucleotides (Table 1) were used to characterize the specificity of labelling. Afterwards, one oligonucleotide/mRNA was used in the mapping (Table 4). Each anatomical level was analysed in at least three (and up to five) independent experiments.

The analysis of the labelling pattern was carried out on both film and emulsion autoradiograms. Identification of anatomical structures was carried out after counterstaining the sections with toluidine blue. Definition of anatomical areas in the brain was based on different atlases, including those of Paxinos and Watson (1986) and Swanson (1992).

Relative quantification of the mRNA levels of the different subunits in the mesencephalic dopaminergic nuclei was carried out by means of the Vidas image analyser (Kontron, Munich, FRG) on the autoradiograms. Radioactivity microscales were also exposed to the films in order to ascertain that the grey levels of the specific labelling were in the linear portion of the image analyser response curve. Two different experiments were considered. Six levels were analysed in one rat brain (bregma –4.7 to –6.3 mm) and four in another (bregma –4.8 to –6.1 mm). After a thresholding procedure which allowed the selective retention of the specific signal, the mean grey value (*MGV*) and the retained area were measured. The total integrated optical density (*TOT*) was then calculated from the integrated optical density of each level as follows:

$$TOT = \Sigma \text{ integrated optical density}$$
$$= \Sigma \text{ retained area} \times \log (MGV_{background}/MGV_{total})$$

After correction (*COR*) for S (for the subunit i, $COR_i = TOT_i/S_i$), the results were expressed as ratio of the β2 subunit values ($COR_i/COR_{\beta 2}$) in each experiment. The value for β2 was arbitrarily chosen as a reference, since it is the most diffuse and relatively homogeneous subunit mRNA in the adult rat brain.

Determination of the K_d and B_{max} values for the α3 probes was performed by hybridizing adjacent brain sections with ten probe concentrations. We plotted optical density against probe concentration (the non-specific binding was determined by adding an excess of cold probe). Fitting a Langmuir isotherm provided the quantitative parameters.

Cell counting of emulsion autoradiograms was performed at bregma –5.3 mm. Recognition of neurons was based on counterstaining of the sections with toluidine blue. Two square fields (380 × 250 μm) were analysed in each region. The sampled fields were placed in the pars compacta of the substantia nigra, or in the centre of the ventral tegmental area (VTA). Neurons were considered positive when they were overlain by at least three times the background density of grains.

Results

Optimization of in situ hybridization procedure with oligodeoxynucleotides

In situ hybridization with oligodeoxynucleotides was selected in order to minimize cross-recognition of different members of the nAChR subunit family and to allow quantitative comparisons between several experiments. This method is less sensitive than cRNA probe-based *in situ* hybridization, as the oligodeoxynucleotide is labelled only at the 3′ end, whereas cRNA probes are labelled over the entire sequence. On the other hand, oligodeoxynucleotides may penetrate more easily in fixed tissue. Apart from the sensitivity issue, oligodeoxynucleotide-based *in situ* hybridization has several advantages over cRNA probe-based *in situ* hybridization, especially when families of genes are investigated.

First, the oligodeoxynucleotides can be chosen within highly specific regions, which is not always true for cRNA probes. For instance, probes used in previous nAChR studies were often directed against the entire coding sequence, and then against some highly conserved parts. In order to generate more efficient probes, authors often cut them to 50–200 nucleotide fragments, a method which probably generates non-specific signal as well as cross-recognition between close members of the same gene family.

Second, the *in situ* hybridization with oligodeoxynucleotides is highly reproducible in every tissue and with every probe. Indeed, the control of the relevant parameters (i.e. probe concentration, specific activity and melting temperature) allows the determination of quantitative characteristics of the binding such as apparent K_d and B_{max} values. For the α3 subunit, a saturation study of the *in situ* binding characteristics of the oligodeoxynucleotides was performed in the medial habenula (N. Le Novère, M. Zoli and J.-P. Changeux, unpublished). Under our experimental conditions (in particular a more stringent wash, T_m –20°C in 0.5 × SSC for 15 min), probe 46 gave a K_d of 0.075 nM with a B_{max} of 0.13 fmol/cm^2 and probe 109 gave a K_d of 0.17 nM with a B_{max} of 0.19 fmol/cm^2. In general, we verified that the difference in labelling provided by the different probes against the same mRNA used in this study was <50%. This figure must be kept in mind when evaluating the relative quantitative differences between labellings shown in Table 4.

Distribution of α6 mRNA labelling in the rat brain

The α6 mRNA signal was detected in a restricted number of brain areas (Table 2). The range of labelling intensity varied from barely detectable to very high. The telencephalon showed no specific labelling. In the other main CNS subdivisions, some nuclei displayed detectable levels of α6 mRNA.

Diencephalon

In the diencephalon, three nuclei were labelled by α6 probes, but with different intensities.

The reticular thalamic nucleus exhibited a rostrocaudal and dorsoventral gradient of α6 labelling (Fig. 1). Labelling intensity was modest even in the rostrodorsal part. Subunits β2 and α4 were also found in the reticular nucleus, although at a rather low level. The β3 subunit was barely detectable in only one of five adult animals (data not shown).

Labelling for α6 was restricted to a small part of the medial habenula (Fig. 2). In the medial habenula, α6 labelling was detected in some medially located cells lining the ventricle. This distribution corresponded to that of β3. However, the labelling intensity for β3 was stronger. The distribution of α6 (or β3) mRNA seemed to be complementary to that of α4 mRNA. In contrast, α3 and β4 labelling was detected at a high level all over the ventral part of the nucleus, whereas α5 labelling was restricted to the ventrolateral part of the nucleus and β2 labelling was present at a moderate level in all parts of the nucleus.

In the lateral habenular nucleus, we detected weak labelling for α6 mRNA, as well as for β3 and β2.

The caudal part of the supramammillary nucleus was lightly labelled by α6 probes. Probes for α5 and β2 revealed weak labelling. Labelling for α4 was absent or very light.

Mesencephalon

The mesencephalon showed three areas containing specific α6 mRNA signal.

