Developmental Regulation of Nicotinic ACh Receptor Subunit mRNAs in the Rat Central and Peripheral Nervous Systems

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In the present study we have investigated the anatomical distribution pattern of nAChR α3, α4, β2, and β4 subunit mRNAs during prenatal and perinatal development of the rat CNS and PNS. Three main developmental patterns have been recognized. (1) In the majority of cases studied (caudal brain, spinal cord, dorsal root ganglia, trigeminal and geniculate ganglia) all four subunit mRNAs are initially (E11–13) detected but, during subsequent prenatal development, the level of some of these subunit mRNAs (α3 and β4 in the brain and spinal cord, α4 and β4 in the dorsal root ganglia, α4 in the visceral sensory ganglia, and α3, α4, and β4 in the somatic sensory ganglia) become undetectable. (2) In the case of the cerebral cortex a pair of subunit mRNAs (α3-β2) is initially (E12–13) expressed followed by a repression of the α3 subunit (E15) and the subsequent (E17–19) induction of the α4 subunit. (3) Only some subunit mRNAs are initially (E13–15) expressed in the retina (α3-α4-β2-β4), parasympathetic or sympathetic motor ganglia (α3-β2-β4), and vestibulo-cochlear ganglia (α4-β2) and their level remains stable throughout prenatal and early postnatal development.

Overall, in most central and peripheral structures the appearance of nAChR subunit mRNAs is precocious and temporally related to the timing of neuronal differentiation. In addition, in several structures the expression of certain subunits (α3, α4 or β4) is transient, although not β2. Finally, the comparison of the different regional distribution patterns suggests that a limited number of structure-specific receptor isoforms are functional during development of CNS and PNS.

[Key words: neuronal nicotinic ACh receptor, development, brain, ganglia, rat, oligodeoxynucleotides]

The neuronal nicotinic acetylcholine receptor (nAChR) belongs to the superfamily of ligand-gated ion channels (Cockcroft et al., 1992; Galzi et al., 1992) largely responsible for fast responses to neurotransmitters. Molecular cloning methods have revealed the existence of homologous genes encoding nAChR

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subunits which have been named α2-8, if carrying a pair of cysteines shown to contribute to the ACh binding site, or β2-4 (Sargent, 1993).

The functional neuronal nAChR results from the assembly of α- and β-chains, with a putative stoichiometry of 2(α):3(β) (Anand et al., 1991; Cooper et al., 1991). Heterologous expression studies in *Xenopus* oocytes and in mouse fibroblasts, as well as patch-clamp studies in brain and ganglionic preparations suggest that multiple subunit combinations may assemble to form functional nAChRs with distinct pharmacological specificities and ionic channel properties (e.g., Listerud et al., 1991; Luetje and Patrick, 1991; Mulle et al., 1991). Accordingly, several different combinations of nAChR subunit mRNAs and/or proteins have been identified in central and peripheral nervous structures (Role, 1992).

The regulation of nAChR gene expression during development remains largely unexplored. In skeletal muscle (reviewed in Salpeter and Loring, 1985; Changeux, 1991), nAChR subunit mRNAs and binding sites appear at the myoblast stage and progressively increase during the formation of myotubes. Upon the arrival of cholinergic motor nerve endings, local clustering, accumulation, and metabolic stabilization of nAChRs occur in the junctional membrane accompanied by a decrease of nAChRs in extrajunctional areas. In some species, a switch in the composition of the receptor (from γ to ε subunit) occurs at around the time of birth (Brehm and Henderson, 1988).

Less is known about developmental changes in neuronal nAChRs, especially in mammals. In mammalian CNS, studies have mainly focussed on the development of nicotinic binding in the postnatal period (see, e.g., Fiedler et al., 1987; Semba, 1992). In the chick lateral spiriform nucleus, the onset of α2 subunit mRNA expression coincides with cholinergic innervation (Daubas et al., 1990). In the chick optic lobe, a transient increase in β2 transcript expression occurs upon arrival of the retinal afferents (Matter et al., 1990).

In the embryonic chick ciliary ganglion, the formation of mature synaptic contacts is paralleled by a progressive increase in several nAChR subunit mRNAs, ACh response and density of nicotinic binding sites (Margiotta and Gurantz, 1989). However, no developmental switch in subunit composition was detected (Corriveau and Berg, 1993). In chick sympathetic ganglia, an increased sensitivity to ACh accompanies the embryonic increase in the number of synaptic boutons (Role 1988; Moss et al., 1989). These data raise the possibility that, in these systems, innervation directly stimulates neuronal nAChR gene expression.

Given the complexity and heterogeneity of the CNS, it appears relevant to assess the region-specific pattern of develop-
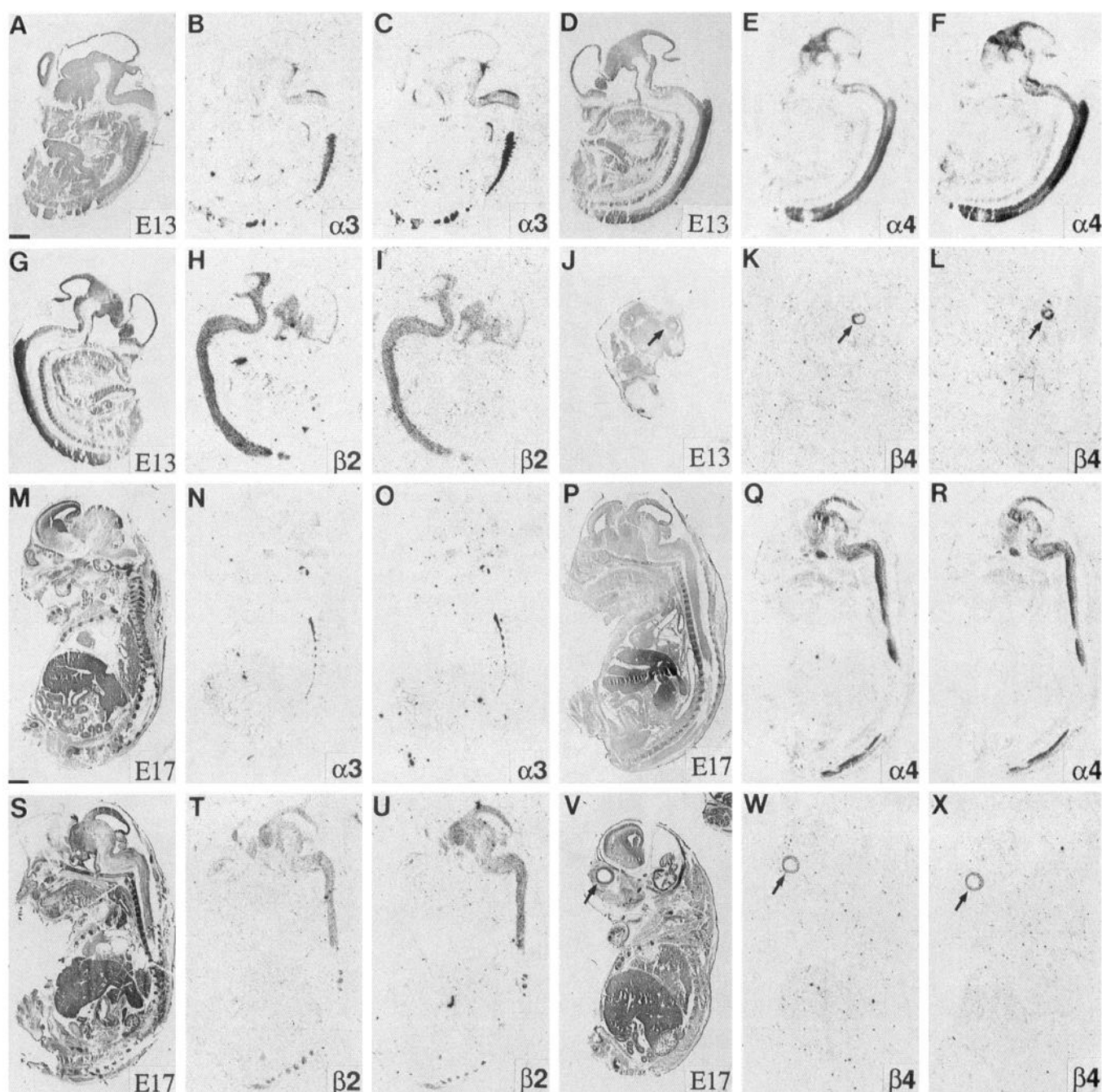
Figure 1. Bright-field microphotographs of film autoradiograms showing hybridization with different oligonucleotide probes against nAChR subunit mRNAs in sagittal sections of E13 (*A–L*) and E17 (*M–X*) rat embryos. The nAChR subunit mRNA labeled is indicated in each panel. *B* and *C*, α3 nAChR subunit mRNA labeled by oligonucleotide 46 (*B*) or 31 (*C*). *E* and *F*, α4 nAChR subunit mRNA labeled by oligonucleotide 47 (*E*) or 49 (*F*). *H* and *I*, β2 nAChR subunit mRNA labeled by oligonucleotide 37 (*H*) or 51 (*I*). *K* and *L*, β4 nAChR subunit mRNA labeled by oligonucleotide 61 (*K*) or 62 (*L*); the *arrow* points to the retina. *N* and *O*, α3 nAChR subunit mRNA labeled by oligonucleotide 109 (*N*) or 110 (*O*). *Q* and *R*, α4 nAChR subunit mRNA labeled by oligonucleotide 49 (*Q*) or 47 (*R*). *T* and *U*, β2 nAChR subunit mRNA labeled by oligonucleotide 51 (*T*) or 37 (*U*). *W* and *X*, β4 nAChR subunit mRNA labeled by oligonucleotide 62 (*W*) or 61 (*X*); the *arrow* points to the retina. Scale bars: *A–L*, 1 mm; *M–X*, 2 mm.

mental expression of the different nAChR subunit mRNAs. Here we report extensive *in situ* hybridization observations on the evolution of α3, α4, β2, and β4 nAChR subunit mRNAs in the rat CNS and several peripheral structures during prenatal and early postnatal development. These subunits are thought to form the predominant nAChR isoforms in the ganglia (α3 and β4,

Halvorsen and Berg, 1990; Listerud et al., 1991) and CNS (α4 and β2, Whiting et al., 1991; Flores et al., 1992).

Materials and Methods

Oligodeoxynucleotide synthesis and labeling. Following analysis for mRNA secondary structure using GCG SEQUENCE ANALYSIS SOFTWARE

Table 1. Characteristics of the oligonucleotides used for *in situ* hybridization

Subunit	Code	Sequence	Length	GC%
α3	31	889–931	43	53
α3	46	987–1031	45	62
α3	109	64–108	45	51
α3	110	472–516	45	58
α4	47	1511–1555	45	62
α4	49	1270–1314	45	55
β2	37	1276–1320	45	60
β2	51	1136–1180	45	55
β4	61	1083–1127	45	55
β4	62	1322–1366	45	53
ChAT	67	1756–1800	45	53
ChAT	68	1827–1871	45	56

cDNA sequences were taken from the following sources: α3, Boulter et al., 1986; α4, Goldman et al., 1987; β2, Deneris et al., 1988; β4, Duvoisin et al., 1989; ChAT, Ishii et al., 1990.

7.1, oligodeoxynucleotide sequences were chosen in unique regions of the mRNAs and synthesized using a Cyclone (Biosearch Inc.) DNA synthesizer. The probe characteristics are reported in Table 1. The oligonucleotide probes were labeled at the 3′ end using ^{35}S-dATP (Amersham) and terminal deoxynucleotidyl transferase (Boehringer-Mannheim) following the specifications of the manufacturer to a specific activity of 100–300 kBcq/pmol. The labeled probes were separated from unincorporated ^{35}S-dATP by means of NucTrap push columns (Stratagene, La Jolla, CA), precipitated in ethanol, and resuspended in distilled water containing 50 mM dithiothreitol.

Animals. Sprague–Dawley rats (Iffacredo, Lyon) were used. Several embryonic and postnatal ages were examined: E10, E11, E12, E13, E15, E17, E19, P0, P4, and adult. Embryos were dated according to Paxinos et al. (1991) so that E0 corresponds to the 24 hr following pairing and P0 to the 24 hr following birth. For each developmental time two to five animals were used. In the case of early embryos (E11–E15), which have abrupt changes in nAChR mRNA levels, at least four animals/group were analyzed. Tissues from embryos (obtained from ether-anesthetized timed-pregnant rats) and postnatal rats (killed by decapitation) were fresh frozen on dry ice.

In situ *hybridization procedure.* Frozen tissues were cut at the cryostat (14 μm thick sections), thaw mounted on poly-L-lysine coated slides and stored at −80°C for 1–3 d. The procedure was carried out according to Young et al. (1986). Briefly, sections were fixed with 4% paraformaldehyde for 5 min at room temperature, washed in phosphate buffered saline (PBS) and then acetylated and delipidated in ethanol and chloroform (5 min). They were prehybridized for 2–4 hr at 37°C and hybridized for 20 hr at 37°C under parafilm coverslips. The composition of the prehybridization and hybridization mixtures was 50% formamide, 0.6 M NaCl, 0.1 M dithiothreitol, 10% dextran sulfate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 × Denhardt's solution (50× = 1% bovine serum albumin, 1% Ficoll, 1% polyvinylpyrrolidone), 0.1 mg/ml polyA (Boehringer), 0.5 mg/ml yeast tRNA (Sigma), 0.05 mg/ml herring sperm DNA (Promega) in 0.02 M Tris-HCl, pH 7.5. Probes were applied at a concentration of 2000–3000 Bcq/30 μl/section (corresponding to around 15 fmol/section). After removal of coverslips and initial rinse in 2 × standard saline citrate (SSC) solution (3 M NaCl,
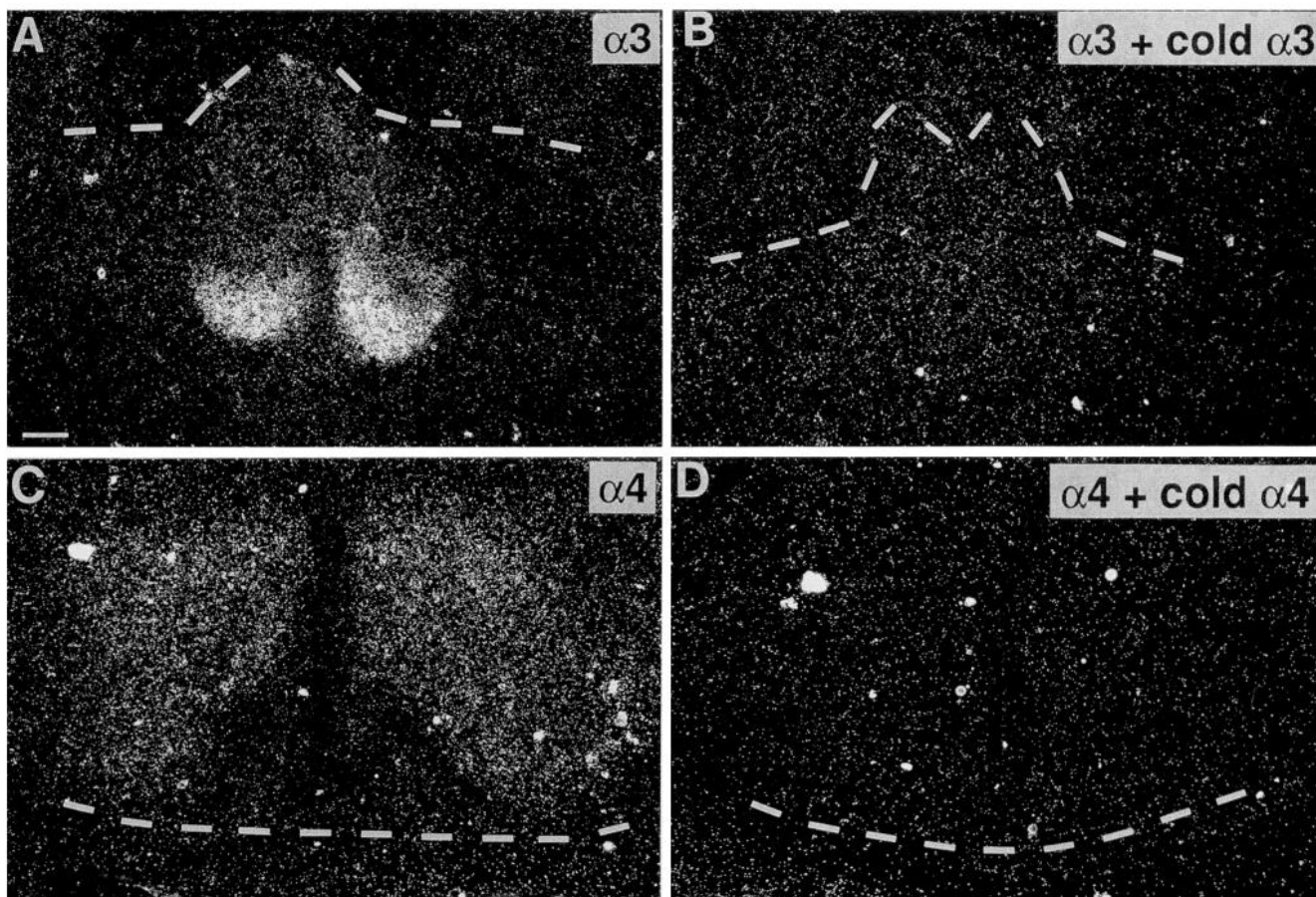
Figure 2. Dark-field microphotographs of emulsion autoradiograms showing α3 nAChR subunit mRNA in coronal sections of the lateral habenula of E17 rat embryo (*A and B*) and α4 nAChR mRNA in coronal sections of the ventromedial hypothalamic nucleus of a E19 rat embryo (*C and D*). In *B* and *D*, sections hybridized with a 20-fold excess of cold probe are shown. The *dashed lines* outline the borders of the brain. Scale bar, 100 μm.

