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Expression of neuronal nicotinic acetylcholine receptor genes in the developing chick visual system

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Expression of the neuronal non-α nicotinic acetylcholine receptor (nα nAChR) gene is transiently stimulated in the chick optic tectum between embryonic days 7 and 16 with a peak value reached around embryonic day 12. This stimulation takes place at the time when optic nerve axons are invading this region of the brain and proceeds along a rostral to caudal gradient. Transcripts of the nα nAChR gene are localized in the superficial layers of the tectum at the time when cells in these layers are forming synapses with retina axons. The transient expression of nα nAChR gene does not take place in the optic tectum of 'eyeless' embryos. The results of our study suggest that the neuronal nα nAChR gene may play a role in neurogenesis of retino-tectal connections.

Key words: acetylcholine receptor/chick embryo/optic tectum/synaptogenesis

Introduction

Synaptogenesis is one of the morphogenic events taking place during development of the nervous system. Although most information about synapse formation comes from the study of the vertebrate neuromuscular junction in which muscle nicotinic acetylcholine receptor (nAChR) is an important link in neuromuscular transmission, the presence of nAChR like molecules in the central nervous system has been revealed by immunological (Swanson *et al.*, 1983, 1987; Jacob *et al.*, 1984; Whiting *et al.*, 1987) and molecular biological techniques (Boulter *et al.*, 1986; Nef *et al.*, 1986, 1988; Goldman *et al.*, 1987; Schoepfer *et al.*, 1988; Wada *et al.*, 1988; Cauley *et al.*, 1989). Neuronal nAChRs are composed of ligand binding (α) and structural (non-α) subunits. In the chick, four genes have been found to encode neuronal α subunits (α2, α3, α4 and α5) and two genes code for neuronal non-α subunits (nα and nα3). Neuronal nAChR genes are expressed in a number of discrete areas of the mature nervous system (Boyd *et al.*, 1988; Deneris *et al.*, 1988; Wada *et al.*, 1988, 1989; Cauley *et al.*, 1989). The cDNAs or the cRNAs of α and non-α subunits form functional AChR when injected in *Xenopus* oocytes (Boulter *et al.*, 1987; Ballivet *et al.*, 1988; Deneris *et al.*, 1988). We chose the retino-tectal system of the chick to explore the possible role of the neuronal nAChR genes during development of the central nervous system. The well defined patterning of connections between retinal axon terminals and tectal cells and the possibility of disrupting, by micro-surgery, the formation of these connections are among the principal advantages offered by the retino-tectal system in such a study. Our data suggest that the nα nAChR gene may participate in the formation of the retino-tectal synapses.

Results

α4 and nα nAChR mRNA levels during development of the optic tectum

Total RNA was isolated from the optic tectum of chick embryos at intervals between embryonic day 6 (E6) and the time of hatching, and at two stages after hatching (Figure 1a). As early as on E6, both α4 and nα nAChR mRNAs could be identified in the optic tectum. In the course of development the level of α4 nAChR mRNA in the tectum remained relatively low and constant, whereas we observed profound changes in the steady state levels of nα mRNA. The level of this mRNA increased ~10 times between E6 and E10 and reached a maximum sometimes between E12 and E13, then it decreased rather abruptly between E13 and E16 and more progressively between E16 and adulthood, when it fell to about one-tenth of the level observed on day 13 (Figure 1b). In the optic tectum of the adult chicken, the levels of α4 and nα nAChR mRNAs were similar (Figure 1b). The optic tectum differentiates according to a rostral to caudal pattern and the development of the most rostral region of the tectum is advanced by ~2 days compared with the caudal region (LaVail and Cowan, 1971; McLoon, 1985). We have determined when the expression of the nα nAChR was stimulated in each of these regions of the tectum. The levels of nα and α4 nAChR mRNAs were determined in the rostral and caudal thirds of the tectum from E7 and E9½ embryos (Figure 1c). On E7 the level of nα nAChR mRNA was found to be ~10 times higher in the rostral region than in the caudal one, and by E9½ similar levels of nα nAChR mRNA were observed in both regions of the tectum. Our data indicate that the transient increase in the steady state level of nα nAChR mRNA proceeds along a rostral to caudal gradient and coincides with the period of development when retinal axons invade the optic tectum (Goldberg, 1974; Crossland *et al.*, 1975; McLoon, 1985).