Mesencephalic dopaminergic cell groups showed a wide variety of nAChR subunit mRNAs (Fig. 6). Labelling for α6 was very high in the substantia nigra (A9), pars compacta (SNc), VTA (A10) and retrorubral area (A8) (Fig. 3). Scattered cells showing high α6 labelling intensity were also present in the mesencephalic periventricular zone and in the substantia nigra pars reticulata (SNr), possibly corresponding to the periventricular mesencephalic dopamine system and to the dopamine neurons of the SNr. Neurons of the SNc and VTA were rather homogeneously labelled in the different parts of these nuclei. The β3 subunit was detected at a somewhat lower, but still very high, level (Fig. 4). In order to determine if α6 and β3 subunit mRNAs are localized in dopamine cells, we counted the neurons labelled by probes directed against the two nAChR subunits or against tyrosine hydroxylase, the dopamine biosynthetic enzyme, after counterstaining of the *in situ* hybridization sections with toluidine blue (Fig. 5). Approximately the same percentage of neurons (80%) in the SN and in the VTA contained specific α6, β3 or tyrosine hydroxylase labelling (Table 3). This finding demonstrates that at least 50% of tyrosine hydroxylase-positive (i.e. dopaminergic) neurons also contain α6 and β3 mRNA. However, the extensive overlap at the regional level between cells containing α6, β3 and tyrosine hydroxylase mRNA suggests that these three mRNAs are present in the same neuronal population. Signals for α4, α5 and β2 subunit mRNAs were also found in dopaminergic nuclei but at moderate levels (Fig. 6). The specific signal for α3 mRNA was very weak. A relative quantification of the transcripts was performed on the autoradiograms according to the method described in Materials and methods. The results, shown in Table 4, defined four classes of subunits. Subunits α2, α7 and β4 were not detected. Labelling for α3 was weak, three times lower than that for β2. Intensities of labelling for α4, α5 and β2 were similar. Lastly, α6 and β3 labelling intensities were several times higher (seven and four times more, respectively) than that for β2. Moreover, α6 labelling intensity was probably underestimated, since the autoradiographic films were partially saturated. No relevant differences were found between the A9 and A8/A10 nuclei for the ratio of labelling between the different subunits.

Some parts of the interpeduncular nucleus were labelled by α6 probes (Fig. 3). Positive cells were located in the most caudal part of the central and lateral subnuclei. This distribution did not match those of α2, α3, α4, α5 or β4 but overlapped that of β2. The β3 subunit had the same distribution as α6, but with a very low labelling intensity (detected in only one of five adult animals; data not shown).

The neurons of the trigeminal mesencephalic nucleus were found to be labelled by α6 probes (Fig. 7). This labelling was less intense than that of β3 but stronger than that of α4 and β2. The cells labelled by α6 probes belonged only to a caudal subpopulation, just rostral to the locus coeruleus, whereas every cell of the nucleus, even around the mesencephalic central grey matter, was found to be positive for β3 probes.

Rhombencephalon

In the rhombencephalon, the locus coeruleus was the only nucleus labelled by α6 probes (Fig. 7). The intensity of labelling for α6 was extremely high and at the same level as that of dopaminergic cells of the mesencephalon. Several other nAChR subunit labellings were found in this nucleus, β2 and β3 at moderate level, α3 at a weak level, and α5 and α4 at very low levels.

TABLE 2. Distribution and relative intensity of the labelling for α6 and β3 mRNAs

Structure	α6	β3
Diencephalon		
reticular thalamic nucleus	++	+
medial habenula		
dorsal part	–	–
ventrolateral part	–	–
ventromedial part	+	+++
lateral habenula	(+)	+
supramammillary	(+)	–
Mesencephalon		
substantia nigra		
pars compacta	+++	++
pars lateralis	+++	++
pars reticulata (scattered cells)	+++	++
ventral tegmental area	+++	++
interpeduncular nucleus		
apical part	–	–
central part	+	(+)
lateral part	+	(+)
rostral part	–	–
mesencephalic V nucleus	++	+++
Rhombencephalon		
locus coeruleus	+++	++

(+), barely detectable signal; +, weak signal; ++, moderate signal; +++, strong signal.

Discussion

Since the first report on the α6 subunit (Lamar *et al.*, 1990), our knowledge of the functional significance of this subunit has not progressed significantly. Here we show that α6 mRNA is restricted to a few specific nuclei throughout the brain. The amount of α6 mRNA is particularly high in some catecholaminergic nuclei (the A6, A8, A9 and A10 nuclei) and significant, though lower, in the