Table 2. Development of nAChR subunit mRNAs in the brain

	α3	α4	β2	β4
E11				
Telencephalon	−	−	−	−
Diencephalon	−	−	−	−
Mesencephalon	−/+	−/+	−/+	−
Rhombencephalon	−/+	−/+	−/+	−/+
E12				
Telencephalon				
Ventral part	−/+	−/+	−/++	−
Dorsal part	−/+	−	−	−
Diencephalon				
Ventral part	−	−/+	−/++	−
Dorsal part	−	−	−	−
Meso-diencephalic junction	++	++	++	−
Mesencephalon				
Ventral part	+	++	++	−/+
Dorsal part	−	+	+	−
Isthmus region	++	++	++	+
Metencephalon[a]				
Medioventral part	−/+	++	++	−
Lateral part	++	++	++	−/++
Mediodorsal part	−	−	−	−
Myelencephalon[b]				
Medioventral part	+++	++	+++	+
Lateral part	+++	++	+++	++
Mediodorsal part	−	−	−	−
Caudal part	+++	+++	+++	++
E13				
Telencephalon				
Cerebral cortex				
External part	+	−	+	−
Internal part	−	−	−	−
Hippocampal formation	−/+	−	−/+	−
Septum	+	+	++	−
Basal ganglia	−	−	++	−
Forebrain ni[c]	−	−	++	−
Preoptic area	−/+	+	++	−
Diencephalon				
Ventral part	−/++	++	+++	−/+
Dorsal part	−	−	−	−
Mesodiencephalic junction	+	+++	+++	−
Mesencephalon				
Ventral part	++	+++	+++	−
Dorsal part	−	+	++	−
Isthmus region	++	++	+++	−/+
Metencephalon[a]				
Medioventral part	−/+	++	+++	−
Lateral part	+/++	++/+++	+++	−
Mediodorsal part	−	−	−	−
Myelencephalon[b]				
Medioventral part	++/+++	++/+++	+++	−/+
Lateral part	++/+++	++/+++	+++	−/+
Mediodorsal part	−	−	−	−
Caudal part	++/+++	+++	+++	−/+
E15				
Telencephalon				
Cerebral cortex				
Plate	−	−	++	−
Intermediate layers	−	−	+	−
Neuroepithelium	−	−	−	−

Table 2. Continued

	α3	α4	β2	β4
Hippocampal formation	−	+	+	−
Septum	−	+	+++	−
Basal ganglia	−	−	+++	−
Forebrain ni[c]	−	−	+++	−
Preoptic area	+	+	+++	−
Diencephalon				
Thalamus				
Habenula	++	+	+++	+++
Anterior	−	+	+++	−
Intermediate	−	+	+++	−
Posterior	−	++	+++	−
Hypothalamus				
Anterior	+	+/++	+++	−
Posterior	+	+/++	+++	−
Mammillary area	+	+	++	+
Mesencephalon				
Ventral	+	++	+++	−/+
Dorsal	−	+	++	−
Metencephalon				
Ventral cochlear nu	++	+	+++	−
Motor nu 5	+	+	+++	−
Other pontine ni	+	+/++	+++	−
Cerebellar neuroepithelium	++	−	−	++
Myelencephalon				
Motor ni	+	+	+++	−
Other bulbar ni	+	+/++	+++	−
E17				
Telencephalon				
Cerebral cortex				
Plate	−	−	++	−
Subplate	−	+	+	−
Intermediate layer	−	−	+	−
Subventricular layer	−	−	+	−
Neuroepithelium	−	−	−	−
Claustrum	−	++	++	−
Olfactory bulb	−	−	++	−
Primary olfactory cortex	−	+	++	−
Hippocampal formation	−	+	++	−
Septum	−	+	++	−
Basal ganglia	−	−	++	−
Basal forebrain ni	−	+	++	−
Dorsomedial forebrain ni	−	+	++	−
Preoptic area	−	+	++	−
Amygdala				
Medial	−	+	++	−
Other ni	−	−	++	−
Diencephalon				
Pineal gland	−	−	−	+
Thalamus				
Medial habenula	+++	+	+	+++
Anterior group ni	−	+	++	−
Mediodorsal nu	−	+	++	−
Intralaminar group ni	−	+	++	−
Midline group ni	−	+	++	−
Lat geniculate dorsal	−	++	++	−
Lat geniculate ventral	−	+	++	−
Med geniculate	−	+	++	−
Other ni	−	++	++	−

Table 2. Continued

	α3	α4	β2	β4
Hypothalamus				
Anterobasal area	−	+ +	+ +	−
Ventromedial nu	+	+ + +	+ +	−
Arcuate nu	+	+	+ +	−
Lat mammillary area	+	+ +	+ +	−
Other ni	+	+	+	−
Brainstem				
Superior colliculus				
External layers	−	+	+ +	−
Internal layers	−	+ +	+ +	−
Pretectum	−	+ +	+ +	−
Central grey	−	+ +	+ +	−
Locus coeruleus	−	+ +	+ +	−
Cranial nerve motor ni	+	+	+ + +	−
Ventral cochlear nu	+ +	+ +	+ +	+
Other ni	+	+	+ +	−
Cerebellum				
Neuroepithelium	+ +	−	−	+
Purkinje cell layer	−	+	+ +	−
Deep ni	−	+ +	+ +	−
E19				
Telencephalon				
Cerebral cortex				
Plate	−	+	+ +	−
Subplate	−	+ +	+	−
Intermediate layer	−	−	+	−
Subventricular layer	−	−	+	−
Neuroepithelium	−	−	−	−
Claustrum	−	+ +	+ +	−
Olfactory bulb	−	−	+ +	−
Primary olfactory cortex	−	+	+ +	−
Hippocampal formation				
Pyramidal layer	−	+	+ +	−
Other layers	−	−	+	−
Septum	−	+	+ +	−
Basal ganglia	−	−	+ +	−
Basal forebrain ni	−	+	+ +	−
Dorsomedial forebrain ni	−	+	+ +	−
Preoptic area	−	+	+ +	−
Amygdala	−	+	+ +	−
Diencephalon				
Pineal gland	−	−	−	+ + +
Thalamus				
Medial habenula	+ + +	+ +	+ +	+ + +
Anterior group ni	−	+	+ +	−
Mediodorsal nu	−	+	+ +	−
Intralaminar group ni	−	+	+ +	−
Midline group ni	−	+	+ +	−
Ventral group ni	−	+ + +	+ + +	−
Lat geniculate dorsal	−	+ +	+ +	−
Lat geniculate ventral	−	+	+ +	−
Med geniculate	−	+	+ +	−
Other ni	−	+ +	+ +	−
Zona incerta	−	+ +	+ +	−
Hypothalamus				
Anterior ni	−	+ +	+	−
Ventromedial nu	−	+ + +	+ +	−
Arcuate nu	−	−	+ +	−
Lat mammillary ni	−	+ +	+ +	−
Med mammillary ni	−	+	+ +	−
Other ni	−	+	+	−

Table 2. Continued

	α3	α4	β2	β4
Brainstem				
Superior colliculus				
External layers	−	+	+ +	−
Internal layers	−	+ +	+ +	−
Pretectum	−	+ +	+ +	−
Substantia nigra/VTA	−	+ +	+ +	−
Central grey	−	+ +	+ +	−
Red nu	−	+ +	+ +	−
Cranial nerve motor ni	−	+	+ + +	−
Locus coeruleus	−	+ +	+ +	−
Ventral cochlear nu	+	+ +	+ + +	+
Prepositus hypoglossal nu	−	+ +	+ +	−
Inferior olive	−	+ +	+ +	−
Other ni	−	+	+ +	−
Cerebellum				
External germinal layer	+	−	−	+
Purkinje cell layer	−	+	+ +	−
Deep ni	−	+ +	+ +	−
P0				
Telencephalon				
Cerebral cortex				
Plate	−	+	+ +	−
Subplate	−	+ +	+ +	−
Claustrum	−	+ +	+ +	−
Endopiriform nu	−	+ +	+ +	−
Olfactory bulb				
Glomerular layer	−	−	−	−
Ext plexiform layer	−	−	+	−
Mitral cell layer	−	−	+ + +	−
Int plexiform layer	−	−	+	−
Anterior olfactory nu	−	+ +	+ +	−
Primary olfactory cortex	−	+ +	+ +	−
Olfactory tubercle	−	−	+ +	−
Hippocampal gyrus				
Pyramidal layer	−	+	+ +	−
Other layers	−	−	+	−
Dentate gyrus				
Granular layer	−	+	+ +	−
Other layers	−	−	+	−
Subiculum	−	+	+ +	−
Entorhinal cortex	−	+	+ +	−
Septum	−	+	+ +	−
Basal ganglia				
Caudate putamen	−	−	+ +	−
Accumbens nu	−	−	+ +	−
Globus pallidus	−	+	+ +	−
Basal forebrain ni	−	+	+ +	−
Dorsomedial forebrain ni	−	+	+ +	−
Preoptic area	−	+	+ +	−
Amygdala	−	+	+	−
Diencephalon				
Pineal gland	+	−	+	+ + +
Thalamus				
Medial habenula	+ + +	+ +	+ +	+ + +
Lateral habenula	−	−	+	−
Anterior group ni	−	+	+ +	−
Mediodorsal nu	−	+	+ +	−
Midline group ni	−	+ +	+	−
Paraventricular nu	−	+	+ +	−
Centromedian nu	−	+	+ +	−
Posterior nu	−	+ + +	+ +	−

Table 2. Continued

	α3	α4	β2	β4
Ventral group ni	−	+++	+++	−
Lat geniculate dorsal	−	++	++	−
Lat geniculate ventral	−	+	++	−
Med geniculate	−	+	++	−
Other ni	−	++	++	−
Zona incerta	−	++	+	−
Hypothalamus				
Arcuate nu	−	−	++	−
Ventromedial nu	−	+++	++	−
Dorsomedial nu	−	+	++	−
Lat mammillary ni	−	+	++	−
Med mammillary ni	−	−	++	−
Other ni	−	+	+	−
Mesencephalon				
Superior colliculus				
External layers	−	+	++	−
Internal layers	−	++	++	−
Pretectum	−	++	++	−
Substantia nigra/VTA	−	++	+	−
Red nu	−	++	+	−
Other ni	−	+	+	−
Pons				
Cranial nerve motor ni	−	+	++	−
Pontine ni	−	+	++	−
Ventral nu lat lemniscus	−	+	++	−
Locus coeruleus	−	++	++	−
Ventral cochlear nu	+	++	++	+
Vestibular ni	−	++	+	−
Other ni	−	+	+	−
Medulla oblongata				
12 nerve motor nu	−	++	+	−
Inferior olive	−	++	++	−
Prepositus hypoglossal nu	−	++	+	−
Other ni	−	+	+	−
Cerebellum				
External germinal layer	+	−	−	−
Purkinje cell layer	−	+	++	−
Deep ni	−	+++	++	−

Scores were attributed as follows: +, low level; ++, intermediate level; +++, high level. At E11–12 the regions considered are often not homogeneously labeled. We therefore decided to assign the scores as follows. When the majority of the region under examination is specifically labeled, only one score is given, referring to the average intensity of specific labeling. When the specific labeling covers a lesser portion of the structure considered, two scores are given: the first one is a minus and the second one refers to the average intensity of positive clusters of cells. At E13–P0, when two scores are given, the first one refers to the average labeling in the region, and the second one to some nonidentified clusters of cells. Abbreviations: ext, external; int, internal; lat, lateral; med, medial; ne, neuroepithelium; ni, nuclei; nu, nucleus; VTA, 'ventral tegmental area.

a The mediodorsal part corresponds to the roof of the IV ventricle.

b The myelencephalon is subdivided into a smaller caudal part, roughly corresponding to the region caudal to the IV ventricle, and a larger rostral part, in turn composed of a medioventral, a lateral, and a mediodorsal part. This last corresponds to the roof of the IV ventricle.

c The term forebrain nuclei indicates the clusters of neurons in the subcortical forebrain which will form the basal and dorsomedial forebrain nuclei at later ages.

0.3 M sodium citrate) at room temperature (two times for 5 min), sections were washed four times for 15 min in 2 × SSC, 50% formamide at 42°C, and then two times for 30 min in 1 × SSC at room temperature; 1 mM dithiothreitol was added to all washing solutions. After rinsing in ice-cold distilled water and drying, they were exposed for 10–20 d to Hyperfilm-³H (Amersham) and then to a photographic emulsion (NTB2, Kodak) for 1–2 months.

Analysis of histological preparations. The analysis of the labeling pattern for the different mRNAs was carried out both on film and emul-

sion autoradiograms. Identification of anatomical structures was carried out after counterstaining of the serial sections of the entire embryos with toluidine blue. Definition of anatomical areas in the brain and recognition of PNS structures was based on different atlases, including *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson, 1986), the *Atlas of Developing Rat Brain* (Paxinos et al., 1991), the *Atlas of Mouse Development* (Kaufman, 1992), and the *Atlas of the Prenatal Mouse Brain* (Schambra et al., 1992). For cranial nerve ganglia development, the plates and descriptions from Altman and Bayer (1982) were
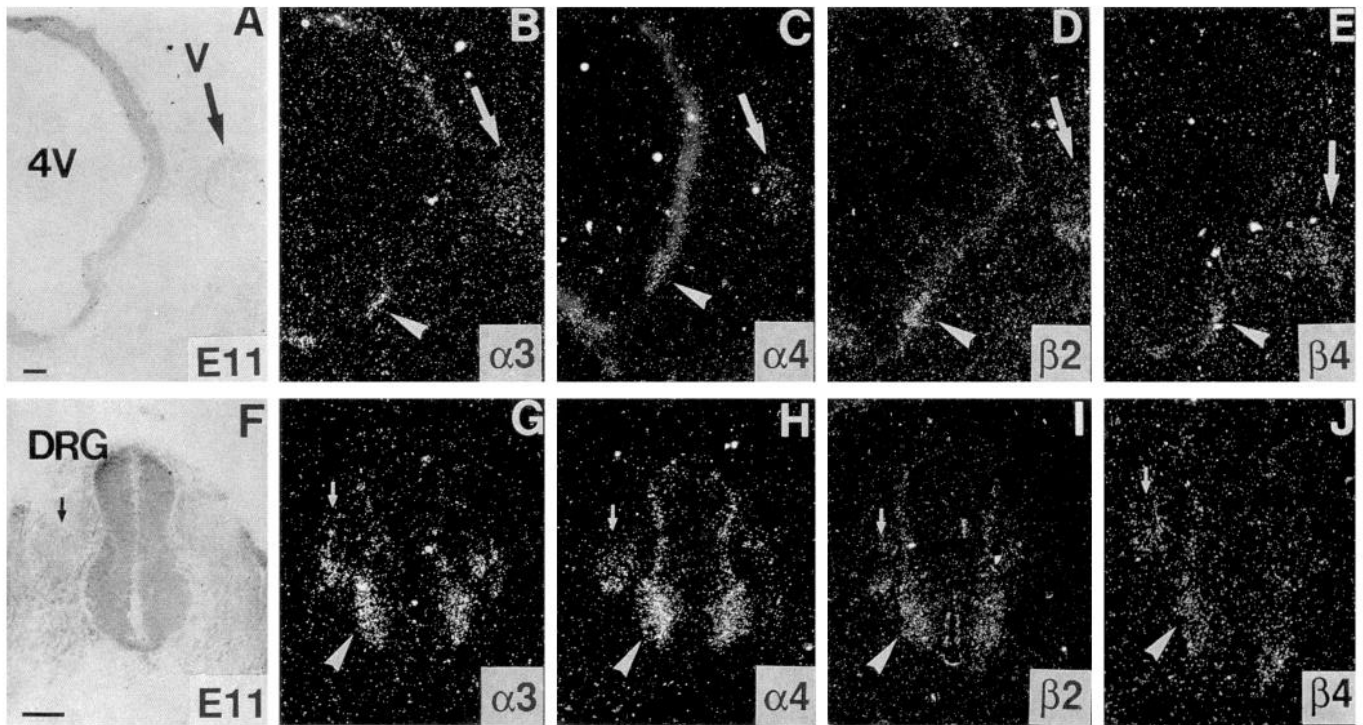
Figure 3. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs at rhombencephalic (*A–E*) and spinal cord (*F–J*) levels of a E11 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* and *F* show toluidine blue staining. In *A–E*, the *arrow* points to the primordium of the trigeminal ganglion (*V*), and the *arrowhead* to clusters of specific grains overlain the external side of the ventral rhombencephalic neuroepithelium. In *F–J*, the *arrow* points to the primordium of the dorsal root ganglion (*DRG*), and the arrowhead to the specific grains located in the ventrolateral part the spinal cord. This is the first area in the spinal cord containing differentiated neurons. *4V*, fourth ventricle. Scale bars, 100 μm.

consulted. In order to confirm the identification of some central and peripheral structures (e.g., cranial nerve motor nuclei, autonomic motor ganglia) *in situ* hybridization for choline acetyltransferase (AChE) was performed on some sections.