α4 and nα nAChR mRNA levels during development of the retina

Total RNA was isolated from the retina of chick embryos at the same stage of development as for the optic tectum analysis (Figure 2a). Non-α nAChR mRNA was first detected on E6, then the level of this mRNA increased between E6 and E10 to remain constant in the course of further development. In the adult retina the level of nα AChR mRNA was found to be slightly higher than during embryonic development (Figure 2a). The transient increase in the level of nα nAChR mRNA observed in the tectum between E10 and E16 (Figure 1) was not detected in the developing retina. Very low levels of α4 nAChR mRNA

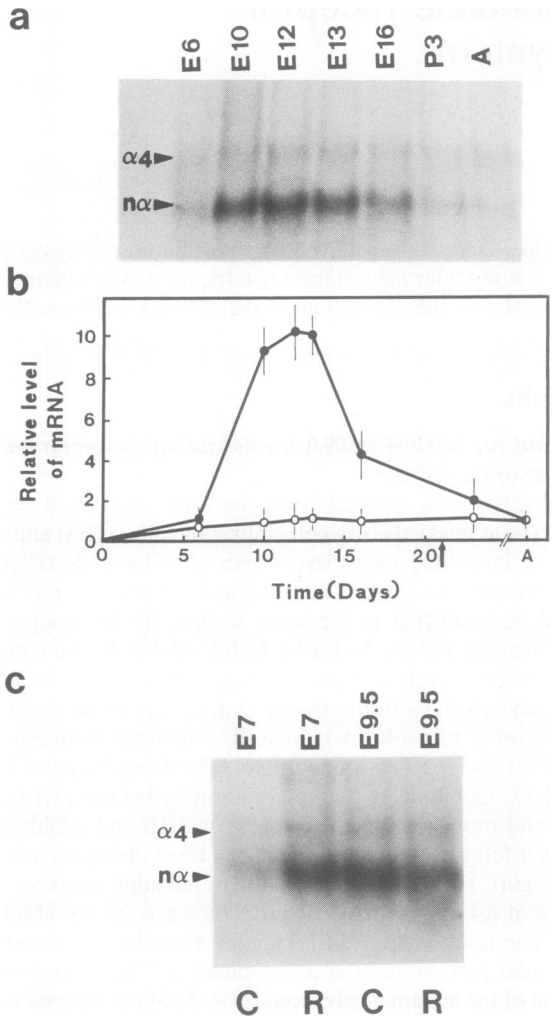

Fig. 1. Levels of the α4 and nα nAChR mRNAs in the developing and adult optic tectum. (**a**) Total RNA (5 μg/sample) extracted from optic tectum at different stages of development was fractionated by gel electrophoresis, blotted and hybridized with nick translated ^{32}P-labelled probes from α4 and nα nAChR cDNAs. Exposure time was 4 days. Arrowheads indicate positions of α4 and nα nAChR mRNAs. (**b**) Levels of nα (●) and α4 (○) nAChR mRNAs on autoradiograms were quantified by scanning laser densitometry. Each point represents the mean value obtained in three independent experiments. Level of nα nAChR mRNA at E13 was chosen arbitrarily as the 10 value. The time scale in days refers to embryonic (E) and posthatch (P) ages, the arrow and 'A' indicate, respectively, hatching and adulthood. Error bars, ±SEM. (**c**) Total RNA (5 μg/sample) extracted from the rostral third (R) and the caudal third (C) of optic tectum at E7 and E9½ was hybridized with ^{32}P-labelled probes from α4 and nα nAChR cDNAs. Exposure time was 5 days.

could be detected at all stages of embryonic development (data not shown). In the adult retina the level of this mRNA increased but remained lower than that of nα nAChR mRNA (Figure 2b).