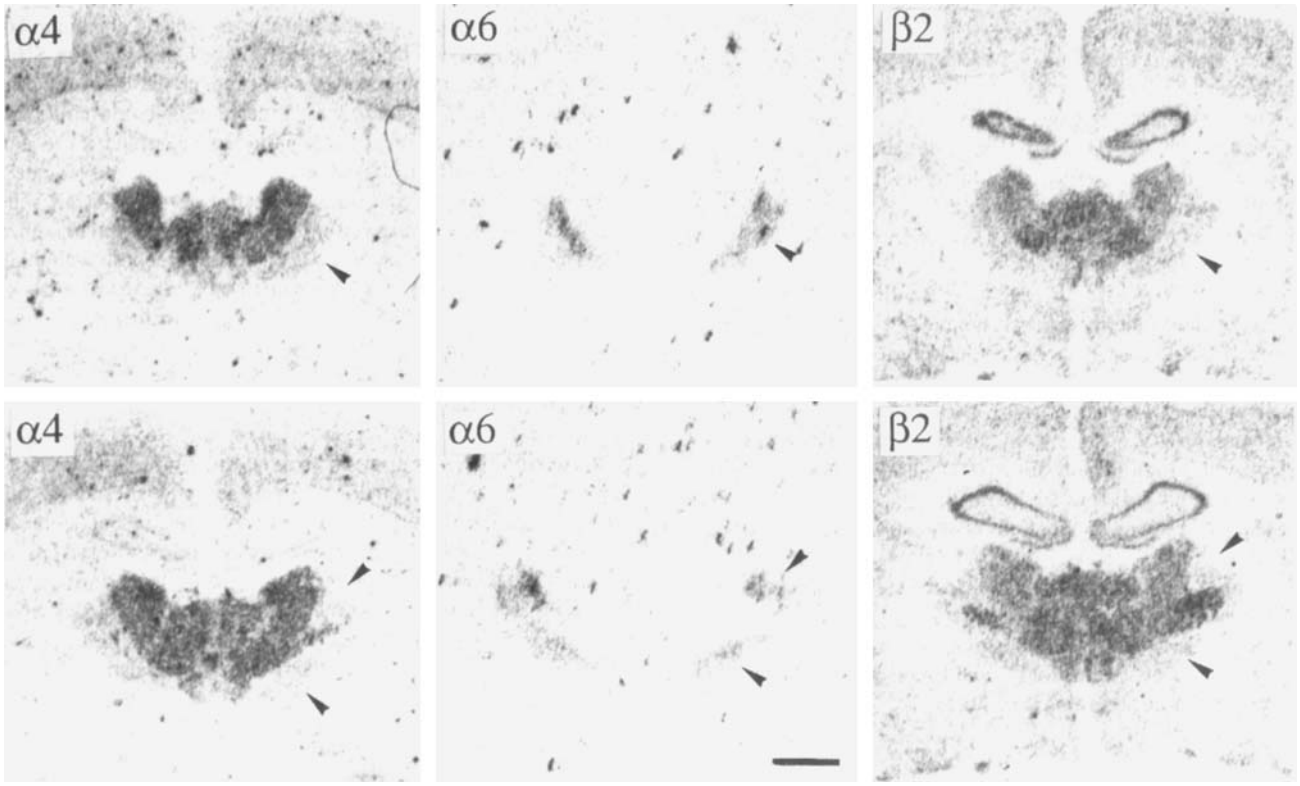

FIG. 1. Bright-field photographs of film autoradiograms showing distributions of α4 (S = 4648), α6 (S = 1623) and β2 (S = 3000) nAChR subunit mRNAs in adjacent sections of the anterior (upper panels) and posterior (lower panels) reticular thalamic nucleus. For details of S values see Materials and methods. Upper panels, bregma –1.4 mm. Lower panels, bregma –2 mm. Arrowheads indicate the reticular thalamic nucleus. Scale bar, 1 mm.

TABLE 3. Count of neurons positive for tyrosine hydroxylase, α6 and β3 in the pars compacta of the substantia nigra and the ventral tegmental area

	Substantia nigra	Ventral tegmental area
Tyrosine hydroxylase	76.6 ± 2.3	86.4 ± 1.2
α6	83.8 ± 2.3	80.9 ± 1.8
β3	83.4 ± 2.3	83.0 ± 3.4

Results (means of two counts/labelling ± SD) are expressed as the percentage of the total number of neurons. For details of analysis see Materials and methods

reticular thalamic nucleus, the medial and lateral habenula and the supramammillary nucleus. This distribution corresponds to that shown in the table of a previous paper (Dineley-Miller and Patrick, 1992). In addition, other nuclei were found to be positive for α6 labelling, such as the interpeduncular nucleus and the mesencephalic V nucleus. Contrary to what is reported in the abstract of Goldner *et al.* (1993), no labelling for α6 mRNA was detected in the anterior pretectal area. This discrepancy is difficult to interpret as this localization was presented in an abstract without figures and methodological details.

Methodological aspects

In this paper we used *in situ* hybridization with oligodeoxynucleotides, a method widely used in the LGIC field (Zhang *et al.*, 1991; Monyer *et al.*, 1992; Poulter *et al.*, 1992; Watanabe *et al.*, 1992; Wisden *et al.*, 1992; Zoli *et al.*, 1995), although different from the *in situ* hybridization method with cRNA probes that has been used until now to map the nAChR subunit mRNAs in the adult mammalian CNS (Deneris *et al.*, 1989; Wada *et al.*, 1989, 1990; Dineley-Miller and Patrick 1992; Séguéla *et al.*, 1993; Rubboli *et al.*, 1994; Rust *et al.*, 1994). In general, our studies with oligonucleotide probes are in good agreement with previous studies with cRNA probes (with a few notable exceptions, such as α5 in the medial habenula). However, a clear discrepancy with previous results was observed for α6 mRNA labelling in a few specific nuclei. In particular, we saw a strong α6 signal and almost no α3 signal in some regions previously thought to be rich in α3 mRNA [i.e. the ventral mesencephalon and the locus coeruleus (Wada *et al.*, 1989)]. This discrepancy cannot be explained on the basis of a different sensitivity of the two techniques, as in most brain areas our mapping agrees well with a previous mapping of α3 (Wada *et al.*, 1989). Interestingly, α3 and α6 are phylogenetically the youngest nAChR subunits, and appeared around the time of the split between bony fishes and tetrapods (Le Novère and Changeux, 1995). Since, according to the present study, α6 is almost 20-fold more concentrated than α3 in some regions, it is possible that a cRNA probe directed against the entire α3 sequence may also label α6 mRNA.

Colocalizations

Our findings demonstrate that α6 and β3 mRNA labellings display a closely similar distribution in the brain. Some preliminary evidence (N. Le Novère, M. Zoli and J.-P. Changeux, unpublished results) indicates that this is also the case in the peripheral nervous system. For instance, in the postnatal retina and somatosensory ganglia (such as the trigeminal ganglion), both α6 and β3 labellings were detected. In the brain, colocalization of α6 and β3 was achieved in most labelled regions (Table 2). Labelling for β3 was sometimes detected at a lower level (thalamic reticular nucleus, interpeduncular nucleus,

