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Nicotinic acetylcholine receptor gene expression in developing chick autonomic ganglia

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Abstract

The developmental expression patterns of ten genes encoding nicotinic acetylcholine receptor subunits were analyzed using Northern blots and in situ hybridization in chick peripheral ganglia of neural crest, placodal and dual embryonic origin. The superior cervical and ciliary ganglia were investigated in detail because they accumulated relatively abundant transcripts of the α3, β4, α5 and α7 genes. In the superior cervical ganglion, these four mRNA species had similar developmental time-courses. They appeared at embryonic day 8 (E8), increased steadily until E16 and maintained a rather high plateau level until E18. In the ciliary ganglion, α7 transcripts were already abundant at E6, increased until E10, and considerably decreased thereafter. High-resolution in situ hybridization showed that α7 transcripts were present in all cell types of the E6 ciliary ganglion, whereas they were restricted to large neuronal somas at E16. Transfections with a reporter gene under the control of the α7 promoter demonstrated that a sharp developmental divide occurred at E11-12, after which stage the promoter was activatable in neurons exclusively. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Eleven different genes encoding neuronal nicotinic acetylcholine receptor subunits have been cloned in vertebrates. Their distinct, but sometimes overlapping expression patterns in the nervous system, provide a molecular basis for the observed developmental and functional diversity of the neuronal nicotinic acetylcholine receptors. However, it has rarely been possible to correlate the physiological and pharmacological profiles of reconstituted channels with those of native receptors, and therefore to infer the subunit composition of the latter (reviewed in Sargent, 1993; Role and Berg, 1996).

The functional properties of neuronal nAChRs are subject to developmental regulation. The acetylcholine sensitivity of chick ciliary and sympathetic lumbar chain neurons increases during development (Margiotta and Gurantz, 1989; Engisch and Fischbach, 1990, 1992; Gardette et al., 1991). There are also changes in the single-channel properties and cAMP dependent regulation of ganglionic nAChRs (Margiotta and Gurantz, 1989; Moss et al., 1989). The overall increase in acetylcholine sensitivity of ciliary neurons results, at least in part, from an increase in the density of nAChR molecules in the postsynaptic membrane (Margiotta and Gurantz, 1989; Jacob 1991). This is accompanied by the accumulation of certain neuronal nAChR mRNAs (Boyd et al., 1988; Corriveau and Berg, 1993), coincident with the maturation of synapses in the ciliary ganglion (Jacob, 1991; Blumenthal et al., 1999), and dependent upon innervation and target tissue interactions (Arenella et al., 1993; Levey and Jacob, 1996). Similarly, the development of acetylcholine sensitivity in sympathetic lumbar chain neurons is influenced by innervation (Role, 1988; Gardette et al., 1991).

The accumulation of certain nAChR transcripts in the central nervous system also coincides with synaptogenesis (Couturier et al., 1990b; Daubas et al., 1990; Matter et al., 1990). Furthermore, nAChR transcript levels increase upon target innervation (Levey et al., 1995) and decline following early deafferentation in both the central and peripheral nervous systems (Matter et al., 1990; Zhou et al., 1998), suggesting that nAChR transcript accumulation might be controlled by synaptic inputs. Conversely, those nAChRs

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that are highly permeable to Ca^{2+} (Galzi et al., 1992; Vernino et al., 1992; Bertrand et al., 1993; Séguéla et al., 1993) may control a number of developmental and physiological processes in the nervous system (Pugh and Berg, 1994; Chang and Berg, 1999).

In this report, we investigate the developmental time-course of nAChR transcript accumulation in the chick superior cervical and ciliary ganglia by combined Northern blot analysis and in situ hybridization histochemistry. We focus our study on the α3, β4, α5 and α7 transcripts because of their relative abundance and proven functional roles in the peripheral nervous system (Boyd et al., 1988; Couturier et al., 1990a; Listerud et al., 1991; Corriveau and Berg, 1993). In addition, we use transfection with the α7 promoter and a reporter gene to show that in the ciliary ganglion a sharp developmental divide occurs at E10-12. Whereas prior to that stage α7 mRNA transcription takes place in all cells of the ganglion, it becomes exclusively restricted to the neuronal lineages thereafter.