Distribution of nα AChR transcripts in the optic tectum

We have analysed by *in situ* hybridization the distribution of nα AChR transcripts during the development of the optic tectum. Tissue sections from tectum dissected at stages E6, E12 and E19 were hybridized with labelled nα nAChR antisense probe. The radioactive signal in E6 tectum was present at background level (Figure 3a), while in tissue

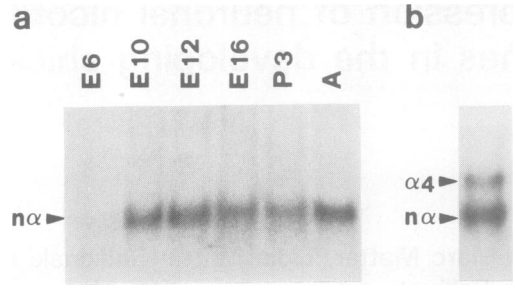

Fig. 2. Levels of the α4 and nα nAChR mRNAs in the developing and adult retina. (**a**) Total RNA (5 μg/sample) extracted from the retina at different stages of development was hybridized with ^{32}P-labelled probe from nα nAChR cDNA. (**b**) Total RNA (5 μg) extracted from adult retina was hybridized with ^{32}P-labelled probes from α4 and nα nAChR cDNAs. Exposure time was 7 days.

sections prepared from E12 embryos the superficial layers of the tectum were found to be strongly labelled (Figure 3b). This labelling was mainly confined to layers v–ix [designation of the layers of the developing tectum conforms to that described by LaVail and Cowan (1971)]. In the most superficial layers of the tectum (layers x, xi and the *stratum opticum*) and in the deeper ones (layers i–iv) the intensity of the signal was slightly above the background level. This pattern of labelling has been observed across the whole tectum (Figure 3b, inset) and similar distribution of the radioactive signal has been found on E10 and E13 (data not shown). When adjacent sections of the tectum dissected on E12 were hybridized with labelled nα nAChR sense (control) probe, no labelling of the superficial layers could be detected (data not shown). These results suggested that between E10 and E13 the level of nα nAChR mRNAs was higher in the superficial than in the deeper layers of the tectum. To confirm this conclusion we have dissected the outer and inner layers of E12 tectum and extracted total RNA from these two regions. RNA blot analysis then revealed that the level of nα nAChR mRNA was ~15 times higher in the outer than in the inner layers of the tectum (data not shown) and this result was consistent with the findings of *in situ* hybridization. On E19, the intensity of the radioactive labelling in the superficial and deeper layers of the tectum was close to background level (Figure 3c). Altogether, these results suggest that the transient increase in the level of nα nAChR mRNA we observed for total tectum between E9 and E16 (Figure 1) takes place mainly in its superficial layers.

Furthermore, the distribution of silver grains in the superficial layers of E12 tectum was not uniform, displaying sparse, strongly labelled cells (Figures 3b and 4). We have analysed the distribution of these cells in the different layers of the tectum (Table I). On E12, ~85% of the strongly labelled cells were found in layers v, vi and vii. In contrast, on E19 only a few labelled cells have been observed in these layers (Table I). No labelled cells were found when tissue sections of E12 and E19 tectum were hybridized with nα nAChR sense probe (Table I). No radioactive labelling of the superficial layers of the tectum has been observed with the α4 nAChR antisense probe. Layers v, vi and vii correspond, respectively, to layers j, i and h of the *stratum griseum et fibrosum superficiale* of the mature tectum (LaVail and Cowan, 1971) and these layers are known to contain the neurons which form synapses with retinal axons (LaVail