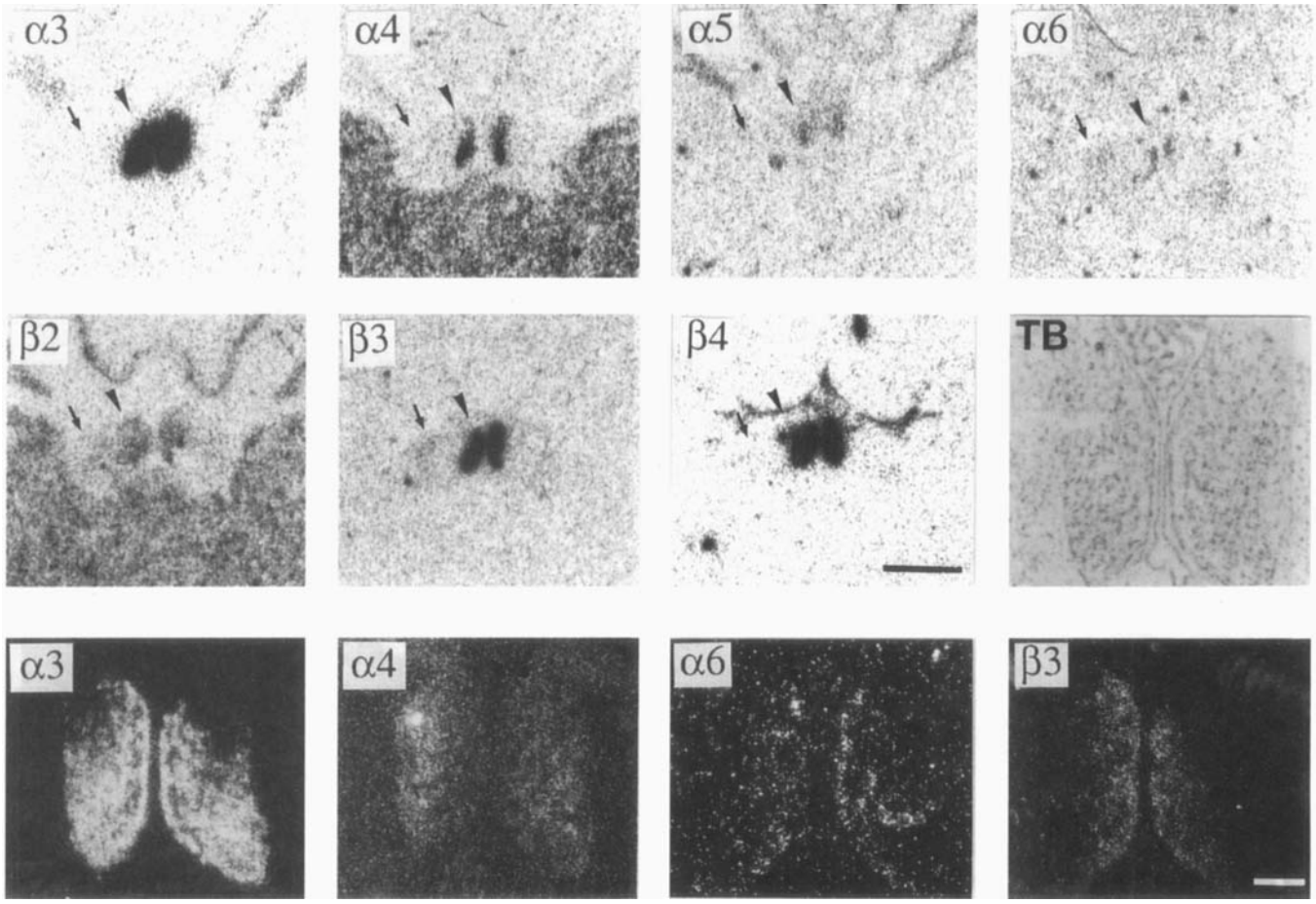

FIG. 2. (Upper panels) Bright-field photographs of film autoradiogram showing distributions of α3 (S = 3469), α4 (S = 4010), α5 (S = 2735), α6 (S = 3227), β2 (S = 4092), β3 (S = 2710) and β4 (S = 3617) nAChR subunit mRNAs in adjacent sections of the habenular nuclei. Arrowheads and arrows indicate the medial and lateral habenula respectively. Scale bar, 1 mm. Note that at this magnification α6 labelling of the medial habenula is barely detectable. (Lower panels) Dark-field microphotographs of emulsion autoradiograms showing distributions of α3 (S = 15462), α4 (S = 7953), α6 (S = 1857) and β3 (S = 3512) nAChR subunit mRNAs in adjacent sections of the medial habenula. The toluidine blue (TB) counterstaining is shown in the last panel of the intermediate row. Scale bar, 200 μm. Note that the distributions of α6/β3 and α4 are complementary. For details of S values see Materials and methods. Bregma –3.3 mm.

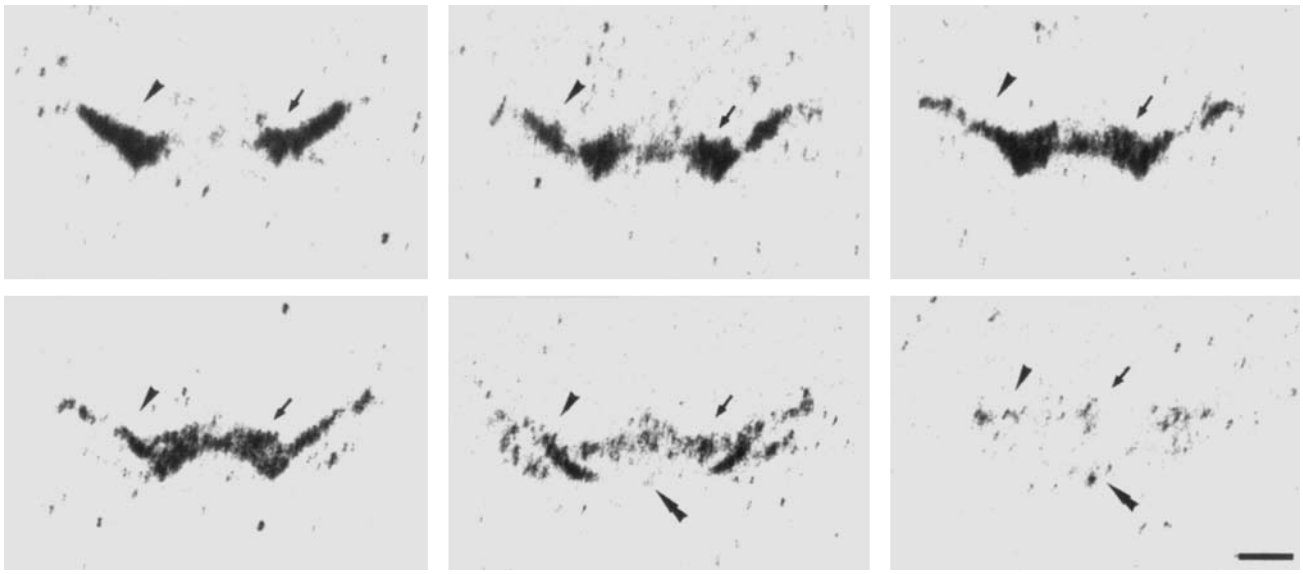

FIG. 3. Bright-field photographs of film autoradiograms showing α6 (S = 1623) nAChR subunit mRNA distribution at six coronal levels (bregma levels –4.8, –5.2, –5.3, –5.7, –6.1 and –6.4 mm) of the ventral mesencephalon. For details of S values see Materials and methods. Arrowhead, arrows and double arrowheads indicate the ventral tegmental area, substantia nigra pars compacta and interpeduncular nucleus respectively. Scale bar, 1 mm.