A score from 1+ (low intensity) to 3+ (high intensity) was assigned to the labeling of the anatomical structures based on the subjective evaluation of two experimenters. Background labeling was considered the density of grains in non-neural tissues with high cellularity (such as the liver and the muscles) or with high density of extracellular matrix (such as cartilage) or the density of labeling over neural structures after displacement with 20× cold probe. In the absence of grain counting at the cellular level, the scores must be regarded with caution. For instance, decreases in labeling intensity of a developing structure may be due to dispersion of positive cells in the structure caused by multiplication of negative cells or formation of neuronal processes. Though the oligonucleotides had the same length and they were labeled according to the same protocol, no attempt to compare the signal intensity of different transcripts was made. Unless specified otherwise, the labeling shown in the pictures has been obtained by using oligonucleotides no. 31 (α3), 47 (α4), 51 (β2), and 62 (β4) (see Table 1 for oligonucleotide characteristics).

Specificity controls. For each mRNA, two to four oligonucleotides were selected in unique parts of the sequence (e.g., the putative cytoplasmic loop between M3 and M4 for nAChR subunits). An initial assessment of specificity was performed by searching for possible homology with other known sequences in GenBank/EMBL. As histolog-
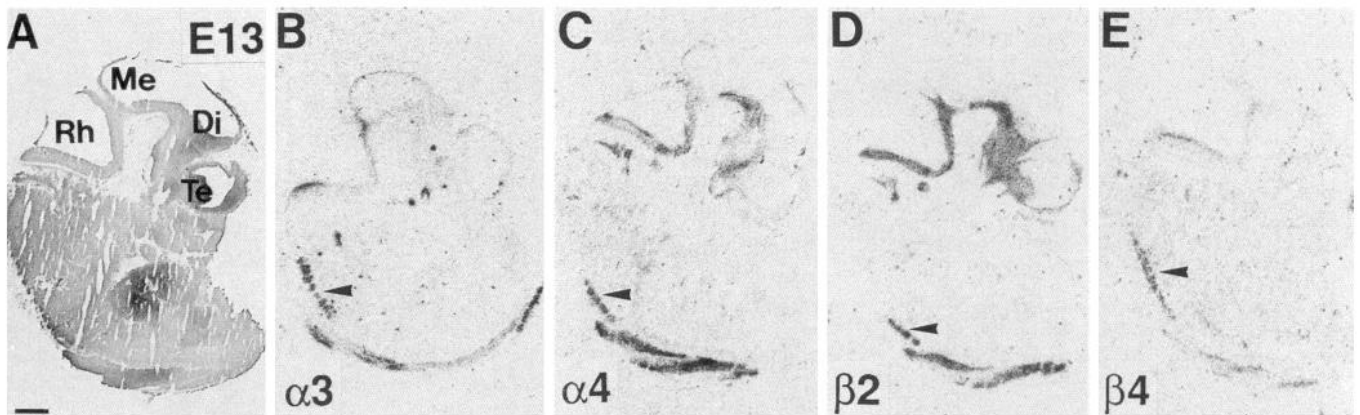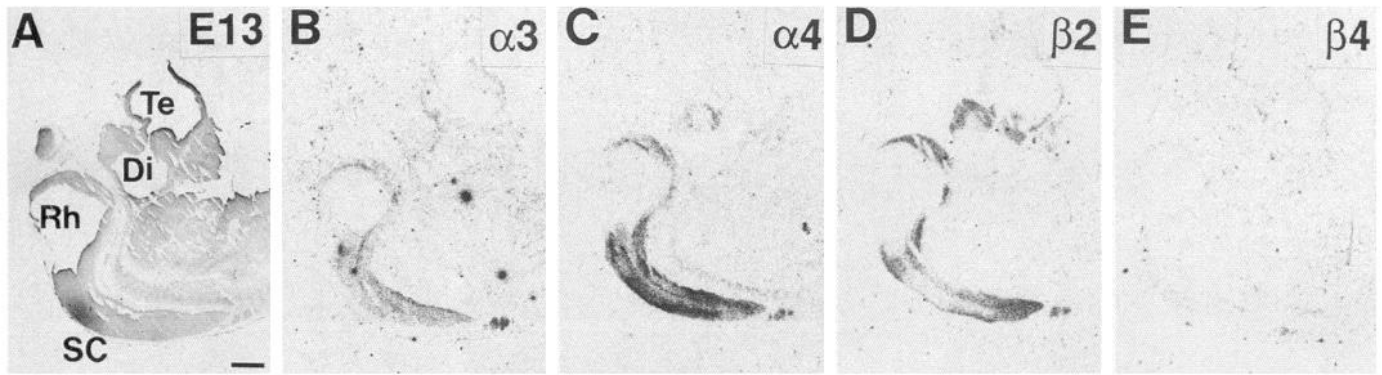

Figure 4. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E13 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. *Arrowheads* point to dorsal root ganglia. Abbreviations: *Di*, diencephalon; *Me*, mesencephalon; *Rh*, rhombencephalon; *Te*, telencephalon. Scale bar, 1 mm.

Figure 5. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E13 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. Abbreviations: *Di*, diencephalon; *Rh*, rhombencephalon; *SC*, spinal cord; *Te*, telencephalon. Scale bar, 1 mm.

ical tests for specificity we considered the following. (1) Two or more oligonucleotide probes for each mRNA gave the same hybridization pattern (Fig. 1). (2) The pattern of labeling in central structures in the adult rat was in agreement with that observed by other authors (Wada et al., 1989; Dineley-Miller and Patrick, 1992). (3) Given that most oligonucleotides used were 45-mers with similar GC content (Table 1), each oligonucleotide probe constituted a control for the specificity of the others. (4) The addition to the hybridization mixture of a 20-fold

excess of cold probe produced a complete disappearance of the labeling (Fig. 2).

The oligonucleotide probes used fulfilled all these criteria, with the exception of the four probes against α3 mRNA, which did not satisfy criterion 2. Previous studies based on cRNA probes showed a relatively widespread distribution of this subunit mRNA in adult rats, notably high levels in the cerebral cortex layer IV, entorhinal cortex layer II, anterior and ventral thalamic nuclei, medial and lateral geniculate nuclei, medial
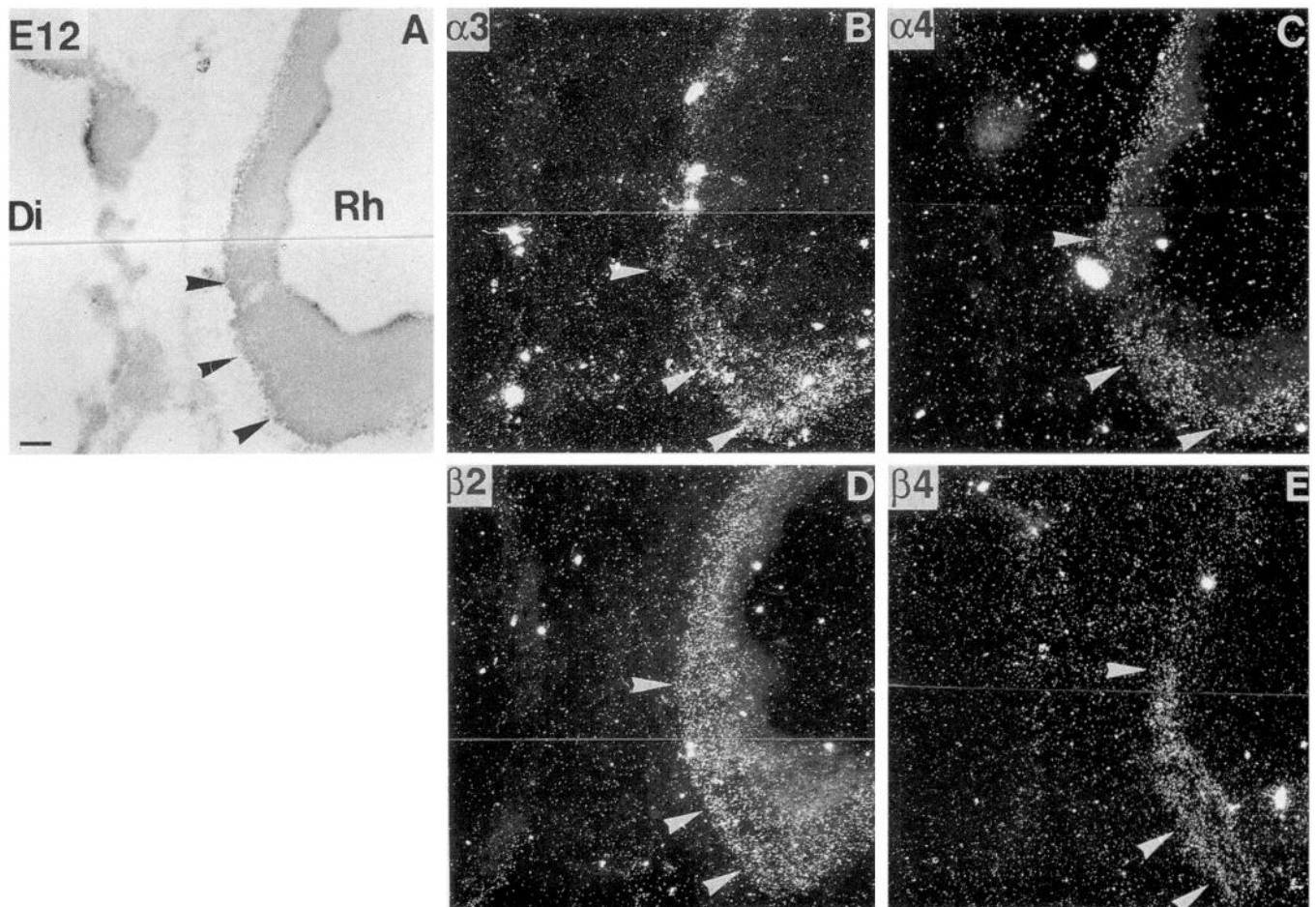
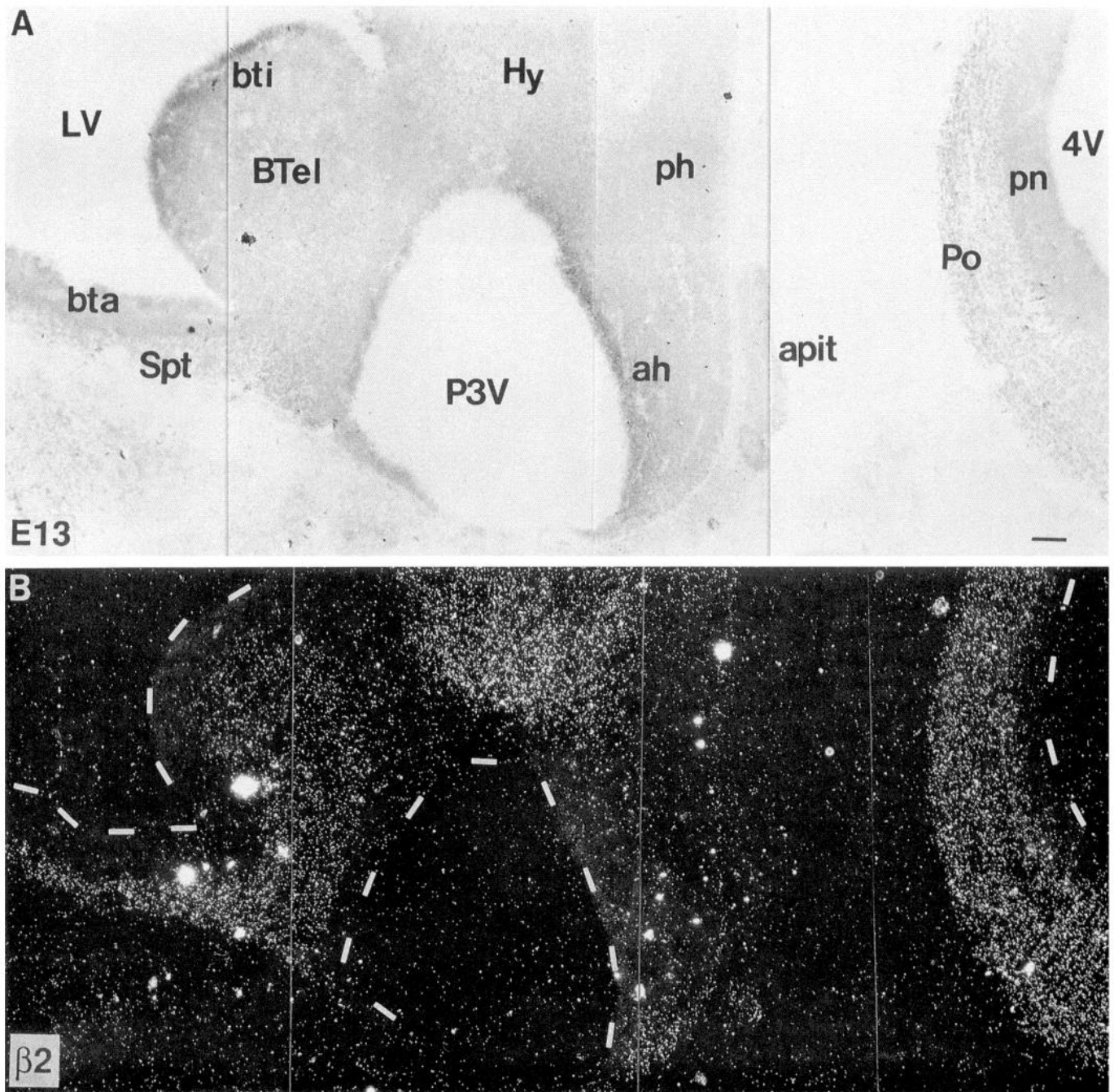*Figure 6.* Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in sagittal sections of the rostral rhomben-cephalon (*Rh*) and caudal diencephalon (*Di*) of a E12 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. *Arrowheads* point to the external side of the lateral metencephalon, indicating a similar distribution of the four transcripts in this region. Scale bar, 100 µm.
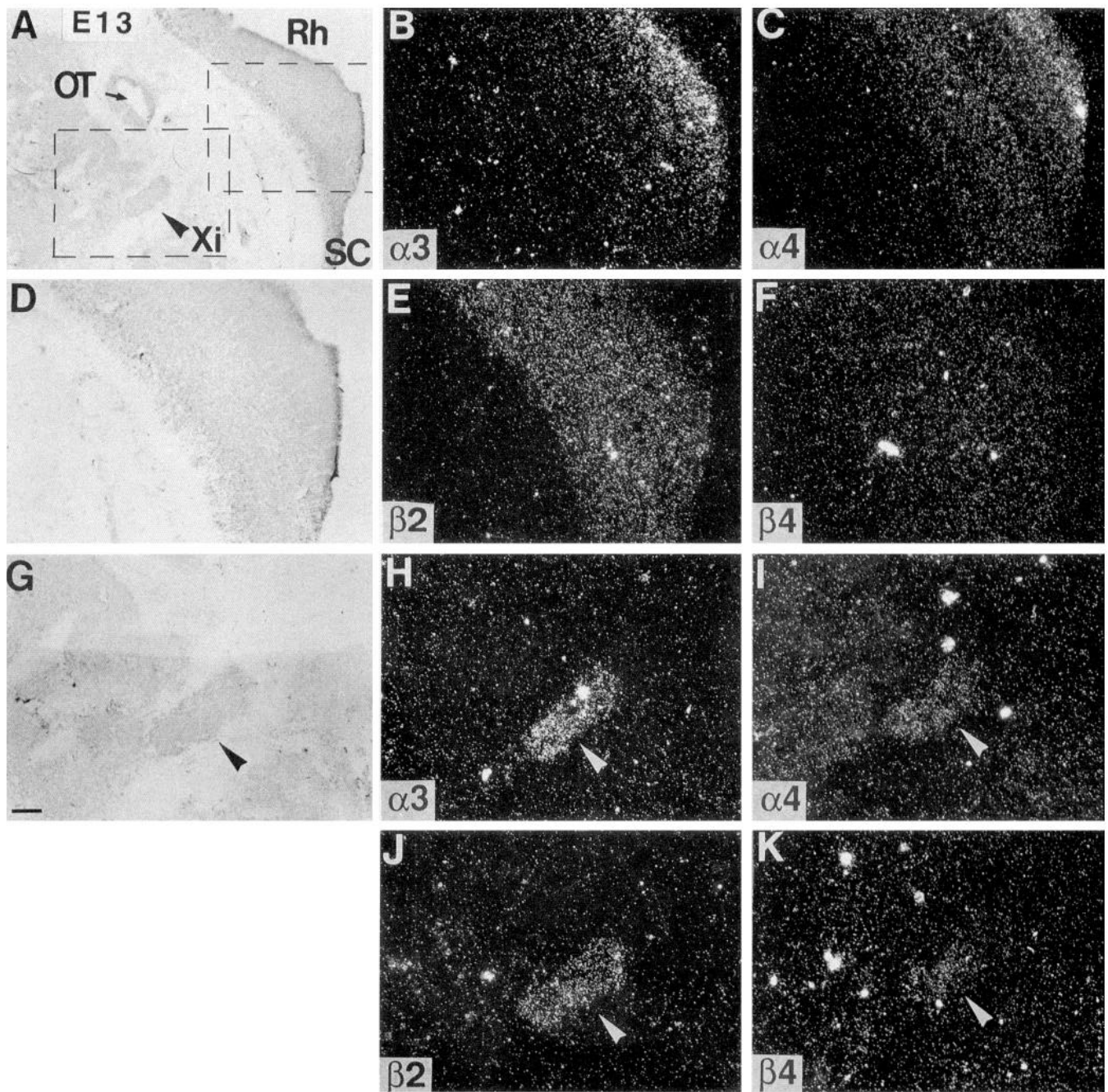
Figure 7. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in sagittal sections of the caudal rhombencephalon (*Rh, B–F*) and inferior X ganglion (*Xi, G–K*) of a E13 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A, D,* and *G* show toluidine blue staining. In *A* the *rectangular frames* delineate the areas which are magnified in the other panels. In *A* and *G–K*, the *arrowhead* points to the Xi. Abbreviations: *OT*, otic vesicle; *SC*, spinal cord. Scale bar, 100 μm.