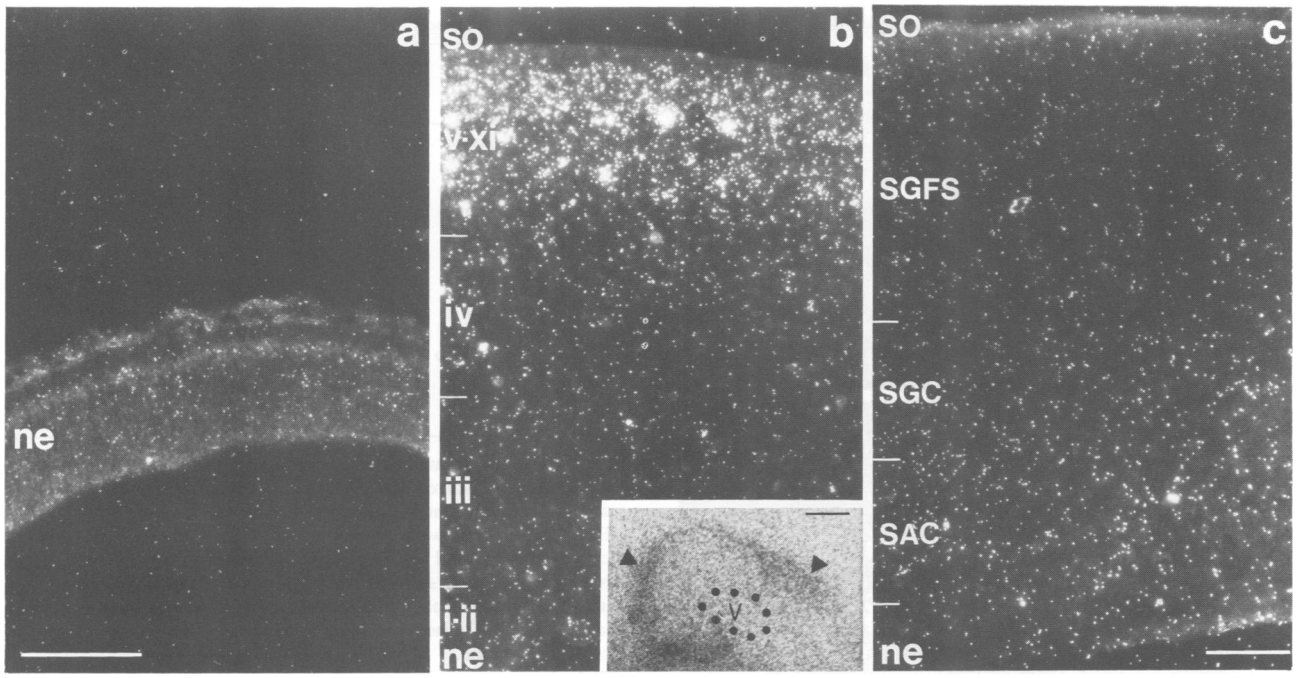

Fig. 3. Topographic distribution of the nα nAChR transcript in the optic tectum. Tissue sections across E6 (**a**), E12 (**b**) and E19 (**c**) tectum were mounted on the same slide and hybridized with ^{35}S-labelled antisense nα nAChR probe. Exposure time was 15 days. SO, *stratum opticum*; SGFS, *stratum griseum et fibrosum superficiale*; SGC, *stratum griseum centrale*; SAC, *stratum album centrale*; ne, neuroepithelium. **Inset**: section through E12 tectum was hybridized with ^{32}P-labelled antisense nα nAChR probe; exposure time was 3 days. Dotted line and arrowheads indicate, respectively, the ventricle (V) and the pial surface. Bars: 200 μm (**a** and **b**); 250 μm (**c**); 500 μm (inset).

and Cowan, 1971; McLoon, 1985). The high level of expression of the nα nAChR gene detected in cells of layers v, vi and vii coincides with the period of development when neurons of these layers form their connections with retinal axons.

