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Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex

(trophic factors/neural development/cell death/brain/transient neurons)

KAREN L. ALLENDOERFER*, DAVID L. SHELTON†, ERIC M. SHOOTER, AND CARLA J. SHATZ

Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Nerve growth factor and its receptor (NGFR) are known to be present in diverse embryonic and neonatal central nervous system tissues, including the cerebral cortex. However, the identity of the cortical cells expressing NGFR immunoreactivity has not been established. We have used immunolabeling coupled with [^{3}H]thymidine autoradiography to identify such cells in ferret and cat brain. Polyclonal antibodies raised against a synthetic peptide corresponding to a conserved amino acid sequence of the NGFR were used for this purpose. Western (immunologic) blot analyses show that these antibodies specifically recognize NGFR and precursor proteins. In both species, NGFR immunoreactivity is primarily associated with the early generated and transient subplate neuron population of the developing neocortex, as indicated by the following evidence: the immunoreactive cells (*i*) are located directly beneath the developing cortical plate, (*ii*) frequently have the inverted pyramid shape characteristic of subplate neurons, and (*iii*) can be labeled by an injection of [^{3}H]thymidine on embryonic day (E) 28, a time when only subplate neurons are being generated. Intense NGFR immunostaining is seen on the cell bodies of these neurons as early as E30, several days after their last round of cell division, and this immunostaining remains strong for ≈3 weeks. The NGFR immunoreactivity begins to decline around E52 and has disappeared from the region altogether by E60, at which time subplate neurons begin to die. The cellular localization and timing of expression suggest that the NGFR may play a role in the maintenance of subplate neurons and in the maturation of the cerebral cortex.**

During the development of the mammalian brain, the formation of the adult six-layered cerebral cortex is preceded by a period in which a special class of transient neurons is generated (1–3). These neurons are among the first postmitotic cells of the telencephalon and, in higher mammals, undergo their final round of cell division well before the neurons of the adult cortical plate [cat at embryonic day (E) 24–30 (1, 4); ferret at E20–24 (3)]. These earliest generated cells then migrate away from the germinal zone and initially take up positions immediately beneath the pial surface. However, as the neurons of the future cortical plate migrate out and assume their final locations, the early generated neuronal population is split into two parts, the subplate, which is located directly beneath the developing cortical plate, and the marginal zone, which is located directly above it (4).

Subplate neurons mature rapidly and express immunoreactivity not only to neurotransmitters but also to microtubule-associated protein 2 (MAP2) long before the cortical neurons do (5, 6). Furthermore, they receive functional synaptic inputs (refs. 5 and 7; E. Friauf, S. K. McConnell, and C.J.S., unpublished data) and pioneer elaborate projections to cortical and subcortical targets (5, 8). Subplate neurons are also thought to interact with the many ingrowing axonal systems from the thalamus and cortex that are known to wait in the subplate zone (2, 9, 10) before finally invading the cortical plate, at ≈1 week before birth (11). After the waiting period ends, subplate neurons begin a programmed cell death, which eliminates over 90% of the population, until finally only ≈10% remain by 3 months of age (12, 13). Here we examine the possibility that nerve growth factor (NGF) plays a role in the maintenance of subplate neurons during early cortical development and in their subsequent death. We studied the distribution of NGF receptor (NGFR) immunoreactivity of the subplate neurons from the time they become postmitotic until after they begin to die, in view of recent reports that the receptor is likely to be transiently expressed in the central nervous system and, in particular, in the telencephalon, during embryonic and neonatal life (14–17).

MATERIALS AND METHODS

Eight cat brains between E35 and E60 and four ferret brains, ages postnatal days (P)2 and 10 were studied. Cat fetal tissue was obtained by sterile surgery on timed pregnant cats, as described (1, 4). Timed pregnant sable ferrets were obtained from Marshall Laboratories (North Rose, NY). As discussed below, the pace and pattern of development of cat and ferret cortex are strikingly similar.