habenula, posterior hypothalamus and supramammillary nuclei, pineal gland, motor nuclei of the V and VII nerves, locus coeruleus, nucleus ambiguus, and area postrema (Wada et al., 1989). At variance with these observations, in adult rats we could detect high levels of α3 mRNA signal only in the medial habenula, intermediate in the pineal gland, area postrema, motor nucleus of the V nerve and cerebellum, low in a few thalamic nuclei and locus coeruleus. Part of the discrepancy may be ascribed to a lower sensitivity of oligonucleotide probes versus riboprobes. However, considering the difficulty of carrying out specificity controls for cRNA probes, especially when hydrolysis of the probe is performed in the histological procedure (Wada et al., 1989), it is possible that some labeling previously attributed to α3 mRNA actually derives from hybridization to other (nAChR?) RNA sequences.

Results

No specific labeling for any of the mRNAs studied was detected in E10 embryos by *in situ* hybridization. Starting with E11, labeling for all nAChR subunit mRNAs studied was noticed in several central and peripheral nervous structures of the embryos (Tables 2–6, Figs. 3–24).

Central nervous system

Overall patterns for each individual subunit. α3 subunit mRNA exhibits early and transient expression in both brain and spinal

Figure 8. Dark-field microphotograph (*B*) of emulsion autoradiograms showing β2 nAChR subunit mRNA in sagittal sections of the basal telencephalon, diencephalon and pons of a E13 rat embryo. *Dashed lines* outline the borders of the ventricles. Abbreviations: *ah*, anterior hypothalamic neuroepithelium; *apit*, anterior pituitary; *bta*, basal telencephalic neuroepithelium, anterior part; *BTel*, basal telencephalic nuclei; *bti*, basal telencephalic neuroepithelium, intermediate part; *Hy*, hypothalamic nuclei; *LV*, lateral ventricle; *P3V*, preoptic recess of the third ventricle; *ph*, posterior hypothalamic neuroepithelium; *pn*, pontine neuroepithelium; *Po*, pontine nuclei; *Spt*, septal nuclei; *4V*, fourth ventricle. Scale bar, 100 μm.

cord. It is first detected at E11 in caudal CNS regions and, subsequently, in rostral CNS, reaching moderate to high levels at E12–13. Transcript levels markedly decrease at E15–17 to become undetectable at later ages in nearly all CNS areas. Yet, α3 subunit mRNA persists at a high level in the medial habenula up to perinatal (and adult) ages; in addition, low to moderate labeling is present at perinatal ages in the cerebellar primordium and in the ventral cochlear nucleus.

Initial expression of α4 subunit mRNA proceeds from caudal

to rostral regions of the CNS. It first appears in the spinal cord and rhombencephalon (E11), then in the mesencephalon (E11–12), ventral diencephalon and basal telencephalon (E12–13), dorsal diencephalon (E15) and finally in the cerebral cortex (E17–19). In subsequent prenatal and perinatal development, α4 mRNA is heterogeneously, but rather stably, expressed in many CNS nuclei.

β2 subunit mRNA exhibits early, widespread and stable expression in the CNS. Starting from E11–12 it appears in nearly
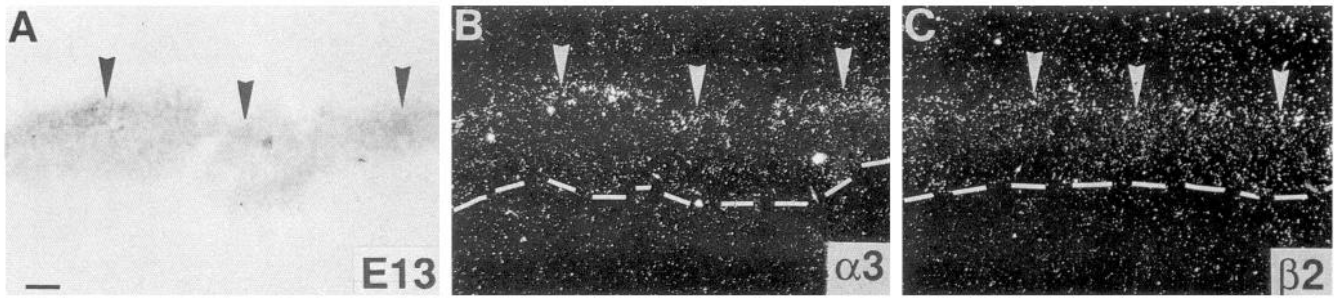
Figure 9. Dark-field microphotographs of emulsion autoradiograms showing α3 (*B*) and β2 (*C*) nAChR subunit mRNAs in sagittal sections of the cerebral cortex of a E13 rat embryo. *Arrows* point to the external side (preplate) of the developing cerebral cortex. *Dashed lines* outline the internal border of the cerebral cortex. Scale bar, 100 μm.

all CNS regions where postmitotic cells are present and in a relatively homogeneous manner. The intensity of labeling is highest between E13 and E15 and thereafter slightly declines.

β4 subunit mRNA is initially expressed in the spinal cord and caudal parts of the brain similarly to the other subunits. However, this expression is transient; β4 transcript becomes undetectable after E13 in spinal cord and brain, with the exception of medial habenula, pineal gland, and a few other areas (corresponding to those expressing low to moderate levels of α3 transcript, see above).

Brain. At E11, we detected all four nAChR subunit mRNAs in the rhombencephalon and α3, α4, and β2 mRNAs in the mesencephalon (see Table 2, Fig. 3). Patches of specific labeling for all subunit mRNAs were observed on the external side of

the neural tube. Yet, α3, α4, and β2 transcripts were present both ventrally and dorsally, whereas β4 labeling was restricted to the ventral part of the brainstem. No specific labeling was detected at diencephalic and telencephalic levels.

From E12 to E13, α3, α4, and β2 mRNA levels strikingly increased in the brain. On the other hand, β4 mRNA levels peaked at E12 and were already reduced at E13 (Table 2, Figs. 4–9). In addition, the distribution of β4 mRNA appeared more restricted than that of the other transcripts. Overall, β2 mRNA was detected in most brain areas where differentiated cells were present (Fig. 8).

For instance, at E12, and more clearly at E13, β2 was detected in the ventral diencephalon, where many postmitotic cells are present, but not in the dorsal diencephalon where postmitotic
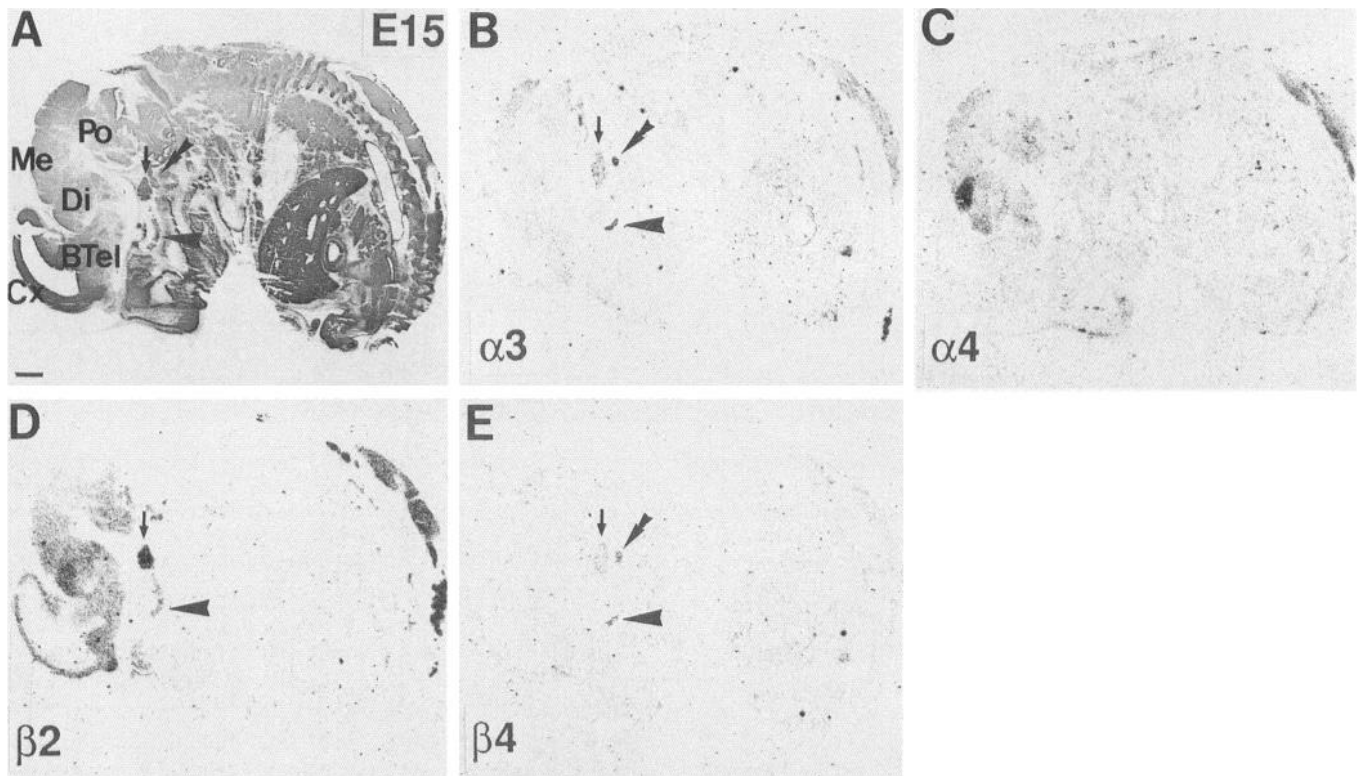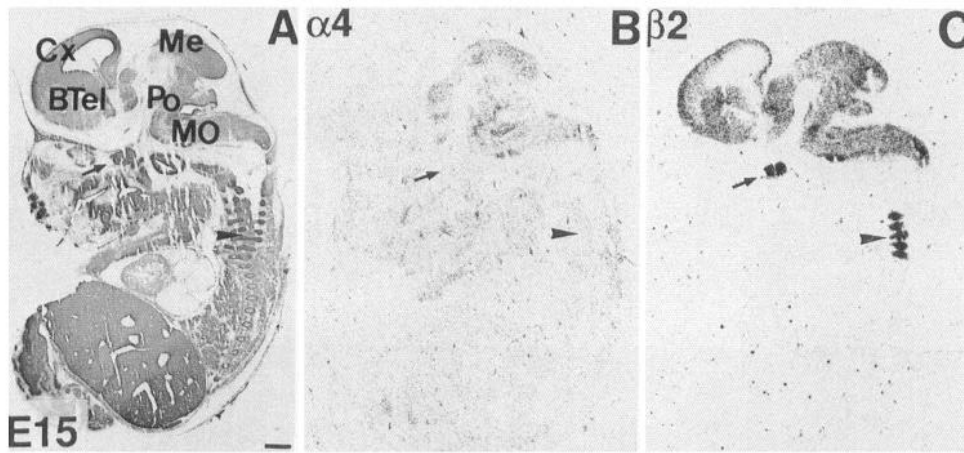

Figure 10. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E15 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. *Arrows, arrowheads,* and *double arrowheads* point to the trigeminal ganglion, the sphenopalatine ganglion and the otic ganglion, respectively. Abbreviations: *BTel,* basal telencephalon; *Cx,* cerebral cortex; *Di,* diencephalon; *Me,* mesencephalon; *Po,* pons. Scale bar, 1 mm.

Figure 11. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E15 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. As no labeling for α3 and β4 mRNAs was visible at this anatomical level, the respective panels have been omitted. *Arrows* point to the trigeminal ganglion and *arrowheads* to the dorsal root ganglia. Abbreviations: *BTel*, Basal telencephalon; *Cx*, cerebral cortex; *Me*, mesencephalon; *MO*, medulla oblongata; *Po*, pons. Scale bar, 1 mm.

cells are rare (Altman and Bayer, 1978, 1979a,b). α3 and α4 mRNAs showed a more discrete distribution, which partially overlapped that of β2 mRNA.

In the rhombencephalon labeling for nAChR subunits was mainly in the ventral and in the dorsocaudal parts, whereas the roof of the IV ventricle was devoid of labeling for any mRNA. Throughout the brainstem, the labeling had a rather patchy appearance. When a positive patch was identified in several consecutive sections, the labeling for the different mRNAs was found to coincide (see, e.g., Fig. 6). This suggests that developing clusters of cells may coexpress several nAChR subunit mRNAs.

In the ventral diencephalon different intensities of labeling (weak for β4, moderate for α3 and α4, and intense for β2) for all transcripts were noticed at E12 and more clearly at E13. At E13, low to intermediate labeling for α3 and β2, but not α4, was observed in the external part of the embryonic cerebral cortex, probably corresponding to the first cortical postmitotic neurons (preplate) (Fig. 9), and in the hippocampal formation. Specific labeling for α3, α4, and β2 mRNAs was also present in already differentiated basal telencephalic areas (Fig. 8).

At E15, the general pattern of labeling of α4 and β2 mRNAs diverged from that of α3 and β4 (Table 2, Figs. 10–13). α4 and β2 subunit mRNAs continued to be detected in a diffuse pattern whereas labeling for α3 mRNA markedly declined, and that for β4 disappeared from the vast majority of brain areas labeled at earlier developmental days. Moderate to high α3 and β4 mRNA levels were restricted to the medial habenula. Although exhibiting relatively similar patterns of regional distribution, α4 and β2 mRNA labeling displayed some distinctive features. β2 labeling was very high and homogeneous throughout the brain whereas α4 labeling appeared more discrete. In addition, β2 was clearly expressed in the cortical plate and less intensely in the other histogenetic layers of the cerebral cortex, whereas α4 was not detected in these regions (Fig. 12).

At embryonic ages E17 and E19 and in the first postnatal day, the distribution patterns delineated at E15 were substantially maintained (Table 2, Figs. 14–17). β2 remained diffusely distributed throughout the brain. α4 displayed a relatively more heterogeneous distribution, being absent in the basal ganglia and olfactory bulb and highly concentrated in the ventromedial hypothalamic nucleus, several thalamic nuclei and a few brainstem nuclei (Table 2). α3 and β4 were highly expressed in the medial habenula (Figs. 14, 16). In addition, the ventral cochlear nucleus and some portions of the external germinal layer and neuroepithelium of the cerebellum contained low to moderate α3 and β4 transcript levels.