Expression of nα nAChR gene in the optic tectum of 'eyeless' embryos

Our data suggested that the transient expression of the nα nAChR gene may be related to formation of retino-tectal synapses. To test this hypothesis we have analysed expression of nα nAChR gene in the tectum of chick embryos in which embryonic development was not influenced by the outgrowth of the retinal axons. Both optic vesicles were removed by surgery on E2, before the axons of the earliest differentiated retinal ganglion cells reach the optic tectum (O'Leary and Cowan, 1983). The embryos which survived after this bilateral enucleation were raised until E10 or E11, when total RNA was isolated from the tectum. Total RNA was also extracted from the tectum of control, mock-operated embryos raised in parallel. Both on E10 (Figure 5a) and E11 (data not shown) the nα nAChR mRNA was present at very low level in the tectum of 'eyeless' embryos. In contrast, in the tectum of normal mock-operated embryos of the same ages (Figure 5a), the level of this mRNA was 10−15 times higher. The transient expression of the nα nAChR gene did not take place in the tectum of 'eyeless' embryos, although development of the tectum in eye deprived embryos is perfectly normal, at least at the morphological level, during the first 12 embryonic days (Kelly and Cowan, 1972; O'Leary and Cowan, 1983). On E10 the amount of total RNA and the level of β actin mRNA (Figure 5b) were similar in the tectum of 'eyeless' and normal embryos.

Discussion

During development of the optic tectum and retina, the nα nAChR gene exhibits distinct temporal and spatial patterns of expression. In the tectum we have observed a transient increase in the steady state level of nα nAChR mRNA between embryonic days 7 and 16 with a peak value reached around E12. Our data suggest that the expression of this gene was stimulated in the tectum along a rostral to caudal gradient which reflects the pattern of sequential invasion of the tectum by axons of the optic nerve. The first retinal axons reach the rostro-ventral part of the tectum on E6, then axons spread over the surface of the rostral region of the tectum (E10) and finally they grow caudally until the entire tectal surface is covered by axons around E12 (Goldberg, 1974; Crossland *et al.*, 1975; McLoon, 1985). On E12 the large majority of the tectal cells which expressed the nα nAChR gene were localized in the three superficial layers v, vi and vii. The fact that only a small proportion of cells in these layers was found to express simultaneously the nα AChR gene suggests that the expression of this gene was stimulated in particular tectal cells only during very short periods of time between the 7th and 13th days of development. Layers v, vi and vii correspond, respectively, in the mature tectum to layers j, i and h of the *stratum griseum et fibrosum superficiale* (LaVail and Cowan, 1971). Many neurons in these layers have ascending dendrites that extend into the terminal projection field of the retinal axons where they form synapses (LaVail and Cowan, 1971; McGraw and McLaughlin, 1980; McLoon, 1985). The transient increase in the level of nα nAChR mRNA in cells of the superficial layers of the tectum coincides with the period of development when cells in these layers are forming connections with retinal axons. In the optic tectum of 'eyeless' embryos, there