Preparation of Antibody. The peptide YNSRPVNQTPP-PEGEKLHSD (in one-letter code), corresponding to residues 258–276 of the rat low-affinity NGFR (18) plus a terminal tyrosine, was synthesized commercially using FMOC (9-fluorenylmethoxycarbonyl) chemistry. This sequence is part of the intracellular domain of the protein and is completely conserved at the amino acid level between the rat and human low-affinity NGFR (18, 19). The peptide was crosslinked to porcine thyroglobulin by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. One milligram of thyroglobulin conjugate (corresponding to 100 μg of peptide) was mixed with 100 μg of free peptide, emulsified with 3 vol of Freund's complete adjuvant, and injected into 10 intramuscular sites of four female New Zealand White rabbits. The animals were reinjected subcutaneously with the same amount of antigen in Freund's incomplete adjuvant at

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Abbreviations: NGF, nerve growth factor; NGFR, NGF receptor; E, embryonic day; P, postnatal day; MAP2, microtubule-associated protein 2.

*To whom reprint requests should be addressed.
†Present address: Genentech, Inc., 460 Point San Bruno, South San Francisco, CA 94080.

monthly intervals. Rabbits were bled 2 weeks after each reinjection, and antipeptide antibodies were affinity-purified with a column of peptide crosslinked via glutaraldehyde to bovine serum albumin bound to Affi-Gel (Bio-Rad) and eluted with glycine hydrochloride, pH 2.5. Before use, antibodies thus obtained were adsorbed with a similar column containing a NGFR-unrelated peptide bound to bovine serum albumin. All of the experiments in this study were carried out with the antibodies obtained from a single bleeding of one rabbit.

Western (Immunologic) Blots. Telencephalon tissue (including cortical plate and subplate; excluding hippocampus) was dissected immediately after euthanasia of animals of the appropriate age, homogenized in 10.8% sucrose, and centrifuged at 3000 × *g* for 5 min. The pellet was dissolved in 0.1 M phosphate-buffered saline with 0.5% Nonidet P-40 (Sigma), incubated on ice for 10 min, and microcentrifuged. The optical density at 280 μm was read to estimate the protein content of the Nonidet P-40 soluble fraction, and ≈200 μg of protein was applied per lane to an SDS/polyacrylamide gel (7.5%) for electrophoresis at 30 V. The protein was transferred to nitrocellulose, and the blot was incubated with anti-NGFR antibody, iodinated by using Iodogen (Pierce) to a specific ^{125}I activity of 6.5 × 10^8 cpm/μg.

Immunohistochemistry. Brains were prepared for immunohistochemistry by transcardial perfusion of the cat fetuses or ferret neonates with 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer followed by 6–12 hr postfixation in the same solution. Tissue was equilibrated in 20% (wt/vol) sucrose, and coronal cryostat sections (20 μm) were cut and mounted on gelatin/albumin-coated slides. Sections were incubated in 1:1000 anti-MAP2 serum (5, 6) (provided by R. Vallee, Worcester Foundation for Experimental Biology), anti-NGFR antibody at 1 μg/ml, or anti-NGFR antibody at 1 μg/ml blocked by 100-fold excess of the peptide against which the antibody was raised. Antibody binding was visualized by using peroxidase immunohistochemistry (ABC, Vector Laboratories). [^{3}H]Thymidine was injected into fetuses *in utero* at E28 and visualized autoradiographically at E47 by coating mounted brain sections with Kodak NTB-2 emulsion and exposing them in the dark at 4°C for 4 to 6 weeks as described (1). The immunohistochemical results are summarized in Table 1.