2. Materials and methods

2.1. Probes

The following DNA fragments were used for RNA blot hybridization: α3 (X07346), a genomic fragment of 900 bp containing the distal part of exon 5 and sequences from intron 5. β4 (J05643), a cDNA fragment of 700 bp encoding most of the 3′ untranslated region. α5 (J05642), a cDNA fragment of 600 bp encoding amino acids 383-454 and approximately 380 bp of 3′ untranslated sequences. α7 (X68586), a cDNA fragment of 700 bp encoding 3′ untranslated sequences. Double-stranded genomic or cDNA fragments were generated by digestion with the appropriate restriction enzymes, purified on 0.8% agarose slab gels, electroeluted and labeled with α-(^{32}P)dATP (Amersham) using a random primed DNA labeling kit (Boehringer-Mannheim). The radiolabeled probes were purified on Biogel P60 columns (Bio-Rad) and denatured by boiling.

For in situ hybridization, we used 42 to 45-mer antisense and sense oligonucleotides corresponding to amino acids 364–377, 342–355, 378–391, and 394–407 of the α3, β4, α5 and α7 subunits, respectively. Oligonucletides least likely to cross-hybridize were selected by aligning with all known chick neuronal nAChR sequences using programs Clustal and Align (PC/Gene package). The ^{32}P-labeled antisense oligonucleotides recognized the same major bands as cDNA probes when hybridized to RNA (Beyer, 1991) from the appropriate source.

Antisense and sense oligonucleotides were labeled by 3′-tailing with Terminal Transferase (Amersham) and α-(^{35}S)dATP (New England Nuclear; 1200–1400 Ci/mmol) to a final specific activity of $5–10 \times 10^6$ cpm/pmol. The probe was purified on a 2 ml Sephadex G-50 (Pharmacia) column and adjusted to 10^6 cpm/μl.

2.2. RNA extraction

Fertilized White Leghorn eggs were kept in a humidified incubator at 38°C. Embryos were staged according to Hamburger and Hamilton (1951). For simplicity, the developmental stages are reported in embryonic days (E). E6, 8, 10, 12, 14, 16, and 18 correspond to stages 29, 34, 36, 38, 40, 42, and 44, respectively. Ganglia and control tissues at the appropriate developmental stages were dissected, immediately frozen in liquid nitrogen, and stored at −70°C. The RNA extraction procedure was adapted from Maniatis et al. (1982). In order to obtain undegraded RNA, it was essential to disrupt the ganglia quickly and thoroughly. This was achieved by vigorous vortexing of 10–50 ganglia in 1.5 ml microfuge tubes containing 0.5 ml baked glass beads (mean diameter 0.45 mm) and 250 μl lysis buffer (10 mM Tris–Cl pH 7.4, 1 mM $MgCl_2$, 10 mM NaCl, 1% SDS, 1 mg/ml proteinase K). After adjusting the buffer volume to 500 μl, the homogenates were incubated 30 min at 37°C, extracted twice with phenol:chloroform (1:1), once with chloroform and precipitated with ethanol. The pellet was dissolved in TE (10 mM Tris–Cl pH 7.4, EDTA 1 mM), and centrifuged for 16 h over a cushion of 5.7 M CsCl at 34,000 rpm, in the TLS-55 rotor of a Beckman TL100 ultracentrifuge. The pellet was resuspended, reprecipitated and dissolved in TE at 0.5 μg/μl.

2.3. Northern blots

We used the procedure described by Matter et al. (1990) to blot total RNA (1 μg/lane) to nylon membranes (GeneScreen, Dupont). The blots were prehybridized in sealed bags for 12–16 h at 42°C in 50 mM Tris–Cl (pH 7.4), 50% deionized formamide, 2× Denhardt's solution, 1 M NaCl, 10% dextran sulphate (Pharmacia), 1% sodium dodecylsulphate (SDS) and 100 μg/ml heat-denatured salmon sperm DNA. Hybridization was done in the same solution containing 10^6 dpm/ml denatured probe for 24 h at 42°C. The membranes were washed 15 min at room temperature in 2× SSC (1× SSC: 150 mM NaCl, 15 mM Na-citrate), 2×30 min at 65°C in 2× SSC containing 0.1% SDS, and 30 min at room temperature in 0.1× SSC. They were exposed for 1–10 days to Fuji RX film at −70°C using an intensifying screen.