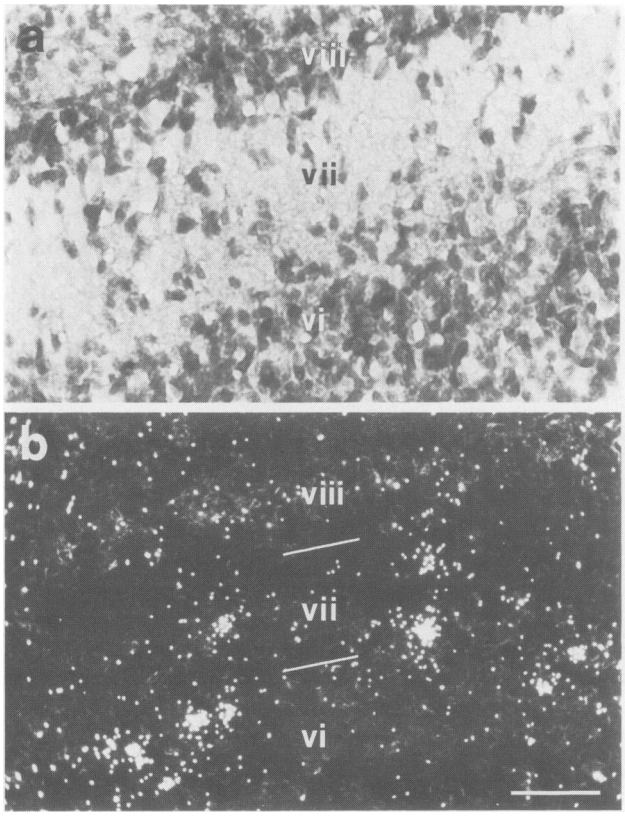

Fig. 4. Topographic distribution of the nα AChR transcript in the superficial layers of the tectum. (**a**) Bright field photomicrograph of tissue section through E12 tectum stained with thionin and (**b**) dark field photomicrograph of an adjacent section hybridized with ^{35}S-labelled antisense nα nAChR probe. Exposure time was 15 days. vi, vii and viii denote three layers of the *stratum griseum et fibrosum superficiale*. Bar: 70 μm.

Table I. Localization of nα nAChR transcripts in the optic tectum

Layers	Age		
	E6	E12	E19
xii−ix/a−f,SO[a]		0(7)	0(6)
viii/g		0(7)	0(6)
vii/h		5 ± 2(7)	0(6)
vi−v/i−j		17 ± 6(7)	4 ± 2(6)
xi−v/SGFS		22 ± 8(7)	4 ± 2(6)
		[1 ± 1(10)]	[0(10)]
iv/SGC		2 ± 1(7)	0(6)
		[1 ± 1(10)]	[0(10)]
iii−i/SAC,SGP,SFP	0(7)	2 ± 1(7)	0(6)
		[0(10)]	[0(10)]
ne	0(7)	0(7)	0(6)

Tissue sections through E6, E12 and E19 tectum were mounted on the same slide, hybridized with ^{35}S-labelled antisense nα nAChR probe and *in situ* hybridization was quantified by cell counting. At E12 and E19, labelled cells were counted in sectors of ~3 mm^2 randomly chosen in the rostral and caudal regions of the tectum. Values represent the mean ± SEM; the number of sections analysed is indicated in parentheses. Values in brackets represent the number of labelled cells in adjacent sections hybridized with the sense (control) nα nAChR probe.

[a]SO, *stratum opticum*; SGFS, *stratum griseum et fibrosum superficiale*; SGC, *stratum griseum centrale*; SAC, *stratum album centrale*; SGP, *stratum griseum periventriculare*; SFP, *stratum fibrosum periventriculare*; ne, neuroepithelium.

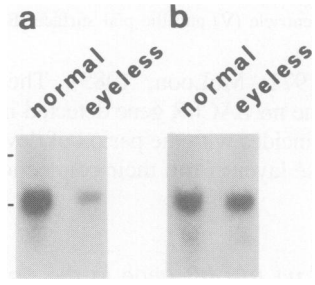

Fig. 5. Levels of nα nAChR mRNA in the optic tectum of 'eyeless' embryo. (**a**) Total RNA (2 μg/sample) extracted from the optic tectum of 'eyeless' or normal embryos at E10 was hybridized with ^{32}P-labelled probe from nα nAChR cDNA. Exposure time was 7 days. (**b**) The nα nAChR cDNA probe was stripped and the membrane was rehybridized with a ^{32}P-labelled β-actin cDNA probe (pA1). Exposure time was 3 days.

is no transient stimulation of expression of the nα nAChR gene. Development of the chick optic tectum in the absence of retinal input has been analysed at the morphological level and, until embryonic day 12, no change in the development of the superficial layers could be detected, except for the lack of the *stratum opticum* (Kelly and Cowan, 1972). Ultrastructural analysis of tectal layers viii and ix in eye-enucleated E9−E11 embryos has only revealed a decrease in the number of synapses (McGraw and McLaughlin, 1980). On the other hand, early eye enucleation does not prevent the tectum from developing retinotopically ordered connections with several other structures in the brainstem with which it is reciprocally connected (O'Leary and Cowan, 1983).