RESULTS

To investigate the presence and distribution of NGFR in the cerebral cortex during development, we immunostained sections from fetal cat brain at ages between E30 and E60 and neonatal ferret brain at postnatal day (P) 2 and P10 (see Table 1). Many previous studies have demonstrated that these two species have a remarkably similar pace of cortical development during fetal life, even though the ferret is born at 41 gestational days (3, 20), whereas the cat is born at 65 days (1) (e.g., a ferret at P2 is developmentally equivalent to a cat at E43). After the ferret is born, its development speeds up in relation to that of the cat, which remains *in utero*, so that by P10 a ferret cortex resembles that of an E60 cat.

Specificity and Cross-Reactivity of the Antibody with the NGFR in Developing Carnivore Telencephalon. The Western blots of Fig. 1 show that the anti-NGFR antibody recognizes a doublet with molecular masses of 80 kDa and 71 kDa in proteins extracted from a rat cell line [PC12 (21)] and a mouse cell line transfected with rat genomic DNA [PCNA15 (18)], both of which are known to express the rat NGFR in large quantities. The 80-kDa band corresponds closely to the molecular mass measured for the rat low-affinity NGFR by other means (22). The 71-kDa band corresponds to a metabolic precursor of the NGFR (D.L.S. and E.M.S., unpublished data). Immunoreactivity corresponding to precursor and receptor bands can also be seen in the ferret and cat protein extract lanes (Fig. 1*a*), indicating that a specific molecule similar or identical to the NGFR is present in ferret and cat telencephalon at early developmental ages. The antibody binding to the pair of bands in all instances can be blocked by incubating the blot with antibody in the presence of 100-fold excess of NGFR peptide (Fig. 1*b*).

Localization of NGFR Immunoreactivity to Subplate Neurons. A section through the neocortex of a P2 ferret immunostained for MAP2, demonstrating the location of the subplate neurons and their processes, is shown in Fig. 2*a*. As in the cat at comparable ages (E43) (6), the heaviest immunostaining is restricted to the subplate and marginal zone neurons.

NGFR immunoreactivity is also present in the subplate region of the neonatal ferret (P2) and fetal cat (E43), as shown in Fig. 2 *b* and *c*. The cortical plate and the marginal zone are