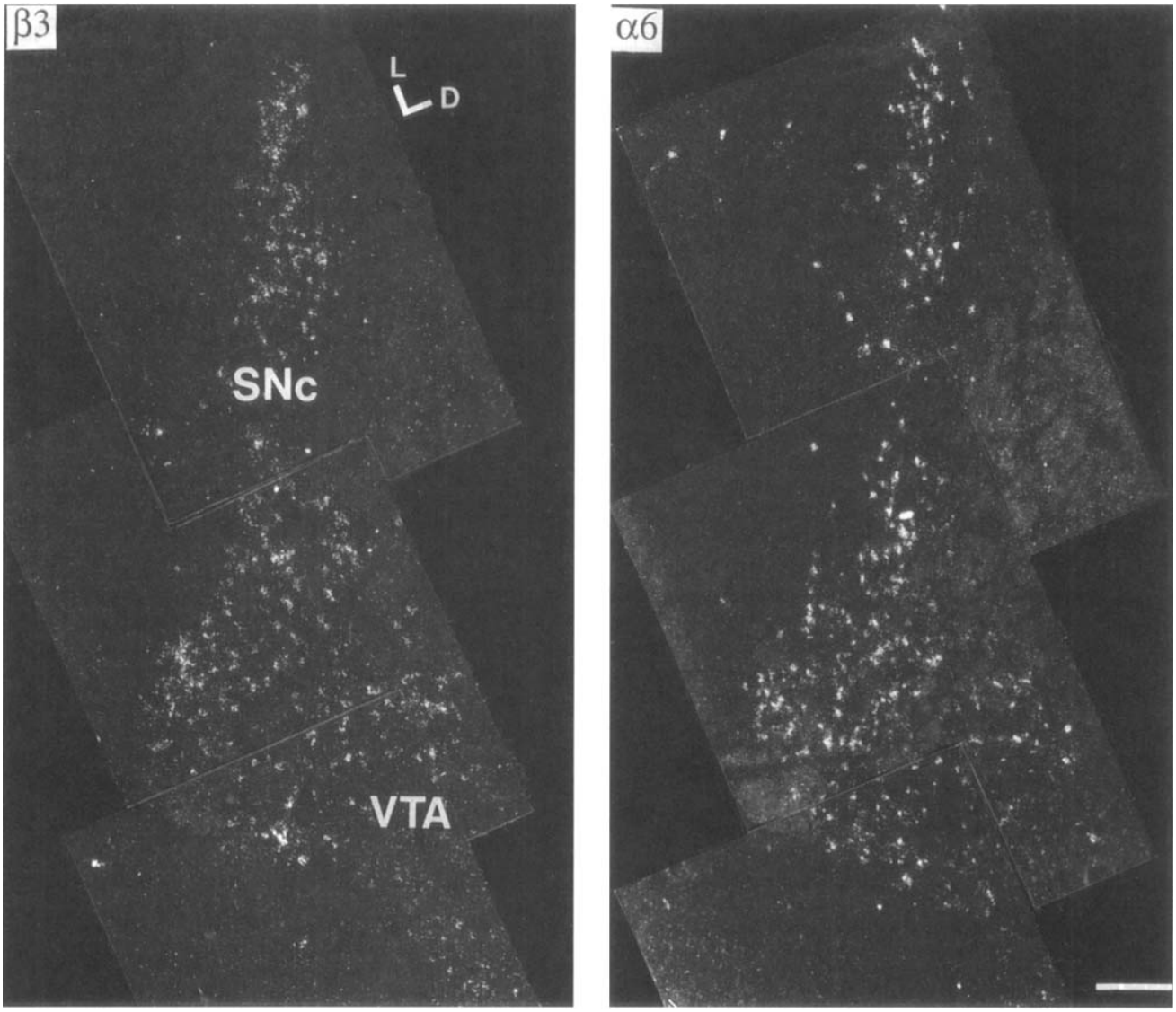

FIG. 4. Dark-field microphotographs of emulsion autoradiograms showing distributions of α6 (S = 1857) and β3 (S = 3512) nAChR subunit mRNAs in adjacent sections of the substantia nigra, pars compacta (SNc) and ventral tegmental area (VTA). For details of S values see Materials and methods. Note the very similar distributions of the two mRNAs in these regions. Bregma –5.3 mm. Scale bar, 200 μm. D, dorsal; L, lateral.

locus coeruleus, dopaminergic nuclei) and sometimes at a higher level (medial habenula, mesencephalic trigeminal nucleus) than α6 labelling. One exception is the supramammillary nucleus, where α6, but not β3, mRNA labelling was detected. In this nucleus, the level of α6 signal was very low, and β3 mRNA levels might be below the detection limit of the technique.

The α6 subunit possesses every residue that has been identified to date as part of the principal component of the acetylcholine binding site (Dennis *et al.*, 1988; Galzi *et al.*, 1990; Galzi and Changeux, 1994). Subunit α6 is thus an 'authentic' α subunit, which may be engaged in acetylcholine binding. The extensive colocalization of α6 and β3 reported here further supports the view that nAChR isoforms assemble into hetero-oligomers, which include both types of subunits. This does not exclude the possibility that α6 may form receptors with other β subunits. A possibility is that β3, like α5, is a third type of subunit (in addition to the standard α and β types) (proposed by Le Novère and Changeux, 1995). These subunits look analogous to the muscle β1 subunit, a subunit which lacks both the principal and the secondary component of the acetylcholine binding site (Galzi and Changeux, 1994; Corringer *et al.*, 1995). Consistent with these conclusions, nAChRs have been identified in the CNS, composed of α4α5β2 (Conroy *et al.*, 1992) or α4β2 (Flores *et al.*, 1992), and in the peripheral nervous system, composed of α3β4 or α3α5β4 (Vernallis *et al.*, 1993; Schwartz Levey *et al.*, 1995). Similarly, we may hypothesize that α6β3β2 and α6β2 complexes are formed in the CNS.

nAChR subunit mRNA in the habenular nuclei

In the lateral habenula, the distribution of α6 mRNA closely parallels that of β2 and β3 subunit mRNAs, although to date there is no direct proof of their colocalization in the same cells. Since the lateral habenula projects to the substantia nigra (Herkenham and Nauta, 1979), and conversely the substantia nigra projects to the lateral habenula (Skagerberg *et al.*, 1984), nAChRs composed of α6β3(β2) may plausibly form in both branches of this bidirectional pathway.