In view of these studies, our results support the idea that the transient stimulation of expression of the nα nAChR gene in tectal cells may be related to the formation of retino-tectal synapses. We suggest that the increase in the level of nα nAChR mRNA in tectal cells is induced by retinal axon terminals.

Although expression of α4 nAChR gene was detected throughout development of the tectum, our results have revealed no evidence of any correlation between expression of this gene and the development of retino-tectal connections.

The subunits encoded by the α4 and nα nAChR genes assemble into the predominant and most ubiquitous form of brain nAChR (Whiting *et al.*, 1987) and form functional nAChR when reconstituted in *Xenopus* oocytes (Ballivet *et al.*, 1988). Two pieces of evidence support the idea that the subunits encoded by these two genes also form nAChR in the mature optic tectum. First, closely similar levels of α4 and nα nAChR mRNAs were present in the adult tectum. Second, the expression of other members of the chicken neuronal nAChR gene family (i.e. α3, nα3 and α5) has not been detected in the tectum (J.-M.Matter and M.Ballivet, unpublished observations). AChR like molecules have been found in the optic tectum of the adult chicken (Swanson *et al.*, 1987), frog (Sargent *et al.*, 1989) and goldfish (Henley *et al.*, 1986) but the authors suggested that in the tectum of these species the AChR like molecules are present in terminals of retinal ganglion cell axons rather than in tectal cells. Their claim was based mainly on the facts that AChR like molecules were found in the retino-tectal pathway and

that immunoreactivity in the tectum disappeared in the mature animals deprived of eyes (Henley *et al.*, 1986; Swanson *et al.*, 1987; Sargent *et al.*, 1989). However, the experiments did not exclude the possibility that the tectal cells containing nAChR like molecules may degenerate in the absence of retinal input (Kelly and Cowan, 1972). We have shown that the tectal cells in the superficial layers of the tectum express the nα nAChR gene and our data suggest that the transport of neuronal nAChR in the optic tract may not represent the only source of receptors in the terminal projection field of retinal fibres.

Differential expression of the nα nAChR gene in the developing tectum suggests a role for the corresponding gene product in neurogenesis of the visual system. The nα nAChR subunit may play this role in association with the α4 subunit—perhaps by driving assembly of the α4/nα nAChR receptor—or with other proteins.

Materials and methods

Probes

For blot hybridization, β1 actin, nα and α4 nAChR cDNA restriction fragments were gel-purified and labelled with [^{32}P]dATP (3000 Ci/mmol; Amersham), by nick translation. For *in situ* hybridization the single-stranded sense and antisense α4 and nα nAChR probes were labelled with [^{32}P]dATP or [^{35}S]dATP (1000 Ci/mmol; Amersham) by primer extension (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia) in conditions yielding labelled DNA of average size 250 nucleotides. The β1 actin probe (pA1) encompassed the complete coding sequence of the protein (Cleveland *et al.*, 1980). The nα sequence (clone C7) used to synthesize single- and double-stranded nα probes was 778 bases in length, beginning 73 bases 5′ of the initiator ATG and extending to the triplet encoding residue 217. The α4 sequence (clone 32.3) used to synthesize single- and double-stranded α4 probes was 804 bases in length, beginning 75 bases 5′ of the initiator ATG and extending to the triplet encoding residue 220 (Nef *et al.*, 1988).