In the cerebral cortex, high levels of β2 mRNA were present in the cortical plate at E17 (Figs. 14, 15), whereas the transient histogenetic cell layers below the cortical plate displayed lower levels of the transcript. This pattern of labeling persisted in the following days. α4 first became detectable in the cortical plate at E19 (Fig. 17). Weak to moderate labeling was, however, observed in the subplate starting from E17. No specific labeling for α3 and β4 was noticed in any layer of the developing cerebral cortex in the late prenatal period.
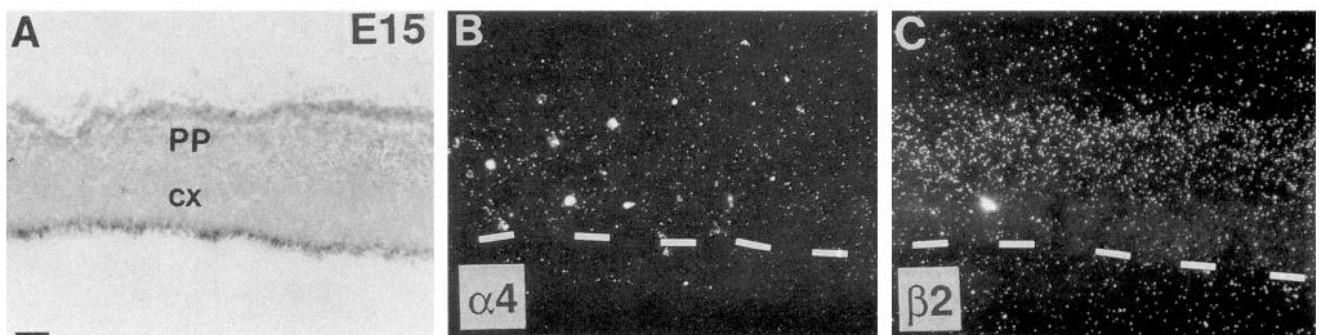

Figure 12. Dark-field microphotographs of emulsion autoradiograms showing α4 (*B*) and β2 (*C*) nAChR subunit mRNAs in sagittal sections of the cerebral cortex of a E15 rat embryo. *Dashed lines* outline the internal border of the cerebral cortex. Abbreviations: *cx*, cortical neuroepithelium; *PP*, cortical preplate. Scale bar, 100 μm.
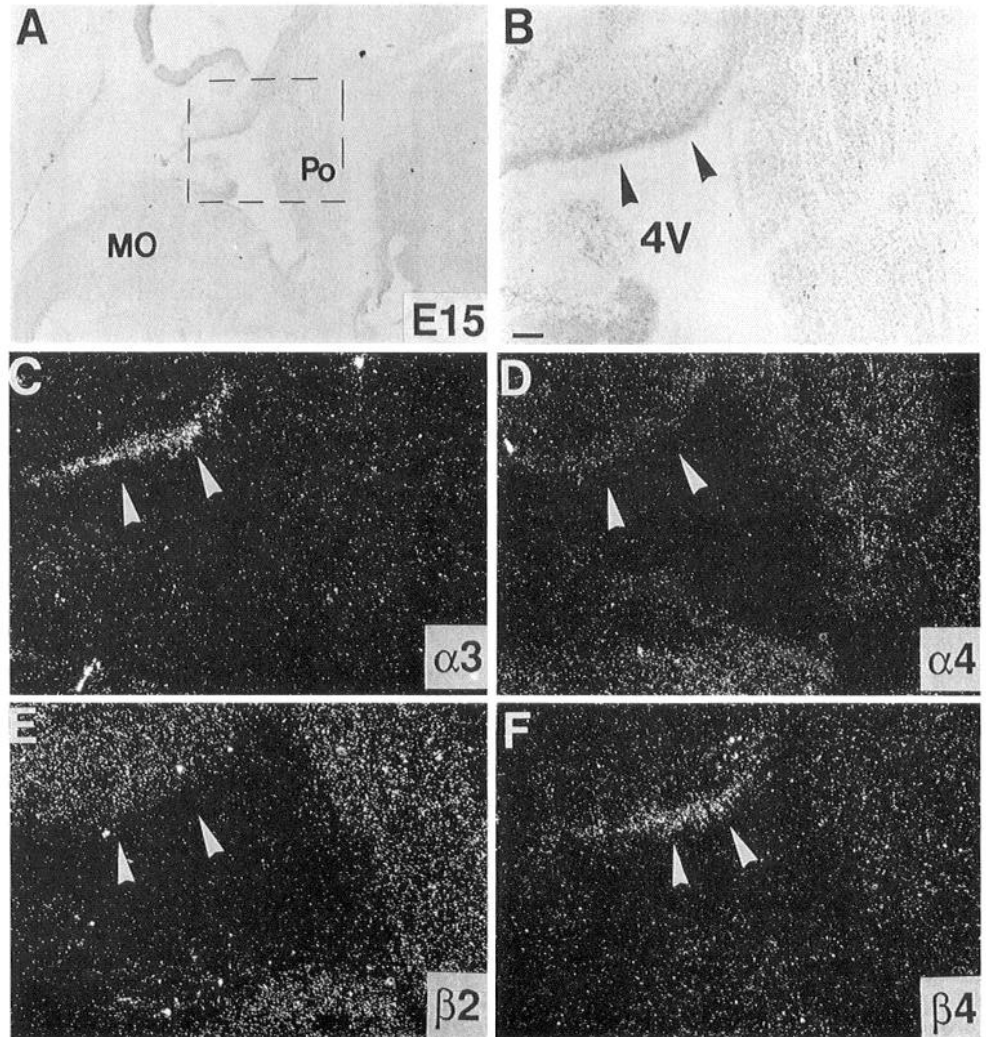
Figure 13. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in sagittal sections of the roof of the IV ventricle (*4V*) of a E15 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* and *B* show toluidine blue staining. In *A* the *rectangular frame* delineates the area which is magnified in the other panels. The *arrowheads* point to the neuroepithelium of the cerebellar primordium. Abbreviations: *MO*, medulla oblongata; *Po*, pons. Scale bar, 100 μm.

Spinal cord. Overall, appearance of nAChR subunit transcripts in the spinal cord was extremely precocious and transient (Table 3). From E11 to E13 (Figs. 3, 5), moderate to very high levels of α3, α4, β2, and β4 mRNAs were present in the spinal cord. At E11, specific labeling for all subunit mRNAs was observed in the ventrolateral part of the spinal cord (Fig. 3), where the first differentiated neurons occur (Kaufman, 1992). A thin layer of cells positive for α3, α4, and β2 was also detected on the lateral side of the dorsal spinal cord. At later embryonic stages (E15–E17), labeling for α3 and α4 markedly and slightly decreased, respectively, whereas β2 mRNA labeling remained high and β4 mRNA became undetectable (see Figs. 10, 14). At P4, no labeling for α3 and β4 mRNAs was present in the spinal cord, whereas α4 and β2 were still weakly and diffusely expressed in both ventral and dorsal horns.

Pineal gland. In this structure of epithalamic origin, β4 was the only subunit mRNA detected at prenatal ages (from E17) (Table 2, Fig. 14). At P0 and P4, α3 and β2 subunit mRNAs were also observed, at high and low levels, respectively. The two epithalamic derivatives, the habenula and the pineal gland, are the only brain structures which express high levels of α3 and β4 mRNAs in the perinatal period. This pattern resembles that of autonomic motor and sensory ganglia (see below). Interestingly, both neurons of the medial habenula and pinealocytes of the pineal gland are morphologically (Gottesfeld, 1983; Bow-

ers et al., 1984) and functionally (at least the pineal gland, Reiter, 1989) linked to the sympathetic system.

Retina

Labeling for all nAChR subunit mRNAs studied was present in the optic stalk in E12–13 embryos (Table 4). At E13, moderate to high labeling for α3 and β4 mRNAs was present in the retina, whereas α4 and β2 mRNAs were not detected. Starting from E15 high labeling for the four mRNAs was concentrated on the vitreal side of the retinal epithelium (Fig. 19). From this age up to P4, in agreement with Hoover and Goldman (1992) data, the four nAChR subunit mRNAs were expressed in the neural retina, though at progressively lower levels. Starting from E15 and up to P0, specific labeling for α4 mRNA of moderate and then weak intensity was detected in the optic nerve (Fig. 20). Specific grains appeared concentrated over cell bodies (Fig. 21) likely representing glial cells (Colello and Guillery, 1992).

Dorsal root ganglia

At E11, the first ganglia appearing at the mid-trunk level, showed high labeling for α3, and moderate labeling for α4, β2 and β4 nAChR subunit mRNAs (Table 5, Figs. 4, 10, 11, 15). At E12 and E13 high labeling for α3, α4, and β2 and moderate for β4 nAChR subunit mRNAs was detected (Fig. 4). After this stage and up to P4, α3 labeling decreased to moderate levels,
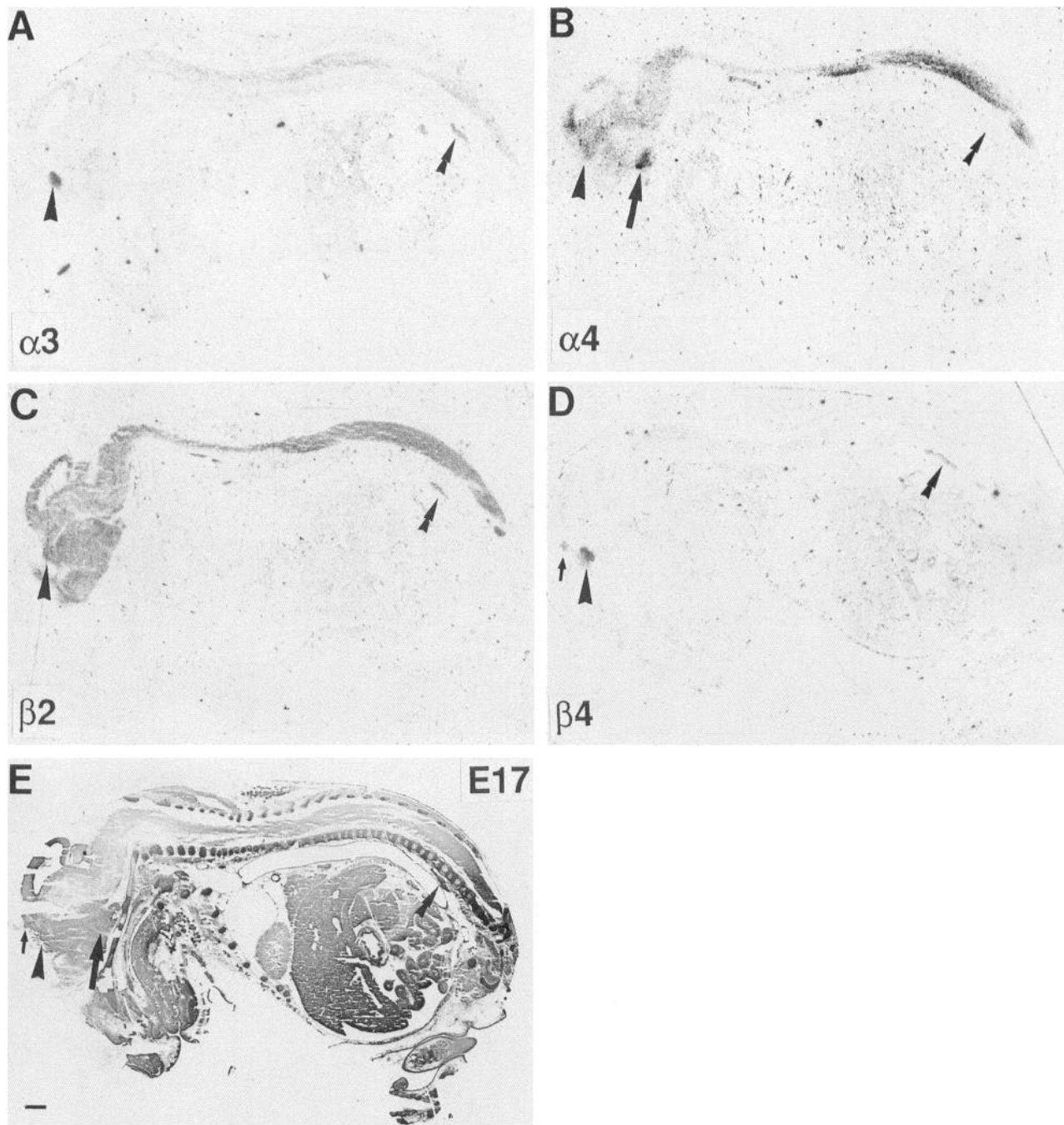
Figure 14. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E17 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *E* shows toluidine blue staining. *Big* and *small arrows, arrowheads,* and *double arrowheads* point to the ventromedial hypothalamic nucleus, the epiphysis, the medial habenula, and lumbar sympathetic ganglia, respectively. Scale bar, 1 mm.

whereas β2 labeling remained stably elevated. On the other hand, α4 and β4 subunit mRNAs became undetectable starting from E15 and E17, respectively.

Cranial nerve and sympathetic ganglia

We observed a wide spectrum of transcript distribution in cranial nerve and sympathetic ganglia (Table 6). As in other neural structures, expression of nAChR subunit mRNAs was precocious and, in some cases, transient.

V nerve. The trigeminal ganglion, a somatic sensory structure,

also containing some visceral sensory neurons projecting to the buccal mucosa, is associated with the V nerve. It exhibited specific labeling for all nAChR mRNAs studied starting from E11 (Fig. 3). α4 and β4 mRNAs were transiently detected peaking at E12–13 and decreasing thereafter to become undetectable at E15–17 (Figs. 10, 11, 16, 22). β2 mRNA labeling reached high intensity at E13 and remained elevated at all subsequent embryonic and postnatal ages investigated (Figs. 10, 11, 16, 22). α3 mRNA exhibited two peaks of expression. High levels were detected at E12–13 followed by a marked decrease to very low
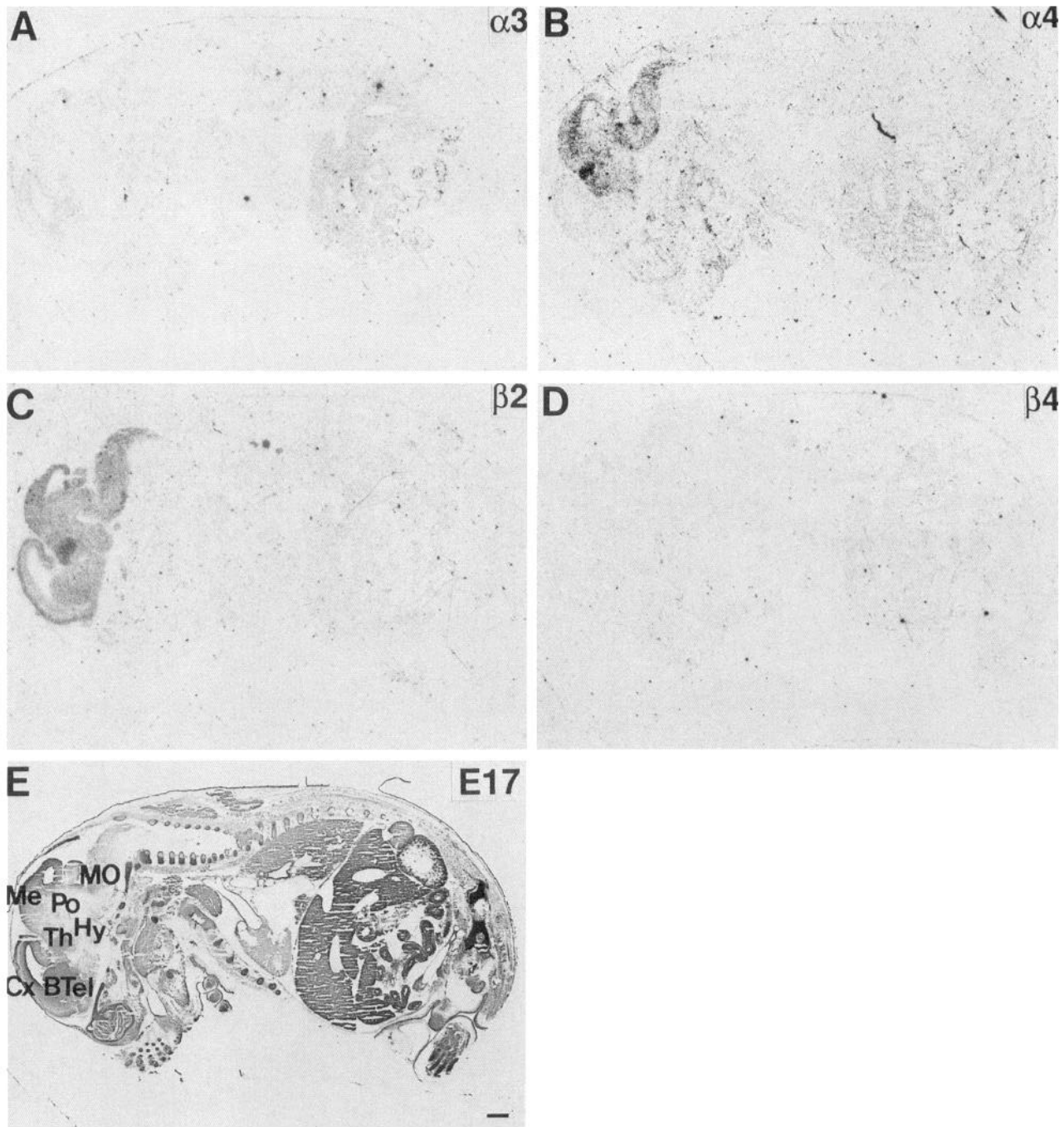
Figure 15. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in sagittal sections of a E17 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *E* shows toluidine blue staining. Abbreviations: *BTel*, basal telencephalon; *Cx*, cerebral cortex; *Hy*, hypothalamus; *Me*, mesencephalon; *MO*, medulla oblongata; *Po*, pons; *Th*, thalamus. Scale bar, 1 mm.

levels in the following prenatal days (Figs. 10, 16, 22) and first postnatal day. At P4, low to moderate levels of α3 mRNA were again visible in the trigeminal ganglion.