2.4. Preparation of tissue sections

Ganglia at different developmental stages were dissected, embedded in OCT compound (Miles) in 2×2 cm Lab-Tek tissue-culture chambers (Miles) or microfuge tubes, frozen in isopentane cooled to −25°C on dry ice, and stored at −70°C. 10 μm cryostat sections were mounted on poly-D-lysine (200 μg/ml) coated slides, thawed for a few seconds on a heating block at 40°C, immediately transferred to −20°C to prevent RNA degradation, and stored for a maximum of 2 weeks at −20°C.

2.5. In situ hybridization

We followed the procedure described by Aubry et al. (1993). The hybridization medium was composed of 50% deionized formamide, 4 × SSC, 1 × Denhardt's solution, 10% dextran sulphate (Pharmacia), 5 mM dithiothreitol, 250 μg/ml yeast tRNA, 5 μg/ml polyA (Boehringer Mannheim), 500 μg/ml salmon sperm DNA, and 10^7 cpm/ml radiolabeled oligonucleotide. The sections were covered with 60 μl of hybridization medium under a 4 cm^2 parafilm ''coverslip'' and incubated overnight at 42°C in a humidified chamber. The next day, the parafilm was removed by dipping the slides in 4 × SSC, and the sections were washed 2 × 30 min in 1 × SSC at 42°C, 30 min in 0.1 × SSC at 42°C, and 30 min in 0.1 × SSC at room temperature. The sections were air-dried after dehydration in 70% and 100% ethanol, and dipped in Kodak NTB-2 emulsion diluted in water (1:1) at 42°C. Following an exposure of 2 to 4 weeks at 4°C, they were developed, fixed, counterstained and mounted by standard procedures.

2.6. Transfections in ciliary ganglion cells

Reporter plasmids α7NP-lacZ and α7NR-lacZ were constructed by placing a 400 bp (NP) or a 1400 bp (NR) segment of the α7 promoter upstream of the β-galactosidase gene. They and the control plasmid pSV-lacZ have been described (Matter-Sadzinski et al. 1992; Hernandez et al., 1995). Ciliary ganglia (30–50, depending on stage) were excised from E8, E10, E11 and E12 embryos, collected in Ca^{2+}-and Mg^{2+}-free Hank's balanced salt solution and incubated with 0.1% trypsin for 25 min at 37°C. Trypsin was inactivated by addition of horse serum to 5% and ganglia were dissociated into single cells by trituration. Dissociated ciliary ganglion cells were pelleted, rinsed in Opti-Mem medium, resuspended in Opti-Mem and subjected to transfection (Matter-Sadzinski et al., 1992). Plasmid DNA (0.5 μg in 50 μl of Opti-Mem) was mixed with Lipofectin Reagent (1.5 μg diluted in 50 μl of Opti-Mem) and added to 400 μl of cell suspension containing 1–1.5 $\times 10^5$ cells. After incubation for 45 min at 37°C, transfection was stopped by adding 0.5 ml of Dulbecco minimum essential medium (DMEM) containing 20% horse serum. Cells were centrifuged, resuspended in DMEM supplemented with 10% chick embryo extract and 10% horse serum, and plated into poly-D-lysine-coated 4-well chambers. Cells were cultured for 48 h at 37°C in a humidified, 5% CO_2 atmosphere. Except as indicated, tissue culture reagents were purchased from BRL and plasticware from Nunc.

3. Results

To study the expression of neuronal nAChR mRNAs in the developing chick peripheral nervous system, we had to adapt RNA extraction and Northern blot procedures to minute sources of material (see Section 2). Initially, we screened RNA blots of peripheral ganglia collected at early and late developmental stages for expression of all known neuronal nAChR mRNAs. Our tissue sampling comprised parasympathetic (ciliary) and sympathetic (superior cervical and lumbar chain) autonomic ganglia, and sensory ganglia of neural crest (dorsal root), placodal (nodose and petrous) and dual (trigeminal) embryonic origin. α2, α6, and α8 mRNAs were low or undetectable at all stages investigated. β3 was expressed at relatively high levels in the trigeminal and dorsal root ganglia, and trace amounts were present in the superior cervical ganglion (Hernandez et al., 1995). α4 was expressed at high levels in trigeminal and dorsal root ganglia, and at low levels in the ciliary ganglion, whereas β2 was present at low, but rather constant levels during the development of all the ganglia (data not shown). In this report, we focus on the remaining, more abundantly expressed transcripts (α3, β4, α5 and α7) showing developmental fluctuations in the ciliary and superior cervical ganglia.