As far as nAChR subunit mRNAs are concerned, the medial

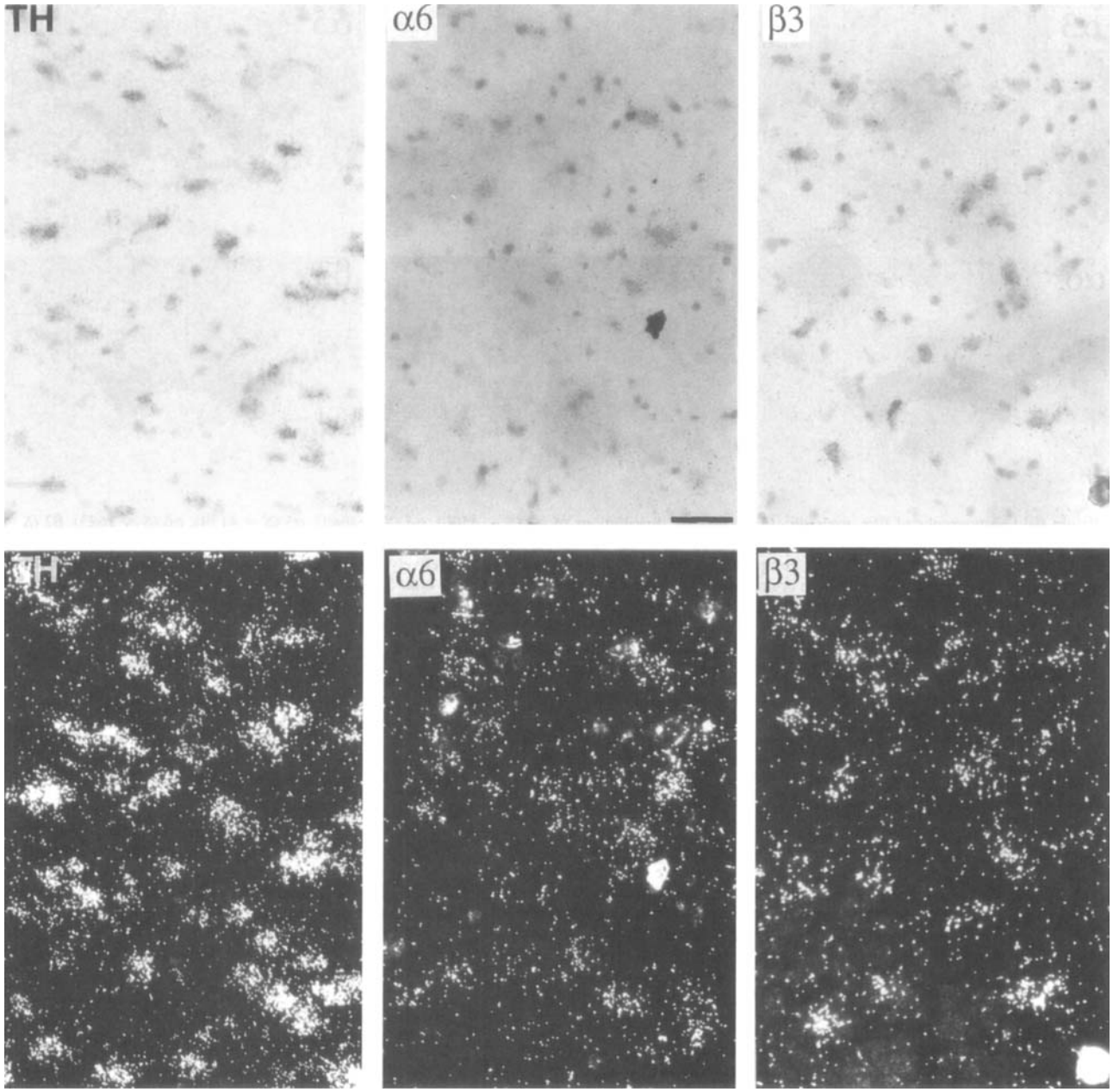

FIG. 5. Bright-field (upper panels) and dark-field (lower panels) microphotographs of emulsion autoradiograms showing distributions of tyrosine hydroxylase (TH, $S = 4028$), and α6 ($S = 1857$) and β3 ($S = 3512$) nAChR subunit mRNAs in adjacent sections of the ventral tegmental area. For details of S values see Materials and methods. Tyrosine hydroxylase oligonucleotide probe was labelled with [^{35}S]dATP (Amersham), which explains the different size of autoradiographic grains with respect to α6 and β3. Scale bar, 40 μm.

habenula can be subdivided into three subnuclei: dorsal, ventromedial and ventrolateral (Fig. 8). Subunits α6 and β3 are localized in the ventromedial subnucleus, with a gradient from the midline to the lateral part (see also Deneris *et al.*, 1989). The more restricted distribution of α6 relative to β3 may be due to the lower level of the α6 mRNA signal. The distribution of α4 mRNA signal is complementary to that of α6 and β3. The α4 subunit was detected at a very low level in the ventromedial subnucleus, at an intermediate level in the dorsal subnucleus and at a high level in the VL subnucleus (see also Wada *et al.*, 1989; Hill *et al.*, 1993). Contrary to Wada *et al.* (1990), we found α5 mRNA labelling in the medial habenula. Its distribution was restricted to the ventrolateral subnucleus. Strong α3 and β4 labelling was detected in the ventromedial and ventrolateral subnuclei, as already described in Wada *et al.* (1989), Dineley-Miller and Patrick (1992) and Hill *et al.* (1993). Labelling for β2 is present almost uniformly in the whole nucleus. The ventromedial and ventrolateral subnuclei correspond to the ventral two-thirds of the medial habenula, which are formed by cholinergic neurons (Levey *et al.*, 1987; Woolf 1995). Accordingly, the distribution of the α3 and β4 subunits overlaps that of choline acetyltransferase-expressing neurons (see Figs 2 and f8b in Oh *et al.*, 1992). The ventrolateral seems richer in choline acetyltransferase than the ventromedial subnucleus (Ichikawa and Hirata, 1986). Lastly, the dorsal subnucleus contains high levels of substance P (Contestabile *et al.*, 1987).