VII nerve. Two ganglia associated with the VII nerve, that is, the geniculate ganglion (gustatory sensory) and the sphenopalatine ganglion (parasympathetic motor) were investigated. At E11–12 the anlage of the VII ganglion could not be distinguished from that of the VIII ganglia. At these ages all four nAChR subunit mRNAs were expressed in the VII/VIII complex. Starting from E13, when a distinct geniculate ganglion

segregated, only the β2 mRNA was detectable (Fig. 22). The sphenopalatine ganglion was clearly recognizable starting from E15. At this age, high labeling for α3, β2, and β4 was found. At all subsequent prenatal and postnatal ages up to P4 (Figs. 10, 23) high levels of α3 and β4 mRNAs (as well as somewhat lower levels for β2) were observed.

VIII nerve. The ganglia associated with the VIII cranial nerve, that is, the cochlear and vestibular ganglia, expressed moderate to high levels of α4 and β2 subunit mRNAs from E13 through P0 (Fig. 22). At E11–12 the aggregate of cells corresponding to
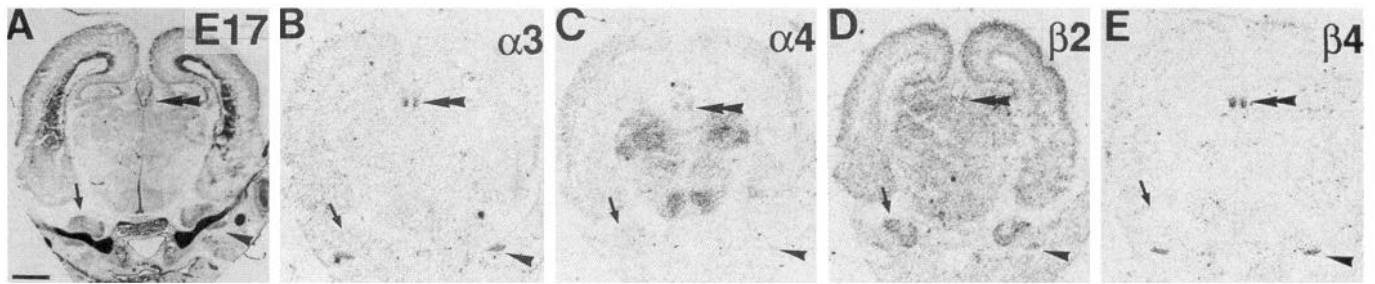
Figure 16. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in coronal sections at midthalamic level of a E17 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. *Arrows* point to the trigeminal ganglion, *arrowheads*, to the otic ganglion and *double arrowheads* to the medial habenula. Scale bar, 1 mm.

the VII and VIII ganglia was positive for all the nAChR subunit mRNAs studied.

IX and X nerves. In adult animals, three ganglia are associated with the IX cranial nerve, that is, the superior (somatic sensory), inferior (visceral sensory), and otic (parasympathetic motor) ganglia. A ganglion is associated with the X nerve, the inferior or nodose (visceral sensory) ganglion. At E11, it was not possible to distinguish the various ganglia associated with the IX and X nerves. At this age low (β2) to high (α3) levels of labeling for the four nAChR subunit mRNAs were present in the primordia of these ganglia. At later ages a different pattern of labeling was observed in the superior (somatic sensory) and two inferior (visceral sensory) ganglia. In IX superior ganglion, the initial appearance of all nAChR subunit mRNAs was followed by the exclusive expression of β2 mRNA (starting from E17) (Fig. 24). On the other hand, with the exception of α4 mRNA labeling which disappeared at E15, the other mRNAs persisted at moderate to high levels up to P4 in the inferior (visceral sensory) IX and X ganglia (Fig. 24). The otic ganglion could

clearly be identified starting from E15. High labeling for α3 and β4 mRNAs, and moderate to low for β2 mRNA, were present at all ages studied (Figs. 16, 22).

Sympathetic system. In every instance in which either cervical or thoracolumbar sympathetic ganglia were identified, at any age, the same pattern of labeling, viz strong labeling for α3 mRNA and moderate to strong labeling for β2 and β4 mRNAs (Figs. 14, 24), was observed.

Overall, during prenatal and perinatal development the patterns of nAChR subunit expression seem specific for the different categories of ganglia (Table 7). In the somatic and gustatory sensory ganglia (trigeminal, geniculate, and superior glossopharyngeal ganglia) the initial expression of all nAChR subunits studied is followed by a restriction to only the β2 transcript. Some expression of α3 mRNA is still detectable in the trigeminal ganglion which also contains some visceral sensory neurons projecting to the buccal mucosa. Interestingly, the same pattern of expression was detected in the dorsal root ganglia (see above) which also contain some visceral sensory neurons. Autonomic
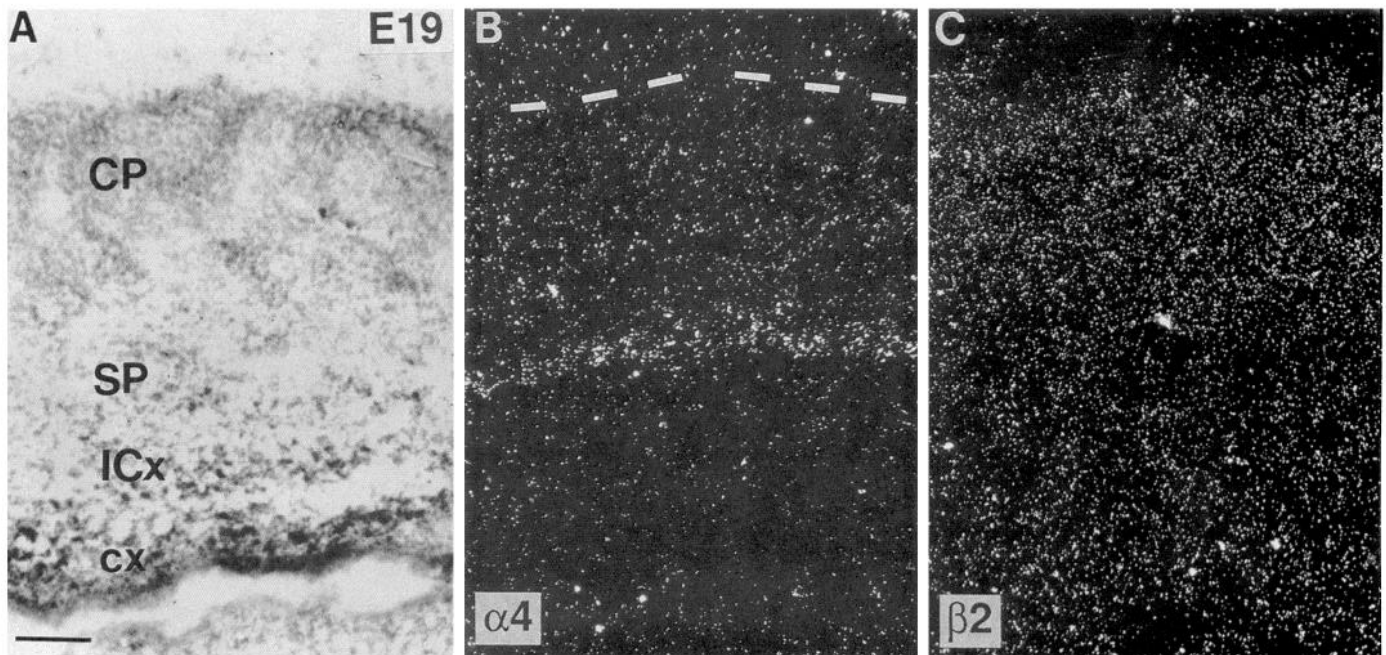

Figure 17. Dark-field microphotographs of emulsion autoradiograms showing α4 (*B*) and β2 (*C*) nAChR subunit mRNAs in coronal sections of the cerebral cortex of a E19 rat embryo. *Dashed lines* outline the external border of the cerebral cortex. Abbreviations: *CP*, cortical plate; *cx*, cortical neuroepithelium; *ICx*, intermediate cortical layer; *SP*, cortical subplate. Scale bar, 100 μm.

Table 3. Development of nAChR subunit mRNAs in the spinal cord

		α3	α4	β2	β4
E11	vent	+++	+++	++	++
	dors	+	++	+	−
E12	vent	+++	+++	+++	++
	dors	+	+++	+	+
E13	vent	+++	+++	+++	++
	dors	+++	+++	++	+
E15	vent	++	++	+++	+
	dors	+	+	+++	−
E17	vent	+	++	+++	−
	dors	+	+	+++	−
P4	vent	−	+	+	−
	dors	−	+	+	−

Scores were attributed as follows: +, low level; ++, intermediate level; +++, high level. Abbreviations: dors, dorsal; vent, ventral.

ganglia, both sensory and motor (sympathetic, sphenopalatine, otic, inferior glossopharyngeal, and vagal ganglia) express α3, β4, and β2 mRNAs. Finally, acoustic and vestibular ganglia only express α4 and β2 mRNAs.

Summing up of regional patterns of nAChR subunit mRNA distribution during development

Although each PNS structure and CNS region studied displayed particular developmental changes in the expression of nAChR subunit mRNAs (see Tables 2–6), three main developmental patterns were recognized:

Pattern A. All four subunit mRNAs are expressed early in development, but, subsequently, during prenatal development, the expression of some of these subunits is repressed.

Pattern B. α3 and β2 subunit mRNAs are detected early in

Table 4. Development of nAChR subunit mRNAs in the retina and optic nerve

	α3	α4	β2	β4
E12				
Optic stalk	++	++	++	+
E13				
Optic stalk	++	+++	+++	+
Retina	+++	−	−	++
E15				
Optic nerve	−	++	−	−
Retina	+++	+++	+++	+++
E17				
Optic nerve	−	++	−	−
Retina	+++	+++	+++	++
E19				
Optic nerve	−	+	−	−
Retina	+++	+++	+++	+
P0				
Optic nerve	−	+	−	−
Retina	++	++	++	+
P4				
Retina	+	++	++	+

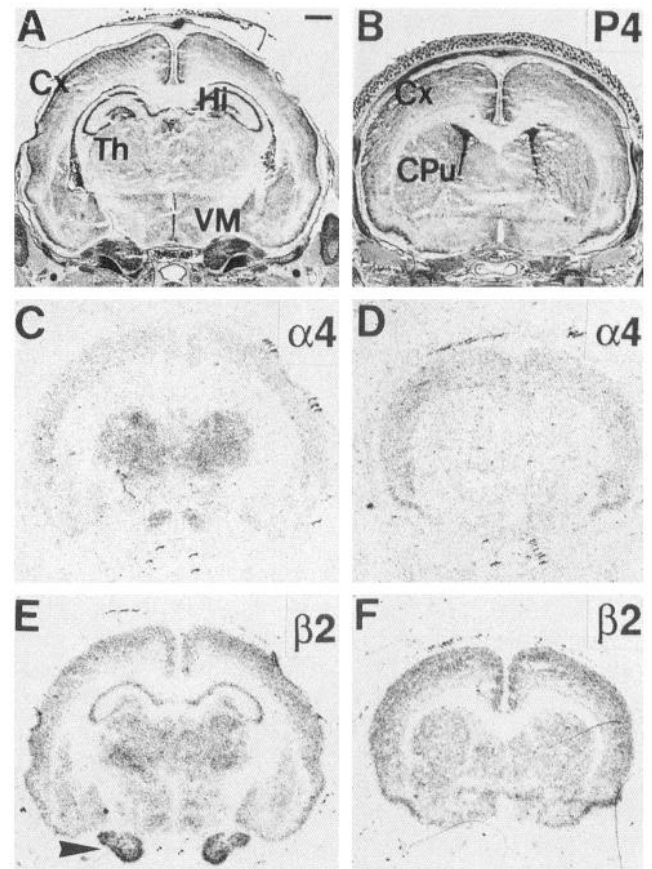Scores were attributed as follows: +, low level; ++, intermediate level; +++, high level.

Figure 18. Bright-field microphotographs of film autoradiograms showing nAChR subunit mRNAs in coronal sections at midthalamic (*A, C, E*) and striatal (*B, D, F*) levels of a P4 rat. The nAChR subunit mRNA labeled is indicated in each panel. *A* and *B* show toluidine blue staining. Note the high β2 mRNA labeling in the trigeminal ganglia (*arrowhead* in *E*). Abbreviations: *CPu*, caudate-putamen; *Cx*, cerebral cortex; *Hi*, hippocampus; *Th*, thalamus; *VM*, ventromedial hypothalamic nucleus. Scale bar, 1 mm.

development, but, subsequently, during prenatal development, the α3 subunit disappears followed by the appearance of the α4 subunit.

Pattern C. The subunit mRNAs which are initially expressed remain stably expressed throughout prenatal and perinatal postnatal development.

Pattern A can be recognized in the majority of neural structures analyzed (caudal brain, spinal cord, dorsal root ganglia, trigeminal, geniculate, glossopharyngeal, and vagal ganglia). In these structures the expression of some nAChR subunit mRNAs (α3 and β4 in the brain and spinal cord, α4 and β4 in the dorsal root ganglia, α4 in the visceral sensory ganglia, and α3, α4, and β4 in the somatic sensory ganglia) was precocious and transient. For instance, in the brain the initial (E11–13) widespread expression of α3 (and to a lesser extent β4) mRNA was followed by a repression to levels undetectable with the present technique. In the late prenatal period high levels of α3 and β4 transcripts were found only in the medial habenula starting at E15 when differentiated cells form in the nucleus (Altman and Bayer, 1979a). In the rostral part of the brain, with the exception of cerebral cortex, only certain subunits (β2, α4, and/or α3) were detected at E12–13. However, similar to what happens in the caudal brain, α3 expression was transient.
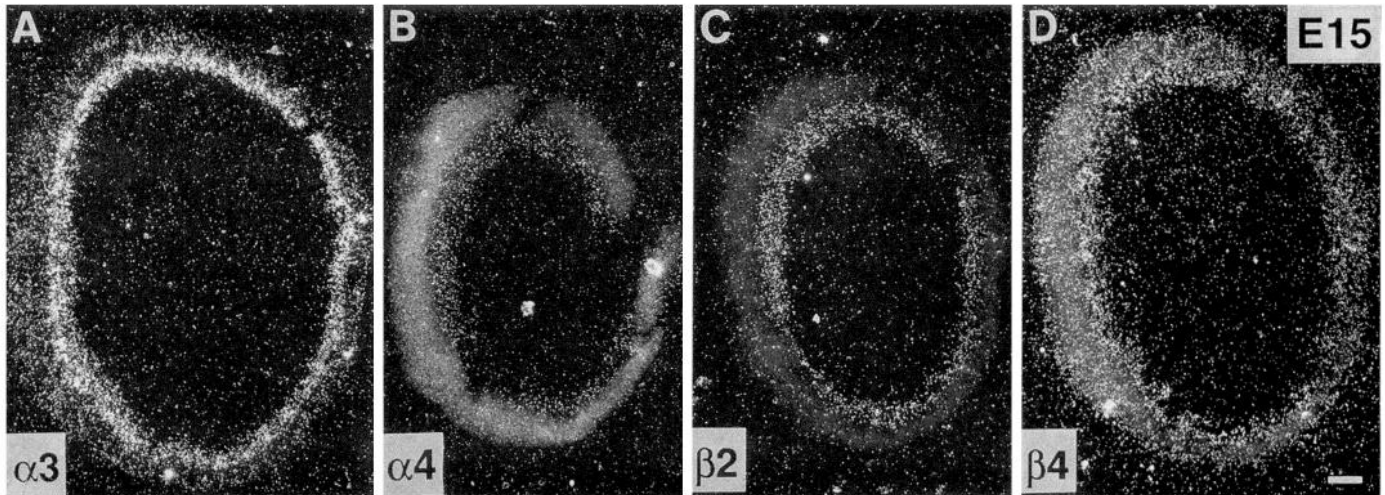
Figure 19. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in sagittal sections of the retina of an E15 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. The images are taken from sections cut from the same animal and processed in the same experiment, in which $\alpha4$ and $\beta2$ labeling was in absolute terms less strong than $\alpha3$ and $\beta4$ labeling. However, the intensity of $\alpha4$ and $\beta2$ labeling in the retina was high when compared to that of the other positive structures in the same experiment. Scale bar, 100 μm.