3.1. Detection of α3, β4, α5 and α7 mRNAs by northern blots

Several non-overlapping genomic or cDNA fragments were initially used to probe for each of the α3, β4, α5 and α7 transcripts. They were seen to yield consistent and specific hybridization patterns, as did the antisense oligonucleotides. The specificity of the probes was further assessed by showing that they did not hybridize to $polyA^+$ RNA from E12 liver and muscle. Total RNA from E12 central nervous system regions (telencephalon, optic tectum, cerebellum, spinal cord and retina) were also challenged with these probes. α7 mRNA was detected in the optic tectum and retina, two tissues known to express the gene at this stage of development (Matter-Sadzinski et al., 1992), and low levels of α3 and β4 mRNAs were detected in the retina, as previously described (Fucile et al., 1998).

Fig. 1 shows hybridizations of α3, β4, α5 and α7 probes to superior cervical ganglion RNA. The α3 probe recognized a major band of 3.5 kb and a minor band of 2 kb. These two bands have also been observed in the chick ciliary ganglion by Boyd et al. (1988). The β4 and α5 probes each detected a unique mRNA species of about 3 and 3.5kb, respectively. α7 mRNA, when abundantly expressed as in the ciliary ganglion (Fig. 3A), gave a more complex pattern consisting of a major band of 7 kb and of a minor band of 3 kb, both of which were recognized by the antisense oligonucleotide probe. The 7- and 3-kb bands and the intervening smear have also been detected in the developing optic tectum of the chick (Couturier et al., 1990b). We postulate that the 4- and 3-kb bands are generated by cleavage of the 7-kb band, as might occur

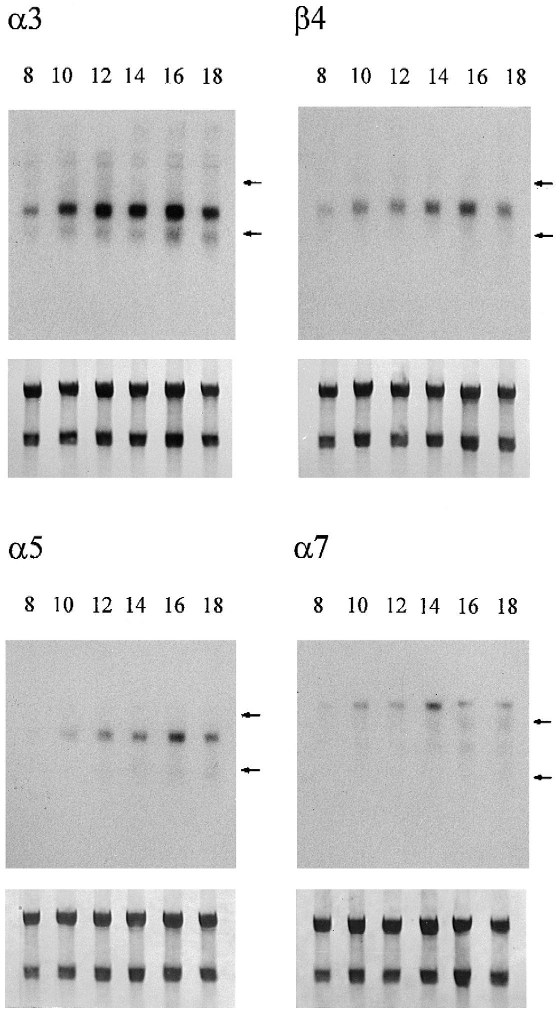

Fig. 1. Determination of α3, β4, α5 and α7 transcript levels in the developing superior cervical ganglion. Total RNA (1 μg/lane) from E8 to E18 was electrophoresed, blotted and hybridized to ^{32}P-labeled genomic or cDNA fragments, as described in Materials and Methods (exposures, 10 days). Arrows mark the positions of the 28S and 18S ribosomal RNAs.

during RNA extraction due to the existence of RNAse hypersensitive sites. Alternatively, the cleavage could be part of a post-transcriptional regulatory process since we notice that the 3 kb species is much more abundant at the earliest developmental stages, in E6 ciliary ganglion (Fig. 3A) or in E5 optic tectum (L. E., unpublished observations). Variable amounts of α3, β4 and α7 mRNAs were also detected in the lumbar sympathetic chain, nodose, petrous, trigeminal and dorsal root ganglia (data not shown). α5 mRNA, in contrast, was not detectable in tissues other than ciliary and superior cervical ganglia under the same experimental conditions.

