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Immunoglobulin Molecules Are Present in Early-Generated Neuronal Populations in the Rat Cerebral Cortex and Retina

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ABSTRACT

Immunoglobulin-like antigens have been identified in neuronal subsets restricted to deeper layers of the developing mammalian cerebral cortex. The pattern is suggestive of selective uptake of immunoglobulin (Ig) from serum or synthesis of Ig or a molecule with Ig-like motifs. To distinguish between these alternatives, biochemical, immunocytochemical, and birthdating analyses were conducted. In neonatal rat cerebral cortex, immunoglobulin-like immunoreactivity was chiefly in subplate neurons, marginal zone neuropil, and processes spanning the cortical plate. Isolated staining was also observed within the ventricular zone. Although most staining in the cortical plate was absent several days after birth, subplate neuron staining persisted until the end of the second postnatal week. Quantitative immunoassays showed that the antigen concentration dropped from 130 ng/mg in cortical cytosol at birth to approximately 80 ng/mg by postnatal day 7 (P7) and remained low thereafter. Two Ig-immunoreactive polypeptides with mobilities similar to heavy and light chains of serum IgG, were identified by Western blotting. The larger band was purified, partially sequenced by Edman degradation, and found to match rat IgG heavy chain. Bromodeoxyuridine birthdating and anti-IgG double-labeling studies showed that most of the Ig-containing cells were early-generated neurons. Outside of the cortex, transient IgG staining was also detected in neurons of the retina and cerebellum. These studies suggest that subplate and other early-generated neurons selectively take up Ig from serum. The IgG may then either be degraded or lost from the central nervous system (CNS) during developmentally regulated cell death. J. Comp. Neurol. 384:271–282, 1997.

Indexing terms: cell death; visual cortex; subplate; macrophage; blood-brain barrier

The mammalian neocortex undergoes remarkable transformations between embryonic and early postnatal periods. Axonal remodeling and neuronal death are known to contribute to these changes, but the underlying cellular and molecular mechanisms are unclear. One population of transient neurons resides in the subplate, beneath the cortical plate. In cats, ferrets, and primates, subplate neurons are widely dispersed within the future white matter (reviewed in Finlay, 1992), but in rodents, they are confined to a thin layer beneath layer 6a, designated 6b or 7 (Marin-Padilla, 1978). These neurons are generated early in cortical neurogenesis (Kostovic and Rakic, 1980; Luskin and Shatz, 1985; Bayer and Altman, 1990), extend pioneering corticofugal axons (Kostovic and Molliver, 1974; McConnell et al., 1989; DeCarlos and O'Leary, 1992), and establish initial synaptic connections with thalamic axons (Molliver et al., 1973; Friauf et al., 1990) and projections from the brainstem before other cortical neurons (Caviness and Korde, 1981; Blue and Parnavelas, 1983a,b; Chun and Shatz, 1988b; Friauf et al., 1990). Subplate neurons also mature electrically (Friauf et al., 1990) and neurochemically (Wahle et al., 1987; Chun and Shatz, 1989) but then undergo a wave of perinatal cell death (Ferrer et al., 1990; Woo et al., 1991; Price et al., 1997).

Evidence suggests that the subplate neurons guide the formation of afferent and efferent cortical projections (Shatz et al., 1988; McConnell et al., 1989; Kostovic and

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Rakic, 1990; DeCarlos and O'Leary, 1992; Miller et al., 1993). In the visual cortex, invading geniculocortical fibers initially extend transient collateral branches into the subplate and establish functional synaptic circuits (Lund and Mustari, 1977; Naegele et al., 1988; Friauf et al., 1990; Ghosh and Shatz, 1992). These branches are later retracted, and new collaterals form within the normal target layers of the cortical plate. The changing axonal branching patterns are accompanied by changes in the distribution of synapses (Blue and Parnavelas, 1983b; Chun and Shatz, 1988b). Concurrent with this phase of axonal and synaptic remodeling, neural cell adhesion molecules and neurotrophin receptors in the subplate are down-regulated, and subplate neurons begin to die (Stewart and Pearlman, 1987; Chun and Shatz, 1988a; Shatz et al., 1988; Koh and Loy, 1989; Antonini and Shatz, 1990; Allendoerfer et al., 1994).

To further study functions of subplate neurons and causes for their elimination, specific markers are needed. One method for marking subplate neurons is by birthdating (Miller and Nowakowski, 1988; Soriano and del Rio, 1991). Recently, hybridoma technology has also been utilized for this purpose (Naegele et al., 1991). Monoclonal antibody (mAb) SP-1 is a reagent that labels subplate neurons of kitten visual cortex during early postnatal periods (Naegele et al., 1991; Wahle et al., 1994). The antigen is found in brain, liver, and plasma and has an apparent molecular weight of 56 kilodaltons (kDa) on Western blots (Naegele et al., 1991; Wahle et al., 1994; Dunn et al., 1995). Partial amino acid sequence of the plasma SP-1 antigen along with the ability of polyclonal antisera against IgG to stain subplate neurons in several vertebrate species strongly suggest that the subplate neurons contain immunoglobulin molecules (Fairen et al., 1992; Henschell and Wahle, 1994; Dunn et al., 1995). Until the present study, however, attempts to purify and obtain the amino acid sequence of the subplate antigen have been unsuccessful.