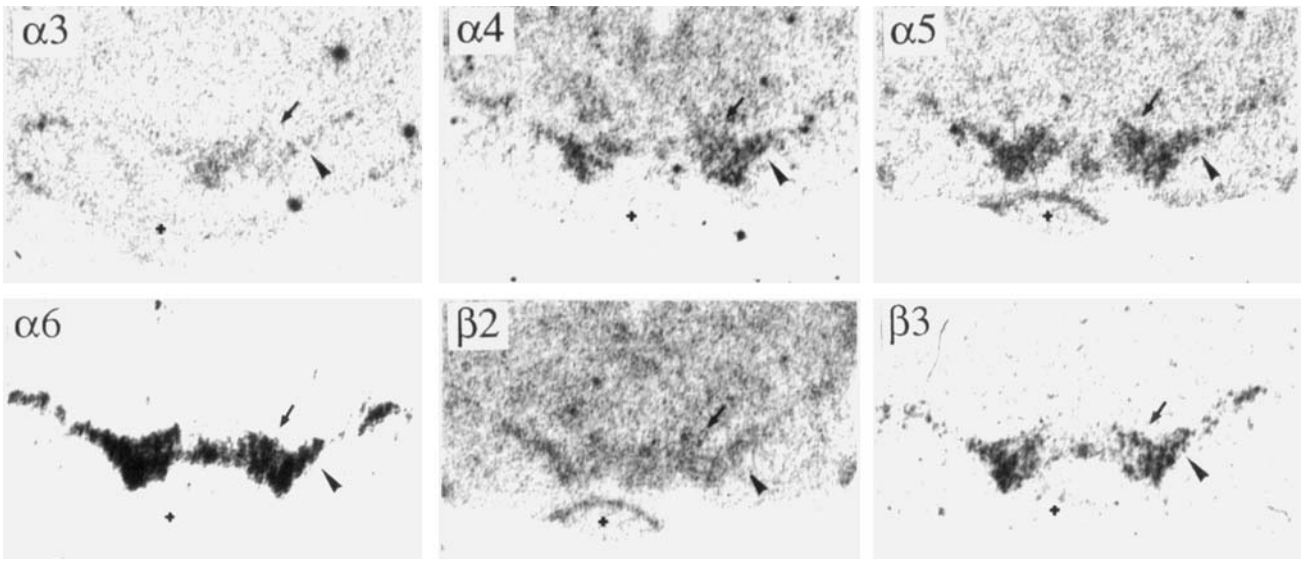

FIG. 6. Bright-field photographs of film autoradiograms showing distributions of α3 (S = 4500), α4 (S = 4648), α5 (S = 4179), α6 (S = 1623), β2 (S = 3000) and β3 (S = 1828) nAChR subunit mRNAs in adjacent sections of the ventral mesencephalon. For details of S values see Materials and methods. Arrowheads and arrows indicate the substantia nigra, pars compacta, and the ventral tegmental area respectively. The small crosses indicate a piece of cerebellum which accidentally moved into the ventral part of the brain during the freezing process. Bregma –5.6 mm. Scale bar, 1 mm.

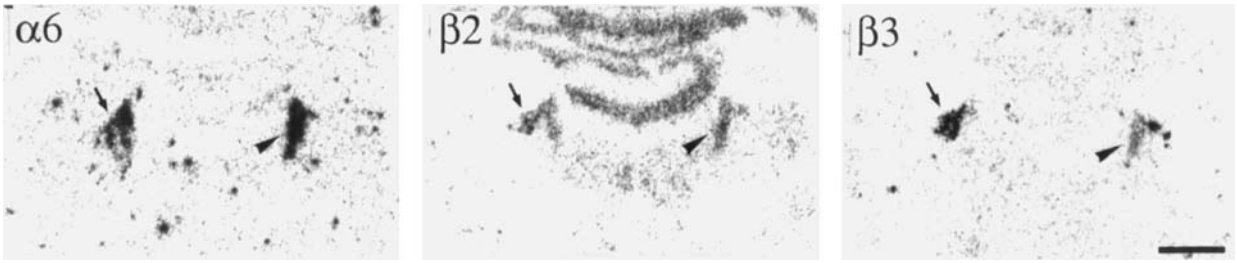

FIG. 7. Bright-field photographs of film autoradiograms showing distributions of α6 (S = 3227), β2 (S = 4092) and β3 (S = 2710) nAChR subunit mRNAs in adjacent sections of the dorsal pons. For details of S values see Materials and methods. Arrowheads and arrows indicate the locus coeruleus and mesencephalic trigeminal nucleus respectively. Bregma –10 mm. Scale bar, 1 mm.

TABLE 4. Densitometry of nAChR mRNA levels in the substantia nigra and ventral tegmental area

	Substantia nigra	Ventral tegmental area
α3	0.39 ± 0.07	0.33 ± 0.14
α4	0.99 ± 0.02	1.15 ± 0.3
α5	1.81 ± 0.83	1.54 ± 0.79
α6	7.23 ± 1.92	7.57 ± 1.19
β2	1	1
β3	4.35 ± 2.42	3.3 ± 1.76

For details of image analysis see Materials and methods. The results, expressed as the ratio of the β2 value, are the mean of two independent experiments (± SD). These experiments were performed with the probes 46, 47 , 51, 58, 59, 62, 131 and 133 (Table 1).