3.2. Developmental patterns of α3, β4, α5 and α7 mRNAs

In Fig. 1, we show the hybridization patterns of the α3, β4, α5 and α7 probes with superior cervical ganglion RNA prepared every other day from E8 to E18. Methylene blue staining of the membranes was included (lower panels) to give an estimate of the loading efficiency and RNA quality at each developmental stage. α7 mRNA levels increased between E8 and E10, peaked at E14 and remained steady until E18. α3, β4 and α5 increased until E16 and slightly decreased thereafter. When examined by in situ hybridization in the superior cervical ganglion at E16, α5 was seen to be expressed throughout the tissue in a punctate pattern such as is usually associated with labeling of large somas (Fig. 2).

α7 mRNA kinetics were quite different in the ciliary ganglion. mRNA was abundant as early as E6, reached a peak at E10–12 and decreased sharply thereafter (Fig. 3A). During the initial phases of ciliary ganglion development (between E6 and E10), when most neurons are already post-mitotic (D'Amico-Martel, 1982) and before the onset of natural neuronal death, the increase in hybridization signals on RNA blots should reflect an increased accumulation of mRNAs in newborn neurons and in proliferating non-neuronal precursors. Naturally occurring neuronal death removes nearly 50% of neurons between E9 and E14 (Landmesser and Pila, 1974), and combined neuronal death and glial proliferation result in a ratio of 8 to 10 supporting cells per neuron in E15 quail ciliary ganglion (Dupin, 1984). In situ hybridization to E16 ciliary ganglion confirmed that expression of α7 mRNA was confined to large

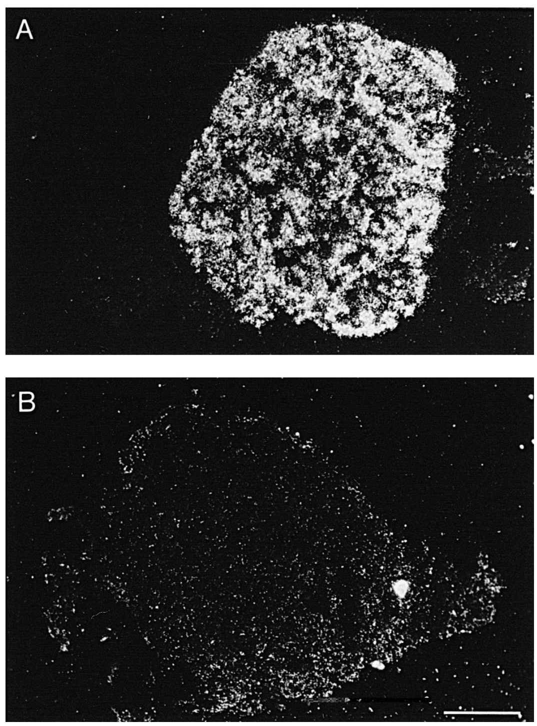

Fig. 2. In situ detection of α5 transcripts in the superior cervical ganglion. Dark field micrographs of E16 sections hybridized with antisense (A) or sense (B) oligonucleotide probes (exposures, 3 weeks; bar, 180 μm).

bodies at later stages, we decided to ascertain whether the transcriptional activity of the gene gained specificity in the course of development.

The promoter element of the $\alpha 7$ gene is contained within a DNA fragment of 1400 bp that encompasses the two major transcription start sites and contains essentially all the information required for tissue-and stage-specific control of $\alpha 7$ gene expression (Matter-Sadzinski et al., 1992). When freshly dissociated ciliary ganglion cells were transfected by plasmids, where the β-galactosidase reporter gene had been placed under the control of the $\alpha 7$ promoter, the nature of the transfected cells varied sharply depending on the stage at which the ganglia had been collected. Numerous neuronal and non-neuronal precursor cells were seen to express the reporter gene in preparations from E8 ganglia. In contrast, only neurons could express the transgene in E12 preparations (Fig. 4 and Table 1). Since transfections using the ubiquitous simian virus 40 (SV40) promoter yielded high percentages of β-galactosidase positive cells of both neuronal and non-neuronal morphologies, the transformation procedure is not biased in favor of a particular cell type. We conclude that ciliary ganglion precursors of all types possess transcription factor complements sufficient for $\alpha 7$ expression at early stages, and that at later stages that ability becomes restricted to neurons only. To determine when this restriction takes