Pattern B is observed in the cerebral cortex. In this region nAChR mRNA development is peculiar in several respects. $\alpha3$ and $\beta2$ were first detected at E12–13 in the preplate. Subsequently, $\alpha3$ mRNA disappeared and $\beta2$ remained the only subunit expressed in the cortical plate (constituted by the neurons which will form the mature cerebral cortex) until E19 when $\alpha4$ started to be detected throughout the plate. $\beta2$ was also expressed in the histogenetic layers of the cortex (subplate, intermediate and subventricular zones) initially alone and then (E17) together with $\alpha4$ (subplate). As in the other structures analyzed, $\beta2$ was highly expressed in cerebral cortex around the time of neuronal differentiation. However, at variance with other CNS areas, $\alpha4$ mRNA appearance was not temporally related to the time of the last mitosis of cortical neurons. The final mitosis of the neurons that will form the cortical plate takes place from E12 to E20 and follows an inside-out gradient (Angevine and Sidman, 1961). However, $\alpha4$ started to be detected at the end of this period with no evident layer-related gradient. Interestingly, $\alpha4$ mRNA appearance seems temporally correlated to the arrival of basal telencephalic afferents which penetrate into the cortical plate in the perinatal period, but are already present in the subplate at E17 (Koh and Loy, 1989; Semba, 1992). This evidence suggests that, similarly to $\alpha2$ and $\beta2$ in chick brain

(Daubas et al., 1990, Matter et al., 1990), innervation may directly stimulate $\alpha4$ subunit gene expression in developing cortical neurons.

Pattern C is present in the retina, in the vestibulo-cochlear ganglia and in both the sympathetic and parasympathetic motor ganglia. In these last ganglia, the subunit transcripts initially detected ($\alpha3$, $\beta2$, and $\beta4$) persisted at a high level throughout prenatal and perinatal development. Accordingly, in both chicken parasympathetic and sympathetic ganglia, the same set of ACh-gated channels are recognized during prenatal development (Margiotta and Gurantz, 1989; Moss et al., 1989). In addition, no evidence for a switch in subunit composition (at least not in chick ciliary ganglion; Corriveau and Berg, 1993) has been presented to date.

At variance with our data in the rat, in chicken parasympathetic and sympathetic ganglia a marked (5–15-fold) increase in nAChR subunit mRNA levels, ACh binding, and response to ACh takes place during prenatal development, and temporally matches the arrival of cholinergic innervation and the formation of mature synapses (Margiotta and Gurantz, 1989; Moss et al., 1989; Corriveau and Berg, 1993). This discrepancy may reflect species differences. In fact, synaptogenesis as well as functional

Figure 20. Dark-field microphotographs of emulsion autoradiograms showing $\alpha4$ (*B*) and $\beta2$ (*C*) nAChR subunit mRNAs in sagittal sections of the optic nerve of a E15 rat embryo. *A* shows toluidine blue staining. The *arrowheads* point to the optic nerve. Abbreviation: *Di*, diencephalon. Scale bar, 100 μm.
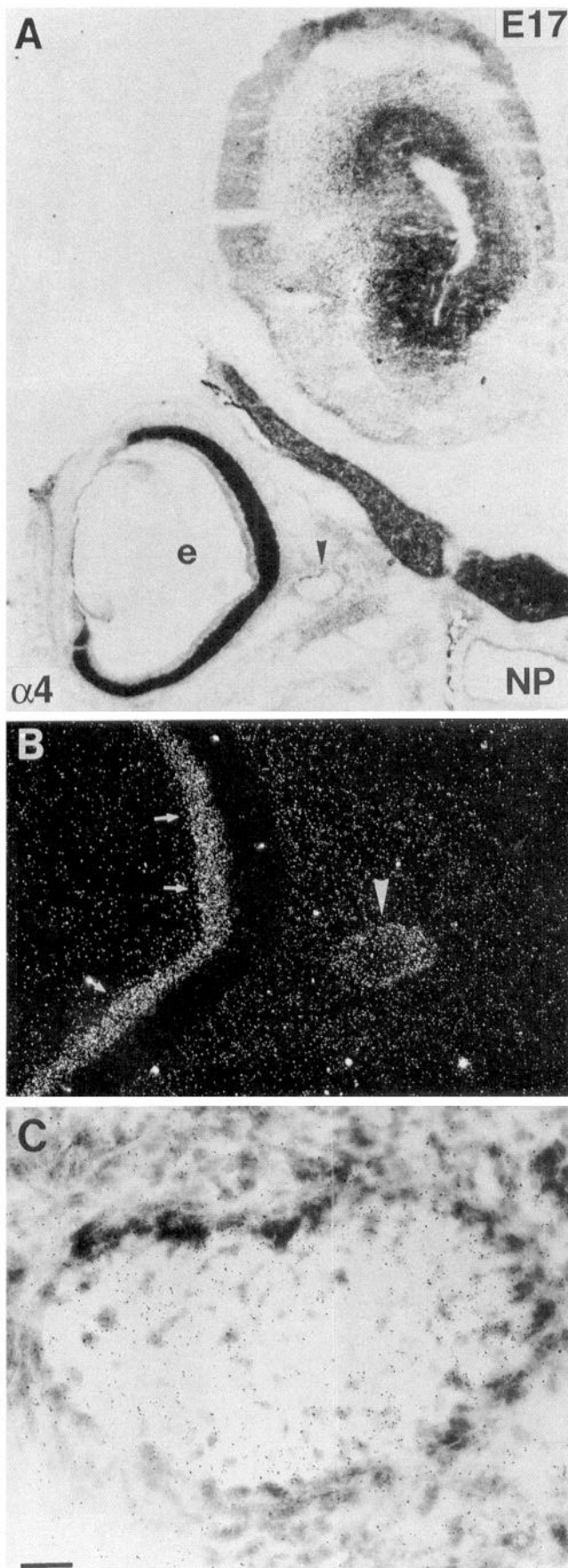
Table 5. Development of nAChR subunit mRNAs in the dorsal root ganglia

	α3	α4	β2	β4
E11	+++	++	++	++
E12	+++	+++	+++	++
E13	+++	+++	+++	++
E15	+	−	+++	+
E17	+	−	+++	−
P4	+	−	+++	−

Scores were attributed as follows: +, low level; ++, intermediate level; +++, high level.

maturation of autonomic ganglia are largely postnatal in the rat (Pappano, 1977; Smolen and Raisman, 1980; Smith et al., 1981).

Discussion

Early expression of nAChR subunit mRNAs

In both CNS and PNS α3, α4, β2, and β4 nAChR subunit mRNAs appear very early, mostly at E11, a stage at which the first neurons differentiate from the central neuroepithelia and from primordia of peripheral ganglia.

The times of the last mitosis of neuronal precursor cells (Altman, 1992) and nAChR subunit mRNA appearance almost coincide in CNS and PNS (see, e.g., the temporally similar caudorostral gradient in the neuraxis). Accordingly, specific labeling was consistently observed in areas containing differentiated cells and not in neuroepithelia (Figs. 3, 8). These data suggest that high expression of nAChR mRNAs is rapidly achieved after the last mitosis in developing neurons.

Early expression of subunit mRNAs seems characteristic of nAChR as other ligand-gated ion channels become expressed somewhat later. For instance, GABA$_A$ receptor α1-3,5-6, β2-3, and γ2 subunit mRNAs were not detected in the rat brain until E14-15 (Laurie et al., 1992; Poulter et al., 1992, 1993) and NMDA receptor ε1-4 and γ1 subunit mRNAs were not observed in the mouse brain until E13 (which corresponds to E14–15 in rat development) (Watanabe et al., 1992) and were very low in rat brain at E14 (Monyer et al., 1994).

In muscle, nAChRs accumulate before the arrival of the motor nerve ending (reviewed in Schuetze and Role, 1987; Changeux, 1991). This increase is accompanied by an elevation in subunit mRNA levels, a process under the control of tissue-specific transcription factors such as MyoD1 (Piette et al., 1990). It is thus possible that onset of neuronal nAChR subunit expression in most brain areas and ganglia is independent of innervation but, rather, is activated by molecules responsible for neuronal differentiation.

Transient expression of nAChR subunit mRNAs

Transient expression of one or more nAChR subunit mRNAs is observed in many CNS and PNS structures. Although in many

←

Figure 21. Bright-field (*A, C*) and dark-field (*B*) microphotographs of α4 mRNA labeling in the optic nerve in a coronal section of the rat head at E17. *B* and *C* are higher magnifications of the optic nerve in *A*. In *A*, the *arrowhead* points to the optic nerve. In *B*, the *arrowhead* and the *arrows* point to the optic nerve and the vitreal side of the retina, respectively. In *C*, note that the specific labeling overlies some cell bodies concentrated in the peripheral part of the nerve and not axonal bundles (which appear white in the picture) concentrated in its central part. Abbreviations: *e*, eye; *NP*, nasopharyngeal cavity. Scale bar, 25 μm.

Table 6. Development of nAChR subunit mRNAs in the ganglia associated with cranial and sympathetic nerves.

Days	Trigeminal g				Sphenopalatine g				Geniculate g				Vestibulo-cochlear gg			
	α3	α4	β2	β4	α3	α4	β2	β4	α3	α4	β2	β4	α3	α4	β2	β4
E11	++	+	+	+	nd	nd	nd	nd	++	++	+	++[1]	++	++	+	++[1]
E12	+++	++	++	++	nd	nd	nd	nd	-	++	++	+[1]	-	++	++	+[1]
E13	+++	+	+++	++	nd	nd	nd	nd	-	-	+++	-	-	+++	++	-
E15	+	-	+++	+	+++	-	++	+++	-	-	+++	-	-	++	+++	-
E17	-	-	+++	-	+++	-	++	+++	-	-	+++	-	-	++	++	-
E19	-	-	+++	-	+++	-	++	+++	-	-	+++	-	-	++	++	-
P0	-	-	+++	-	+++	-	++	++	nd	nd	nd	nd	-	++	++	-
P4	+	-	+++	-	+++	-	++	+++	nd	nd	nd	nd	-	++	++	-

Days	Otic g				Superior IX g				Inferior IX/X gg				Sympathetic gg			
	α3	α4	β2	β4	α3	α4	β2	β4	α3	α4	β2	β4	α3	α4	β2	β4
E11	nd	nd	nd	nd	+++	++	+	++[2]	+++	++	+	++[2]	nd	nd	nd	nd
E12	nd	nd	nd	nd	+++	+	++	++	+++	+	++	++	nd	nd	nd	nd
E13	nd	nd	nd	nd	++	+	++	+	+++	+	++	+	nd	nd	nd	nd
E15	+++	-	++	+++	++	-	++	+	++	-	++	+	+++	-	+++	++
E17	+++	-	++	+++	-	-	++	-	++	-	++	++	+++	-	+++	+++
E19	+++	-	++	+++	-	-	++	-	++	-	++	++	+++	-	+++	+++
P0	+++	-	+	+++	-	-	++	-	++	-	++	++	+++	-	+++	+++
P4	+++	-	+	+++	-	-	+++	-	++	-	+++	++	+++	-	+++	+++

At days E13, E15, and E17 both cervical and lumbar sympathetic ganglia were detected. At days E19, P0, and P4, only cervical sympathetic ganglia were detected. Abbreviations: g, ganglion; gg, ganglia; nd, not detected.
Notes: [1]VII/VIII complex; [2]Superior and inferior IX and X ganglia were not distinguished.

instances several subunit mRNAs were detected in the same clusters of cells (see, e.g., Fig. 6), it could not be determined if nAChR subunit mRNAs in E11–13 embryos are coexpressed in the same cells or segregate in distinct cell populations. In the former case, repression of one or more subunits may cause a shift in the predominance of different nAChR isoforms or a switch in subunit composition from an embryonic to an adult form of the receptor, similar to what occurs in the developing muscle (Brehm and Henderson, 1988; Duclert et al., 1993).

On the other hand, β2 mRNA is continuously and stably expressed in all structures studied, confirming previous data on widespread distribution of β2 mRNA (Wada et al., 1989) and protein (Hill et al., 1993) in the adult rat CNS. Immunocytochemical studies at the electron microscopic level in CNS demonstrated an excess of β2 subunit protein in the cytoplasm relative to the plasma membrane, and it was suggested that the expression of β2 subunit may be relatively unregulated in adult CNS (Hill et al., 1993). The present data further support this notion, as β2 mRNA seems to be the least sensitive nAChR subunit transcript to developmental events.

Transient expression of other ligand-gated ion channel subunits has been observed in rodent CNS during prenatal and/or postnatal development (see, e.g., Zhang et al., 1991; Laurie et

al., 1992; Poulter et al., 1992, 1993; Watanabe et al., 1992). However, the extreme precocity, widespread occurrence, and relative synchrony of nAChR subunit repression during early ontogeny seem characteristic of neuronal nAChR. This phenomenon may be similar to the evolution of muscle extrajunctional nAChR during endplate development, where several subunits are expressed initially and are subsequently repressed in extrajunctional areas by electrical activity after formation of functional motor endplates. Similarly, a widespread signal such as spontaneous electrical activity may serve as a candidate for nAChR subunit repression during neural development.

Diversity of nAChR receptors during development

A physiological role for nAChR expression during embryonic development requires that subunit mRNAs are translated into protein, transported, inserted into the cell membrane and that there exists cholinergic input able to activate them.

Although some authors have argued that development of nicotine binding in the rodent brain is mainly postnatal (Yamada et al., 1986; Fiedler et al., 1987; Kumar and Schliebs, 1992), other papers have documented high levels of nicotine binding during the embryonic period (Lichtensteiger, 1988; Zhang et al., 1990; Naeff et al., 1992). This discrepancy is likely to stem from meth-
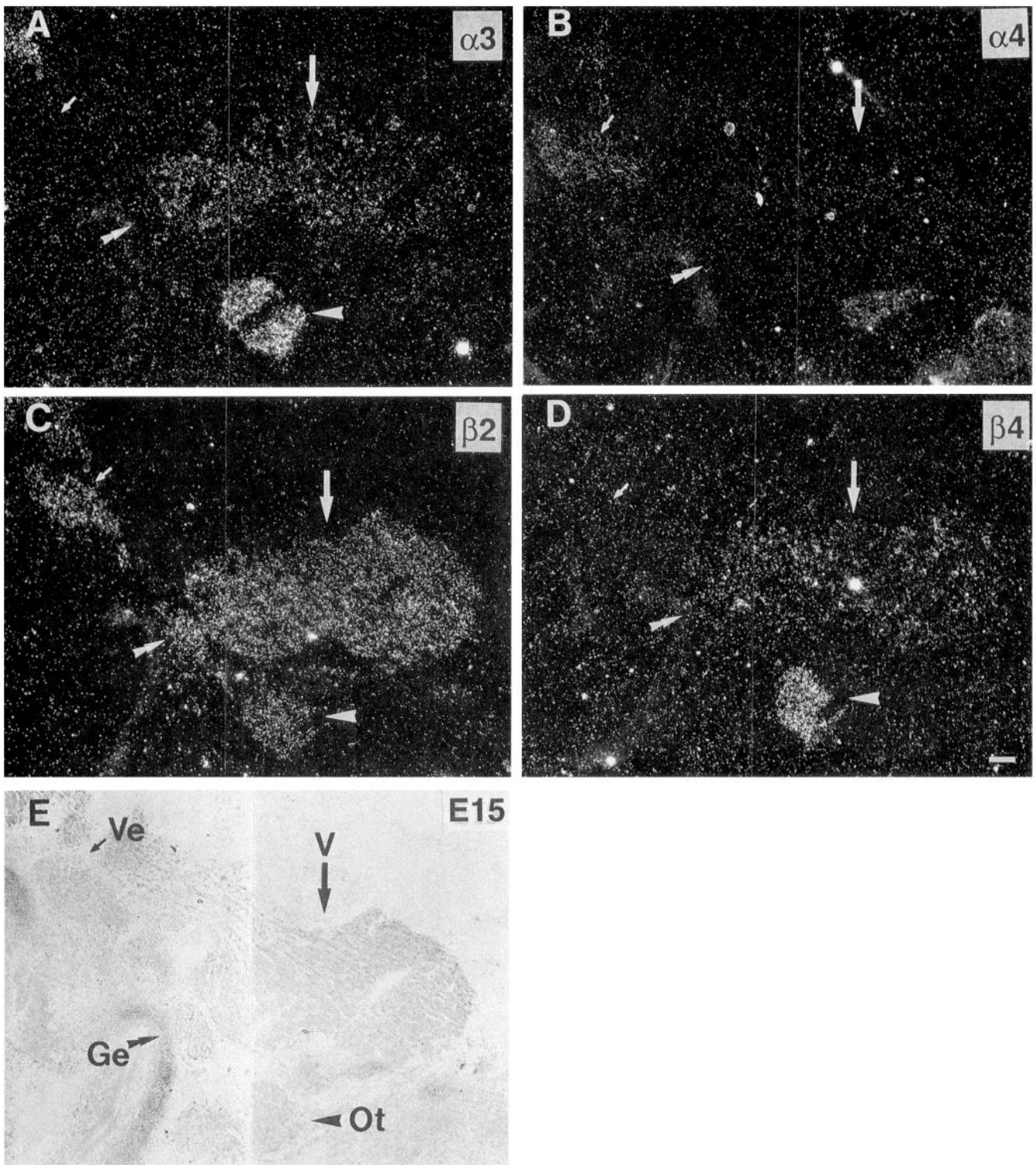
Figure 22. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in several cranial ganglia in sagittal sections of a E15 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *E* shows toluidine blue staining. The *big* and *small arrows* point to the trigeminal (*V*) and vestibular (*Ve*) ganglia, respectively, the *arrowhead* to the otic (*Ot*) ganglion, and the *double arrowhead* to the geniculate (*Ge*) ganglion. Scale bar, 100 μm.