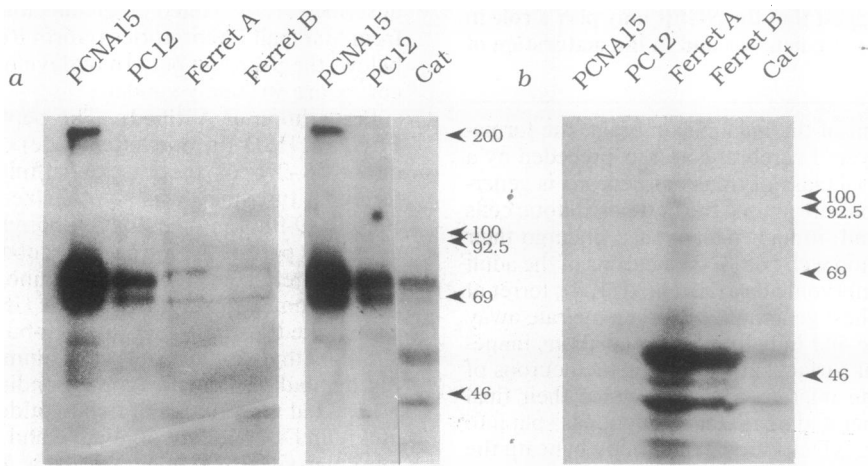

FIG. 1. Western blot reveals that P2 ferret and E43 cat telencephalon contain NGFR-like molecules (*a*), and labeling of the bands corresponding to these molecules can be blocked by incubation with antibody in the presence of excess NGFR peptide (*b*). PCNA15 is a mouse cell line transfected with rat genomic DNA that expresses 1–2 million NGFR molecules per cell (enriched from the PCNA10 cell line described in ref. 18 by further rounds of cell sorting); PC12 is a rat pheochromocytoma cell line known to express NGFR (21). Relative molecular mass in kDa is shown at right. Approximate molecular masses of ferret receptor, 82 kDa; ferret precursor, 68 kDa; cat receptor, 78 kDa; and cat precursor, 68 kDa. Lower molecular mass bands, ≈50 and 37 kDa, were also seen in all cat tissue (Fig. 1 *a* and *b*, cat lanes) and in ferret tissue 2 of 4 times that it was studied (Fig. 1*b*, ferret lanes). Labeling of these bands was not seen consistently (it is absent in Fig. 1*a*, ferret lanes) and seemed to increase in the same tissue preparation with the time of extract storage. Labeling of these bands was not blocked by incubation with antibody in the presence of NGFR peptide (Fig. 1*b*) and therefore represents nonspecific binding.