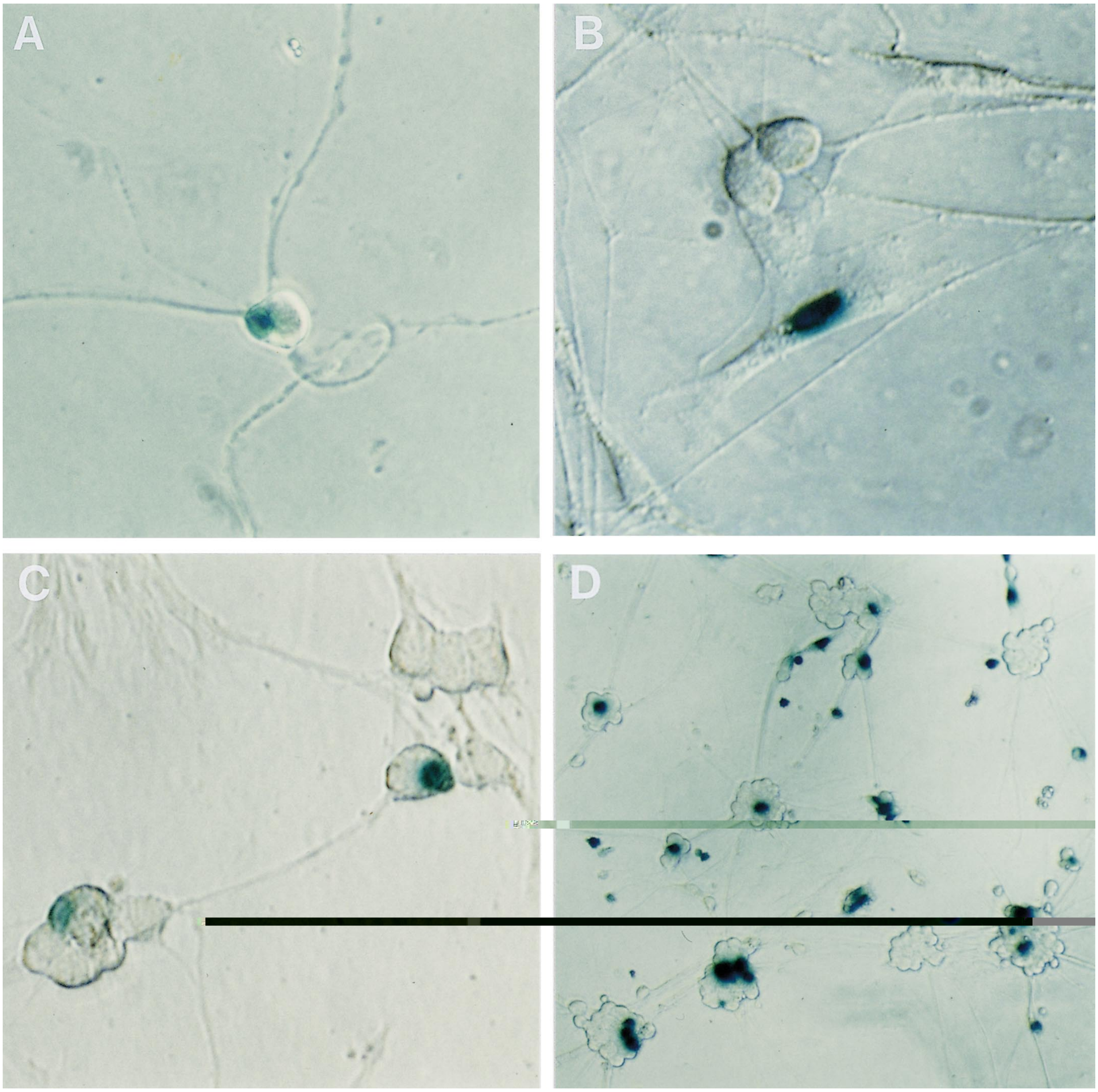

Fig. 4. Activity of the $\alpha 7$ promoter in dissociated ciliary ganglion cells. Cells isolated on E8 (A, B, D) and E12 (C) were transfected with α7NP-lacZ. β-galactosidase positive nuclei were revealed 48 h after plating. Phase-contrast micrographs of β-galactosidase-positive neuronal (A, C) and non-neuronal cells (B). (D) Low magnification micrograph of ciliary ganglion cells transfected with the control plasmid pSVlacZ. 15–20% of the cells were transfected.