The medial habenula was formerly assumed to be a homogeneous nucleus, containing several different nAChR isoforms in the same cells. The different nicotinic currents recorded from habenular neurons were thus assigned to multiple receptor isoforms present in a homogeneous class of cells (Mulle and Changeux, 1990; Mulle *et al.*, 1991; Connolly *et al.*, 1995). The present data, in particular that showing the segregation of α4/α5 and α6/β3 mRNAs, suggest that nAChR isoforms in the medial habenula are, at least in part, cell-specific. Based on the present and previous studies in transfected cultured cells, and *in situ* studies, we may infer that different nAChR isoforms are produced in the different subnuclei: α4β2 in the dorsal subnucleus; α3β4 and/or α3β2 and α6β2β3 in the ventromedial subnucleus; α3β4 and/or α3β4α5 and α4β2 and/or α4β2α5 and α3β2α5 in the ventrolateral subnucleus (Fig. 8).

nAChR subunit mRNAs in catecholaminergic nuclei

The locus coeruleus and the A8, A9 and A10 nuclei displayed the highest levels of α6 subunit mRNA in the CNS. These nuclei contain high levels of tyrosine hydroxylase and are responsible for the major part of the noradrenergic and dopaminergic innervation of the brain. The stimulatory actions of nicotine on dopamine release (Westfall, 1974; Giorguieff *et al.*, 1977; Arqueros *et al.*, 1978) are mediated by the receptors located on terminals (short-term stimulation) and by the receptors located on the dopaminergic soma of the mesencephalon (long-term stimulation). The two sets of receptors could be identical or different.

Involvement of β2

Clarke and Pert (1985) clearly showed the existence of receptors with high affinity for nicotine in the mesencephalic dopaminergic soma,

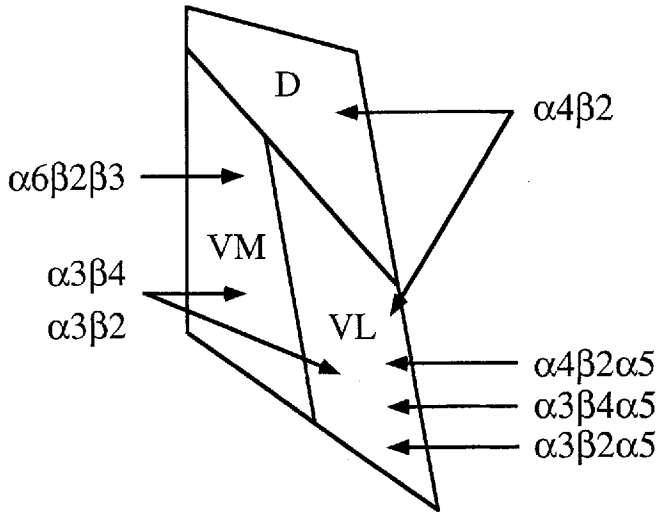

FIG. 8. Schematic drawing of the distributions of nAChR subunit mRNAs in the medial habenula. The subunit combinations for which electrophysiological (in oocytes after heterologous expression of the subunits) and/or biochemical (coprecipitation) evidence has been obtained are indicated for each subnucleus. The location of the putative α6β2β3-containing receptor is also shown. D, dorsal; VL, ventrolateral; VM, ventromedial.

as well as in the striatal dopaminergic terminals. Picciotto *et al.* (1995) demonstrated that inactivation of the β2 subunit gene results in the complete disappearance of receptors with high affinity for nicotine. At least some nAChRs on the dopaminergic soma and terminals are then likely to contain β2. Moreover, β4 mRNA was not detected in dopaminergic cells (Duvoisin *et al.*, 1989; Dineley-Miller and Patrick, 1992; and results presented here).

Subunit α4 seems not to be implicated

The pharmacology of nicotine-enhanced dopamine release indicates that the receptor involved is not α4β2 (Rapier *et al.*, 1990; Grady *et al.*, 1992; Sacaan *et al.*, 1995). In striatal synaptosomes, Grady *et al.* (1992) found that potency was in the order cytisine = nicotine > DMPP = acetylcholine. In striatal slices, Sacaan *et al.* (1995) found the order cytisine > nicotine = DMPP. These pharmacological spectra do not match the properties of an α4β2-containing receptor (Luetje and Patrick, 1991). Moreover, 100 nM neuronal bungarotoxin blocks the nicotine-induced endogenous dopamine release (Schulz and Zigmond, 1989). At this concentration, the toxin is able to inhibit α3β2 but not α4β2 (Luetje *et al.*, 1990).

Subunit α6 is more likely to be implicated than α3

Here we show that α6 labelling is >20-fold more intense than α3 labelling in the dopaminergic cells as well as in the locus coeruleus. We can therefore hypothesize that the pharmacology observed for nAChR-mediated potentiation of dopamine (and noradrenaline) release results from an α6-containing receptor. But nothing is known about the pharmacology of α6-containing nAChRs. However, the sequence similarity between α3 and α6 is very high (61% identity over the entire amino acid sequence, the differences being located mainly in the signal peptide and the cytoplasmic loop). It is noteworthy that every identified amino acid composing the acetylcholine binding site is conserved; in particular the whole of the A and C loops (Galzi and Changeux, 1994) are 100% identical in the two subunits. The participation of the α3 and α6 subunits in the binding site might then be similar and may account for the pharmacological data reported initially.

Involvement of β3

Although protein levels may not parallel mRNA levels, the strong expression of β3 subunit mRNA in the tyrosine hydroxylase-positive neurons supports the notion that this subunit contributes to the nicotinic response of the catecholaminergic nuclei. At this stage no direct evidence exists for protein oligomers containing α6 and β3 in these cells. Yet the extensive colocalization of α6, β2 and β3 mRNA supports the view that the formation of an oligomer containing these three subunits may account for the nicotine-induced catecholamine release.

Conclusion

The presence of high levels of α6 mRNA in the dopamine-containing neurons might be of considerable physiological importance, since the release of dopamine mediated by nicotine is thought to mediate its locomotor and addictive properties (Clarke *et al.*, 1993; Stolerman and Shoaib, 1991). More generally, the catecholaminergic neurons of the locus coeruleus and the A8, A9 and A10 nuclei have been postulated to directly control physical dependence on addictive drugs (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Nestler, 1992; Schulteis and Koob, 1994). The design of drugs specifically directed against the receptor subtypes responsible for these behaviours is an important challenge for modern neuropharmacology. In order to achieve this aim, the exact molecular constitution of these receptors has to be established. Here we propose that α6β2β3 nAChRs are likely candidates for the mediation of nicotine-induced potentiation of catecholamine release.

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Abbreviations

CNS	central nervous system
GABA	γ-aminobutyric acid
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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