In this study, our first goal was to identify and quantify the rat subplate IgG-like antigen by biochemical purification, amino acid sequencing and enzyme-linked immunosorbent assays (ELISA). These investigations showed that the subplate antigen is IgG and that IgG levels in the developing cortex are high at birth, but then they drop rapidly within the first few postnatal days. Our second goal was to determine whether IgG-immunoreactive cell types in the cerebral cortex, and elsewhere, were generated early in neurogenesis. Birthdating methodology combined with immunostaining for IgG showed that, although embryonic day 13 (E13)-generated cortical neurons were IgG immunoreactive, later-generated neurons were not. We conclude that IgG is a reliable marker for developing subplate neurons during the perinatal period of cell death, but it does not remain detectable within the remnants of the subplate population that persist. The presence of IgG in early-generated neurons in multiple regions of the central nervous system (CNS) suggest that it may be taken up selectively by these populations before formation of the blood-brain barrier or may be produced by subpopulations of neurons and then down-regulated.

MATERIALS AND METHODS

Gel electrophoresis and Western blotting

Postnatal day 0, (P0, n = 10), P5 (n = 10), P7 (n = 12), P11 (n = 8), P15 (n = 9), and adult (n = 3) Long Evans rats (Charles River Laboratories, Raleigh, NC) were given an anesthetic overdose of Nembutal (60 mg/kg i.p.) and then perfused transcardially with homogenization buffer (0.005 M HEPES, pH 7.4, containing 0.32 M sucrose and 0.008 M EDTA). The cerebral cortices were removed, and cytosolic fractions were prepared as described previously (Dunn et al., 1995). Thirty micrograms per lane of cortical cytosol fractions were loaded onto 10% SDS-polyacrylamide gels, separated by electrophoresis, and transferred onto nitrocellulose, as described previously (Laemmli, 1970; Towbin et al., 1979; Dunn et al., 1995). The blots were probed overnight at room temperature (RT) with a 1:3,000 dilution of biotinylated goat $F(ab')_2$ anti-rat IgG (Cappell, Durham, NC) in Tris-buffered saline (TBS, 0.01 M Tris, pH 7.5) containing 5% nonfat dry milk. Blots were incubated in streptavidin horseradish peroxidase (HRP, 1:1,000, Vector, Burlingame, CA) for 1 hour at RT. Immunoreactive bands were detected by using chemiluminescence (ECL kit, Amersham, Arlington Heights, IL). Mobilities of immunoreactive bands were calculated by linear regression, and the results were averaged from at least 5 Western blots. Preadsorption controls were performed by adsorbing diluted IgG antisera with 30–40 µg of serum IgG overnight at 4°C. The final concentration of antiserum was adjusted to 1:3,000 before use on Western blots.

Purification of the larger IgG immunoreactive polypeptide

The 53-kDa IgG immunoreactive band was purified from cortical cytosol by sequential chromatography by using DEAE followed by anti-IgG affinity columns. For sequencing, P2 rat cortical cytosol (11 mg total protein/10 ml) was passed over a 10-ml column of the anion exchanger, DEAE Affi-Gel Blue (Econopac, Bio-Rad, Cambridge, MA). Protein was eluted with 0.02 M Tris-HCl, pH 8.0, containing 0.025 M NaCl, and desalted (Centriprep-10, Amicon, Beverly, MA). The partially purified protein was then diluted in homogenization buffer and passed three times over an IgG immunoaffinity column (5 mg goat anti-rat IgG coupled to 2 g cyanogen bromide-activated sepharose 4B (Sigma, St. Louis, MO). Bound antigens were eluted with 3 M guanidine-HCl (Sigma). Fractions were pooled, concentrated to 0.04 mg/ml, and desalted.

The purified sample was electrophoresed through a 10% acrylamide gel, visualized by Coomassie Blue stain, excised from the gel, and subjected to proteolytic digestion. The peptide fragments were separated by reverse-phase liquid chromatography, analyzed by ion-spray mass spectroscopy, and sequenced by Edman degradation (Jeff Hulmes, W. Alton Jones Cell Science Center, Lake Placid, NY). The PIR database (Release 47) was searched for amino acid homology, and an alignment analysis was done with the BioSCAN program (Chapel Hill, NC).

Gels and Western blots were scanned into Adobe Photoshop software by using a Hewlett Packard Scanjet 4c scanner and printed on a dye sublimation printer.