Table 1
α7 Promoter activity at different stages of ciliary ganglion development
Cells dissociated from ciliary ganglia were transfected with either α7NP-lacZ or α7NR-lacZ. β-galactosidase positive cells were revealed and counted 48 h later and expressed as a percentage of positive cells obtained upon transfection with the control plasmid pSV-lacZ. In parentheses, percent β-galactosidase positive cells with typical neuronal morphology.

Ciliary ganglion	α7NP-lacZ	α7NR-lacZ
E8	27 ± 4 (51)	15 ± 3
E10	25 ± 4	16 ± 4
E11	6 ± 1 (95)	3 ± 1
E12	3 ± 2	<1

place, ciliary ganglion cells were transfected on successive days in the critical period between E8 and E12. As shown in Table 1, a very sharp divide in the percentage of transfected cells that were able to activate the α7 promoter occurred between E10 and E11. In addition, whereas at E8 half the cells expressing the reporter gene were of non-neuronal morphology, that proportion fell to 5% at E11.

4. Discussion

In this report, we describe the fluctuations in the expression of the α3, β4, α5 and α7 nAChR genes in developing chick autonomic ganglia, as detected by RNA blot analysis and by in situ hybridization histochemistry. α3, β4 and α5 form a gene cluster spanning 28 kb of the chicken genome (Couturier et al., 1990a). The linkage between the three genes is conserved in all the vertebrate species that have been examined (reviewed in Matter and Ballivet, 2000), and there is mounting evidence that this is due to essential regulatory elements of one gene being embedded within another gene in the cluster (McDonough and Deneris, 1997). Biochemical studies indicate that α3, β4 and α5 are likely to form the ganglionic post-synaptic receptors (Halvorsen and Berg, 1990; Conroy et al., 1992), whereas α7 is a constituent of extrasynaptic α-bungarotoxin binding sites (Couturier et al., 1990b; Vernallis et al., 1993), being detected functionally on soma spines (Shoop et al., 1999) and at presynaptic nerve terminals (Coggan et al., 1997).

We show that in the superior cervical ganglion, α3, β4 and α5 transcripts follow quite similar developmental kinetics, steadily increasing between E8 and E16 and then decreasing slightly before hatching. Such a sustained expression of the major nAChR mRNA species is consistent with the fact that, in chick sympathetic ganglia, neuroblasts proliferate until the post-natal period (Rothman et al., 1978). In addition, the continued and parallel accumulation of α3, β4 and α5 mRNAs suggests that the gene cluster is under tight coordinate transcriptional control. In situ hybridization of E16 ganglia with an α5 probe was homogeneous all over the sections (Fig. 2), and as the probes for the β4 and α3 subunit genes yielded essentially identical in situ hybridization images (L. E., unpublished), a majority of cells must be expressing all three transcripts at this stage.

α7 mRNA displays very different developmental kinetics in the superior cervical and in the ciliary ganglia. In the developing ciliary ganglion, we report that α7 mRNA accumulates very early in development, reaches a peak by E10–12 and declines sharply thereafter. Early detection of α7 mRNA is in keeping with previous observations in the central nervous system (Matter-Sadzinski et al., 1992) and in migrating neural crest cells (Howard et al., 1995). The sharp reduction in α7 mRNA levels we detect after E12 is not associated with a parallel decrease in α-bungarotoxin binding or in α-bungarotoxin sensitive acetylcholine responses (Role and Berg, 1996). We suggest that this is probably due to the fact that α7 mRNA is initially present in all cell types of the immature ganglion. As development proceeds, however, its host-range becomes restricted to the neurons, whose proportion is only about one eighth of the total number of cells. This regulation is effected at the transcriptional level because transfections clearly demonstrate that neurons maintain the ability to express the reporter gene throughout development, whereas non-neuronal cells lose that capacity.

Acknowledgements

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