Surgical manipulations and tissue collection

Embryos and chicks of a White Leghorn strain were used for this study. Embryos were raised in an incubator at 38°C and at a high relative humidity. On day 2 of incubation the embryos were exposed through an opening in the shell, both optic vesicles were ablated with a fine glass needle, the opening in the shell was then sealed with plastic tape and the embryos were returned to the incubator until harvesting time. Mock-operated embryos were treated in the same way except that optic vesicles were not ablated. Embryos were staged according to Hamburger and Hamilton (1951) and, following dissection, optic tectum and retina (without the pigment layer) were immediately frozen in liquid nitrogen and stored at −70°C.

RNA extraction and Northern blot analysis

Total RNA was extracted by a guanidium isothiocyanate/hot phenol method (Feramisco *et al.*, 1982). RNA was denatured with formaldehyde and separated by horizontal electrophoresis through a 1% agarose gel in the presence of 6.7% formaldehyde, 40 mM 3-[N-morpholino]propane-sulphonic acid, pH 7.5, 10 mM sodium acetate, 1 mM EDTA (Lehrach *et al.*, 1977; Maniatis *et al.*, 1982). Samples of 5 and 2 μg of total RNA were loaded, respectively, on large and mini gels. After electrophoresis RNA was electrophoretically blotted (Khandjian, 1986) onto nylon membrane (GeneScreen, Dupont) and UV irradiated (Church and Gilbert, 1984) for 3 min with a Philips TUV 15 W tube at a distance of 10 cm. The 45S pre-rRNA, 28S and 18S rRNAs were visualized by staining the membrane with methylene blue (Maniatis *et al.*, 1982). Blots were prehybridized for 2 h in 1 M NaCl, 2 × Denhardt's solution, 1% SDS, 50 μg/ml of sheared and boiled salmon sperm DNA, 10% dextran sulphate (Pharmacia) and 50% formamide at 42°C. The hybridization buffer had the same composition and contained 5 × 10^5 c.p.m./ml of denatured probe. Hybridization was allowed to proceed for 24 h at 42°C, the blots were washed twice in 2 × SSC at room temperature, twice in 2 × SSC, 0.05% SDS at 65°C and once in 0.1 × SSC at room temperature and exposed to X-ray film (Fuji RX) at −70°C with an intensifying screen (Dupont Cronex Lightning Plus). Before rehybridization, membrane was stripped of ^{32}P-labelled probes as described by the manufacturer. Autoradiograms were quantified by scanning laser densitometry (Shimadzu).

In situ hybridization

Total E6 embryos were fixed in Dulbecco's modified phosphate-buffered saline containing 4% paraformaldehyde (Fluka). The tectum of older embryos was dissected out prior to fixation. After fixation the tissues were dehydrated in ethanol, cleared in xylene and embedded in paraffin (Paraplast, Monoject Scientific, Inc.). Tissue sections, 10 μm thick, were mounted on glass slides and were pretreated as described by Matter and Cowan (1990) except that the acetylation step was omitted. Hybridization was performed in 50% formamide, 5 × SSC, 2 × Denhardt's solution, 0.2% sodium pyrophosphate, 600 μg/ml salmon sperm DNA and 5 × 10^6 c.p.m./ml of probe. Forty microlitres of hybridization solution were pipetted onto the dehydrated tissue sections and covered with a 24 × 60 mm coverslip. Tissue sections were hybridized for 8 h in a humidified chamber at 42°C, then sections were washed for 10 min in 4 × SSC at room temperature and for 30 min each in 2 × SSC at 40°C and in 0.1 × SSC at 50°C. After drying, sections hybridized with ^{32}P-labelled probes were exposed to X-ray film for 1−5 days and sections hybridized with ^{35}S-labelled probe were dipped in Kodak NTB-2 photo emulsion (diluted 1:1 in water), exposed for 7−15 days at 4°C, developed (Kodak D19) and fixed.

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