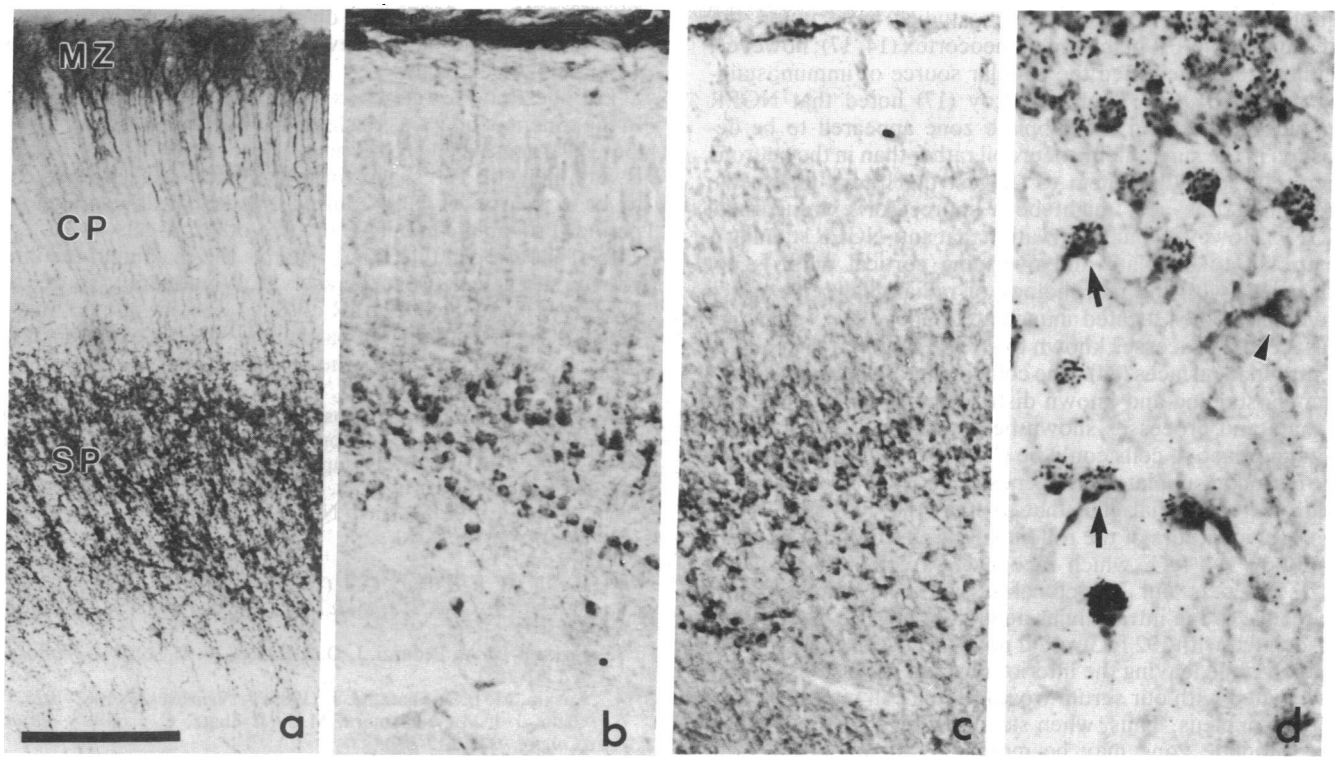

FIG. 2. Location and appearance of ferret (*a* and *b*) and cat (*c* and *d*) subplate neurons in coronal sections. (*a*) MAP2 immunostaining of the cerebral wall in P2 ferret reveals the subplate neurons. SP, subplate; CP, cortical plate; MZ, marginal zone. Section from the same P2 ferret (*b*), E43 cat (*c*), and E47 cat (*d*), each stained with antiserum to NGFR. In *d*, subplate neurons were identified by their birthdates with [^{3}H]thymidine labeling at E28 (arrows). Because thymidine is only available for uptake for 3 hr, not all NGFR-immunostained subplate neurons are thymidine-labeled (arrowhead). [Scale bar = 150 μm (*a*); 100 μm (*b* and *c*); and 40 μm (*d*).]

lightly stained; deeper regions of the developing white matter are not stained. Intense staining is also seen in the pia, as noted previously (16). With the exception of a light band at the pial surface, all immunostaining is blocked by incubation of the sections with antibody in the presence of 100-fold excess of NGFR peptide; sections incubated with normal rabbit serum showed only a light, diffuse background staining of all cells.

Most, if not all, the immunostaining in the subplate zone is associated with cell bodies of the subplate neurons. For example, as shown in the higher magnification view of the subplate from an E47 cat in Fig. 2*d*, many immunoreactive cells have the inverted-pyramid morphology characteristic of subplate neurons (5, 6). It is also noteworthy that subplate neurons are known to send processes into the cortical plate, which may account for the presence of the diffuse immunostaining of the cortical plate noted above (refs. 6 and 23; E. Friauf, S. K. McConnell, and C.J.S., unpublished data). The immunoreactivity cannot be localized to specific axons or dendrites of the subplate neurons. However, the perinuclear distribution of the staining at the soma is consistent with the fact that the antibody was generated to and recognizes an intracellular epitope of the NGFR.

To prove that cells immunoreactive for the NGFR are, indeed, subplate neurons, we took advantage of the fact that, as mentioned above, the subplate neurons have unique birthdates and therefore can be identified conclusively on the basis of [^{3}H]thymidine autoradiography. For example, an injection of [^{3}H]thymidine at E28 labels only subplate neurons (4); therefore, all cells that show silver grains over their nuclei after autoradiography can be identified as belonging to the subplate neuron population (6). The results of such an experiment are included in Fig. 2*d*: all thymidine-labeled (subplate) cells are NGFR-immunoreactive. On the other hand, not all immunoreactive cells are [^{3}H]thymidine labeled, an observation consistent with the fact that the thymidine is only available for uptake for ≈3 hr and, therefore, will only label a fraction of the entire subplate population, which is generated over a week (4).

Time Course of NGFR Expression in the Subplate. Tissue from brains of various developmental ages was stained for NGFR immunoreactivity (Table 1). Staining is present in the cat subplate at early fetal ages (E30), remains intense during the ensuing 2 weeks, and then declines and disappears by ≈1 week before birth (E60). No NGFR immunoreactivity can be found anywhere in the subplate or cortex by this age. Comparable changes are seen in the ferret brain. Thus, NGFR immunostaining in the subplate is transient.