odological differences allowing preferential labeling of low or high affinity nicotine binding sites. Appearance of high affinity (10 nM) nicotine binding in the CNS follows a caudorostral gradient from spinal cord and caudal medulla oblongata (E11) to pons and midbrain (E12), diencephalon (E13), basal telenceph-

alon (E14), and cortical subplate (E19) (Naeff et al., 1992). This gradient closely corresponds to that of the appearance of α4 subunit mRNA observed herein (see Table 2). β2 expression is more precocious in rostral zones and, prior to the appearance of α4, either does not contribute to a functional receptor, or con-
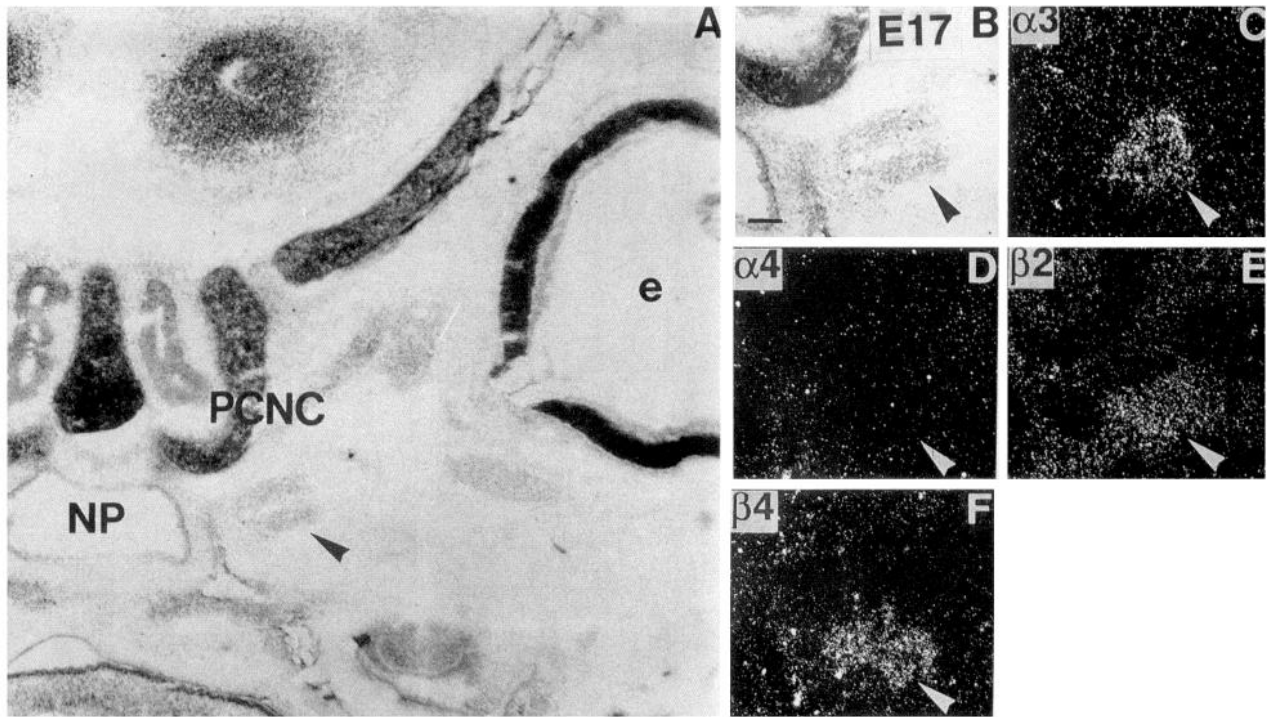
Figure 23. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in the sphenopalatine ganglion (*arrowhead*) in coronal sections of a E17 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* and *B* show toluidine blue staining. In *A*, the *arrowhead* indicates the sphenopalatine ganglion magnified in *B*. Abbreviations: *e*, eye; *NP*, nasopharyngeal cavity; *PCNC*, posterior cupula of the nasal capsule. Scale bar, 100 μm.

stitutes a receptor which is not recognized in nicotine binding experiments (see below). A receptor formed by α4 and β2 (and possibly also α5; see Conroy et al., 1992) is thus likely to be responsible for the high affinity nicotine binding observed in the prenatal development of most brain areas.

Throughout embryonic development, the distribution of α3 and β4 subunit mRNAs display striking correspondence in most central and peripheral structures. β4 mRNA was consistently detected in cerebral regions or ganglia where α3 was present. In addition, α3 was detected in some regions of the early (mesencephalon and telencephalon) and late (trigeminal and dorsal root ganglia) embryonic nervous system where β2 rather than β4 was present. Considering that the expression of α3 and β4 subunits is highly restricted (e.g., cerebellar primordium, medial habenula, pineal gland, sympathetic and parasympathetic ganglia) these data strongly support the view that they form (possibly in combination with other subunits) a distinct neuronal nAChR subtype in the rat. This is in agreement with previous studies showing that a nAChR receptor formed by α3, α5, and β4 mediates nicotinic transmission at the synapses of chick autonomic ganglia (Vernallis et al., 1993).

Based on the coincidence in regional distribution, receptors formed by other combinations of subunits may be functional during development, especially if complex combinations including more α and/or β subunits are considered. A distinct possibility is the existence of a receptor formed by α3 and β2 subunits (see above). However, when α3 and β2 mRNAs are the only subunits detected, such as in the cerebral cortex at E13, no high affinity nicotine binding is detected (Naeff et al., 1992). This could be interpreted, *inter alia,* as a lack of translation or posttranslational processing of the subunits. Alternatively, this

receptor may remain undetected by ^{3}H-nicotine at the concentration used. In fact, *in vitro* α4-β2 or α3-β4 containing receptors have relatively high affinity for nicotine, whereas α3-β2 combination has the lowest affinity for nicotine, though relatively high affinity for ACh (Luetje and Patrick, 1991).

Starting from late prenatal development, the combinations of nAChR subunits appear structure specific (Table 7). The analysis of other nicotinic subunits not considered here should further increase the number of region-specific isoforms. Mediation of nicotinic transmission in different classes of neural structures is thus likely to be based on a variety of specific isoforms of nAChR. This heterogeneity opens up the possibility of a targeted pharmacology based on selective nicotinic drugs.

nAChR receptors and cholinergic circuits during development

The appearance of ChAT immunoreactivity (IR) and AChE activity in the CNS follows a caudorostral gradient (Armstrong et al., 1987; Schambra et al., 1989; Phelps et al., 1991). ChAT IR neurons are first visible in the spinal cord (somatic and then visceral motoneurons at E12–14) and, subsequently, in more rostral structures such as basal forebrain (E17–18). Cholinergic neurons are among the first to differentiate in the CNS regions studied and are thought to play a role in subsequent differentiation of other neuronal populations (Semba and Fibiger, 1988; Semba et al., 1988; Phelps et al., 1990; Barber et al., 1991).

Less is known regarding development of cholinergic innervation. Although the first ChAT IR cell bodies appear relatively early (see above), marked increases in ChAT IR nerve terminals and ChAT activity, especially in the forebrain, occur during the first postnatal weeks (Semba, 1992). However, other markers of cholinergic innervation, such as ACh levels (Coyle and Yama-
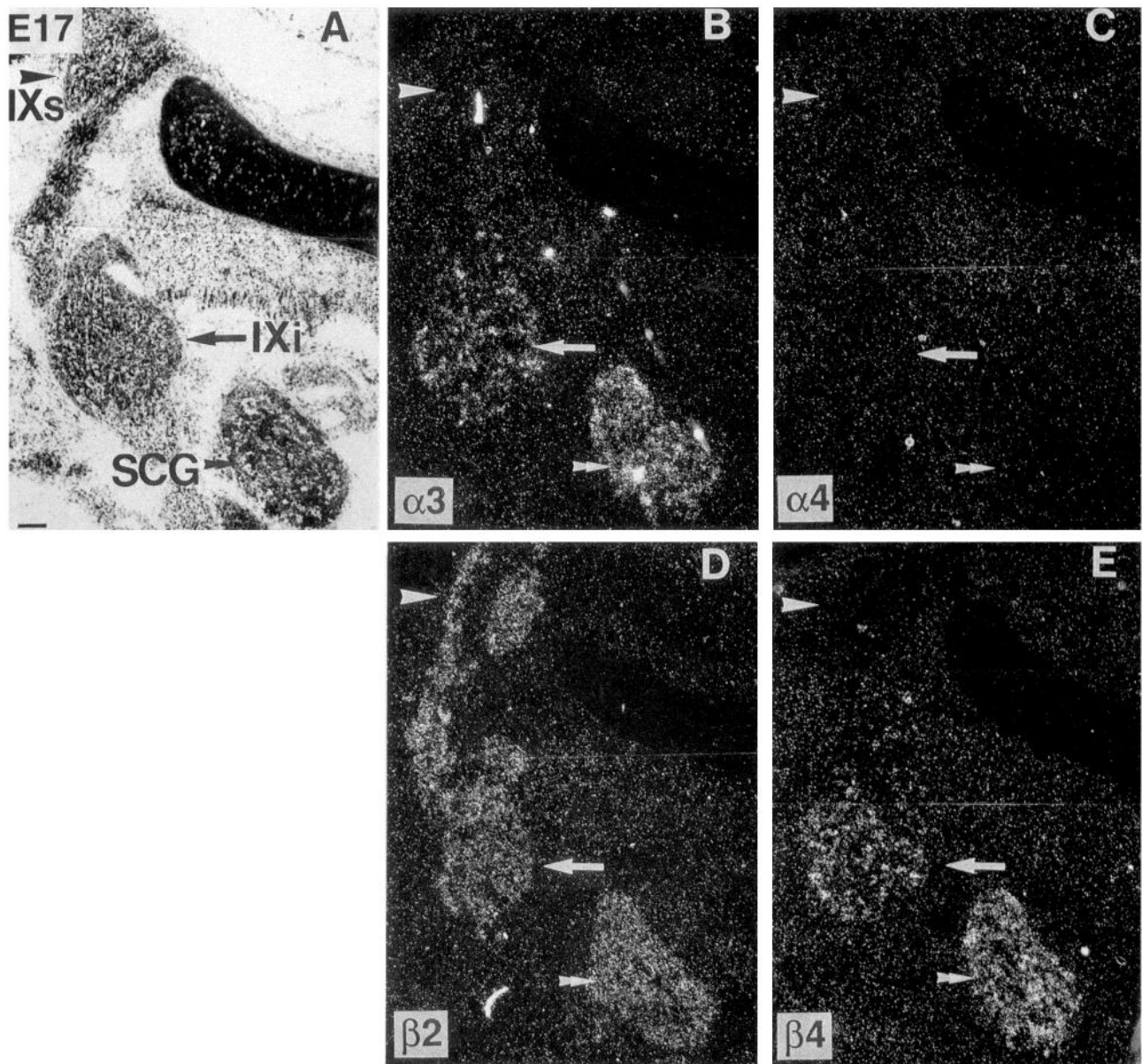
Figure 24. Dark-field microphotographs of emulsion autoradiograms showing nAChR subunit mRNAs in the superior (*IXs, arrowhead*) and inferior (*IXi, arrow*) glossopharyngeal ganglia and the superior cervical ganglion (*SCG, double arrowhead*) in coronal sections of a E17 rat embryo. The nAChR subunit mRNA labeled is indicated in each panel. *A* shows toluidine blue staining. Scale bar, 100 μm.

mura, 1976), AChE (Semba, 1992), NGF receptor (Koh and Loy, 1989) and choline uptake (a better marker of activity in cholinergic terminals than ChAT activity, Zahalka et al., 1993), are more precocious. In particular, ³H-hemicolinium-3 binding, a ligand for the high affinity choline transporter (Happe and Murrin, 1993), is very high in the prenatal brain, suggesting that the limited number of prenatal cholinergic terminals may have higher activity than those of the adult (Zahalka et al., 1993).

The presence of cholinergic circuits in embryonic neural tissue suggests a role for ACh and nAChRs in the development of rat CNS and PNS (Lauder, 1993). *In vitro,* nicotinic antagonists block the ACh-induced inhibition of neurite outgrowth from rat retinal ganglion cells (Lipton et al., 1988) and nicotine (or ACh) causes neurite retraction in chick ciliary ganglion cells (Pugh and Berg, 1994). Moreover, ACh, through neuronal nAChRs, is a chemoattractant for growth cones of *Xenopus* spinal neurons

(Zheng et al., 1994). Much evidence has accumulated on the developmental effects of prenatal or neonatal *in vivo* manipulation of ACh systems and nAChRs (Hagino and Lee, 1985; Slotkin et al., 1987; Navarro et al., 1989; Nordberg et al., 1991). These effects include decrease in cortical cell number, blunted maturation of cortical cholinergic systems and upregulation of nicotine binding sites (Wonnacott, 1990). Our data documenting high levels of nAChR subunit mRNAs in embryonic CNS and PNS further support the notion of a morphogenetic role of ACh and nAChRs in the prenatal period.

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Table 7. Developmental changes in nAChR subunit expression

	Early embryonic period	Late embryonic/early postnatal periods	Main types of neurons[a]
Caudal brain	α3-α4-β2-(β4)	α4-β2	
Rostral brain	α3-α4-β2	α4-β2[b]	
Spinal cord	α3-α4-β2-β4	(α4)-β2	
Retina	α3-α4-β2-β4	α3-α4-β2-(β4)	(vi)
Dorsal root ganglia	α3-α4-β2-β4	(α3)-β2	(SS, VS[c])
Cranial nerve ganglia	α3-(α4)-β2-(β4)	(α3)-β2	Tr (SS,VS[c])
	α3-(α4)-β2-(β4)	β2	Ge (SS,Gu), IXs (SS,Gu)
	α3-β2-β4	α3-β2-β4	SP (PM), Ot (PM)
	α3-(α4)-β2-β4	α3-β2-β4	IXi (VS), Xi (VS)
	α4-β2	α4-β2	Co (Au), Ve (Eq)
Sympathetic ganglia	α3-β2-β4	α3-β2-β4	(SM)
Pineal gland	—	α3-(β2)-β4	

Values within brackets indicate low levels of the transcript. Abbreviations: IXi,s, inferior and superior ganglia of the glossopharyngeal (IX) nerve; Xi, inferior ganglion of the vagus (X) nerve; Au, auditory; Co, cochlear ganglion (VIII nerve); Ge, geniculate ganglion (VII nerve); Gu, gustatory; Ot, otic ganglion (IX nerve); PM, parasympathetic motor; SM, sympathetic motor; SP, sphenopalatine ganglion (VII nerve); SS, somatic sensory; Tr, trigeminal ganglion (V nerve); Ve, vestibular ganglion (VIII nerve); Vi, visual; VS, visceral sensory.

[a] Only in the case of ganglionic cells.

[b] With the exception of medial habenula which expresses α3, α4, β2 and β4 subunits and of some telencephalic areas, like the olfactory bulb and the caudate-putamen which express only β2.

Trigeminal ganglion and dorsal root ganglia mainly contain somatic sensory afferents from the skin of the face and the rest of the body, respectively, but also some visceral sensory afferents from the buccal mucosa and internal organs, respectively.

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