DISCUSSION

The main finding of this study is that NGFR immunoreactivity present within the developing neocortex is largely associated with a special class of transient neurons, the

Table 1. Time course of NGFR immunoreactivity in subplate region

Gestational age		Intensity of anti-NGFR staining	
Cat	Ferret	Cat	Ferret
E30		++	
E35		++	
E43	P2	++	++
E47		++	
E52		+	
E57		+/−	
E60	P10	−	−

++, Strong; +, moderate; +/−, weak; −, absent.

subplate neurons. Two previous studies have noted the presence of NGFR in the rodent neocortex (14, 17); however, neither study identified the cellular source of immunostaining. In their study, Koh and Loy (17) noted that NGFR immunoreactivity in the subplate zone appeared to be deposited in the surrounding neuropil rather than in the neurons themselves, leading them to suggest that radial glial cells, present at this age, might be a source for extraneuronal NGFR. However, our observation that anti-NGFR staining is restricted to subplate and overlying cortical plate is not consistent with the morphology of radial glial cells, which have cell bodies located immediately above the ventricular surface and processes known to stretch between the pial and ventricular surfaces (24). The coincidence between pattern of immunostaining and known distribution of subplate neuron somata and processes shown here and the fact that NGFR immunoreactive cells could be [^{3}H]thymidine-labeled during the period of subplate neurogenesis indicate that it is subplate neurons, not radial glia, that express the NGFR. Furthermore, the authors of the rodent studies used the monoclonal antibody 192 IgG, which recognizes a cell-surface epitope (25), whereas our polyclonal serum was generated to a sequence in the intracellular domain of NGFR. The staining associated with 192 IgG would probably outline neuronal cell bodies while leaving the interior unstained, whereas staining associated with our serum would fill the cell interior, except for the nucleus. Thus, when stained with 192 IgG, NGFR in the subplate zone may be more difficult to localize with certainty.

Subplate neurons are immunoreactive for the NGFR as early as E30, just as the last of these neurons become postmitotic (1, 4) and the first of them begin to project axons to subcortical targets (8). These neurons remain immunoreactive during the month-long period in which they mature and ingrowing axons from thalamus and other regions of cortex accumulate around them. Then NGFR immunoreactivity is lost by E60, a time when the waiting axonal systems have just left the subplate to invade the cortical plate (2) and at a time when subplate neurons, though still present in appreciable numbers, have begun to disappear by cell death (6, 12, 13). The complete loss of NGFR immunoreactivity at this age cannot be due to the disappearance of the neurons themselves because many MAP2-immunoreactive cells and processes can still be seen in the subplate zone at E60, although their density is beginning to decrease (6, 12, 13). Furthermore, between E52 and E57, a progressive decrease in the overall intensity of NGFR immunostaining on each neuron is seen, not a decrease in the total number of NGFR-immunoreactive neurons, suggesting a down-regulation of receptor expression on the cells rather than elimination of NGFR-immunoreactive neurons by cell death at this time.

These correlations raise several possibilities concerning the functional significance of NGFR expression on subplate neurons. (*i*) The presence of its receptor at early ages could indicate that NGF guides the growth of subplate axons in a manner similar to that suggested for the guidance of axons of sympathetic neurons *in vivo* (26) or chicken sensory neurons *in vitro* (refs. 27 and 28; for an alternate view, see ref. 29). (*ii*) NGF has also been shown to regulate the expression of neuropeptide genes in sensory neurons (30) and could do the same for neuropeptides in the subplate. (*iii*) Alternatively, loss of NGFR from subplate neurons may play a role in bringing the axonal waiting period to a close. (*iv*) A final possibility is that, as in the development of the peripheral nervous system (31–33) and perhaps also that of the basal forebrain (34, 35), NGF might act to sustain subplate neurons. If so, the loss of NGFR on subplate neurons may be the first step in a cascade of events that eventually leads to programmed cell death.

The question then arises as to the cellular source of NGF. In the rodent neocortex, the level of NGF mRNA is very low (36, 37) at ages when NGFR immunoreactivity in the subplate is very high (14, 17), suggesting that cortex itself is not likely to be a source of NGF during this early developmental period. Probable candidates include other targets and afferents of the subplate neurons, such as the thalamus, tectum, or even subplate neurons themselves. Whatever the source of NGF, the presence of NGFR on this transient population of neurons suggests that NGF participates in the developmental processes that give rise to the structure of adult cortex.

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