Enzyme-linked immunosorbent assay (ELISA)

Individual and pooled cortical cytosol fractions from postnatal rat pups were prepared by differential centrifugation. Immulon 2 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with goat anti-rat IgG (H + L) at 1 µg/ml (Southern Biotechnology, Birmingham, AL) in coating buffer (15 mM $NaHCO_3$, 35 mM Na_2CO_3, pH

9.6). Plates were incubated at 37°C for 1 hour, and wells were aspirated. Nonspecific binding to the plates was blocked by using coating buffer containing 2% BSA for 1 hour at 37°C. The plates were washed five times with buffer (137 mM NaCl, 2.6 mM KCl, 1.6 mM KH_2PO_4, 1 mM Na_2HPO_4, pH 7.4) containing 0.05% Tween 20. Rat IgG, in known amounts, was used to construct a standard curve to determine the concentration of IgG in rat brain cytosolic fractions. Both were plated in triplicate in binding buffer (0.1 M PBS, 0.05% Tween 20, 0.25% BSA) and incubated for 1 hour at 37°C. The microtiter plates were washed as described above, HRP conjugated goat anti-rat IgG (1: 10,000, Southern Biotechnology) was added for 1 hour at 37°C, and the plates were washed again. Bound antibody was detected by adding TMB substrate (3,3′,5,5′-tetramethylbenzidine, KPL, Gaithersburg, MD). Following a 10-minute incubation at room temperature (RT), the reaction was stopped with 0.5 N HCl. Color development was determined by using a microplate reader (model 3550-UV, BioRad, Hercules, CA) at 450 nm.

Immunocytochemistry

Postnatal animals were anesthetized with Nembutal (60 mg/kg i.p.), perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), postfixed in the same solution for 2 hours, cryoprotected, and stored at −80°C. Ten-micron-thick cryostat sections of retina or brain were incubated in phosphate-buffered saline (PBS, 0.1 M, pH 7.4), containing 5% normal horse serum (NHS) to block nonspecific staining. Ig-immunoreactive cells were visualized by immunoperoxidase staining as described previously (Dunn et al., 1995) with slight modifications; biotinylated anti-rat IgG (1:600–1:1,200, Vector) was used as a primary antibody followed by avidin biotin complex coupled to (ABC) peroxidase (Vector), and diaminobenzidine (DAB) histochemistry.

Two-color fluorescent labeling, to identify retinal ganglion cells and Ig-reactive neurons, was accomplished by immunofluorescent staining. Briefly, retinal sections were incubated in mouse anti-neurofilament antibody (ms-NF-200 Sigma, 1:400) at RT overnight, followed by incubation in Texas Red-conjugated goat anti-rat IgG (Vector, 1:500) at RT overnight. Ms-NF-200 was detected with fluoroisothiocyanate (FITC)-conjugated horse anti-mouse IgG (rat absorbed, 1:500, Vector) for 1 hour. Sections were washed and mounted in Vectashield (Vector).

Bromodeoxyuridine (BrdU) injections and immunocytochemistry

Timed-pregnant rats (Charles River Laboratories) received three intraperitoneal injections of BrdU (50 mg/kg, each) in 0.1 M Tris-buffered saline, pH 7.4, over a 24-hour period. BrdU injections were made into the mother on embryonic days 12 (E12), E13, E14, E15, E16, or E17. After normal delivery, the pups were euthanized with Nembutal (60 mg/kg) on P7, and brains were fixed for immunocytochemistry by transcardiac perfusion, as described above. Tissue was cryoprotected, embedded in freezing media (Polysciences, Warrington, PA), and stored at −80°C until further use. Ten-micron-thick cryostat sections were cut in the parasagittal plane and thaw-mounted onto poly-L-lysine-coated slides. BrdU was detected by the method of Magaud et al. (1989), with slight modifications. Sections were treated with Pronase E (3 μg/ml) for 10 minutes, followed by 2 N HCl treatment for 30 minutes at RT. Serial sections were incubated in mouse anti-BrdU (Sigma, 1:100) overnight at 4°C, washed, and incubated in FITC-conjugated horse anti-mouse IgG. Ig immunoreactivity was detected with Texas Red conjugated rabbit anti-rat IgG (Vector, 1:500), or biotinylated rabbit anti-rat IgG (Vector, 1:1,000), followed by avidin-Neutralite Texas Red (1:300, Molecular Probes, Eugene, OR). Sections were washed in PBS and coverslipped in Vectashield (Vector). Fluorescent double-labeled sections from P7 animals were viewed under epifluorescence, and counts were made of Ig-positive/BrdU positive (IgG+/BrdU+) and IgG−/BrdU+ populations. Counts were made from 4 cortical sections taken from two animals and included a total of 127 BrdU+ neurons.

Color slides of double staining were scanned by using a slide scanner, formatted by using Adobe Photoshop software, and printed with a dye sublimation printer.

The animal study protocols used comply with NIH guidelines on the Care and Use of Animals in Research and were reviewed and approved by the Wesleyan Animal Care and Use Committee.

RESULTS

IgG-immunoreactive cortical antigens correspond to two polypeptides of 53 and 27 kDa

To characterize the immunoreactive antigens, Western blots of exsanguinated rat cerebral cortex were probed with anti-rat IgG, and the results are shown in Figure 1A. At all ages examined, two IgG immunoreactive bands were enriched in cytosolic fractions, with molecular weights of 53 ± 3 and 27 ± 2 kDa. Both bands were present at high levels in the cortex at early postnatal ages (lanes P0, P5, P7, P11), and lower levels were detected by P15 and adulthood. Specificity of these bands was shown by preadsorbing IgG antiserum (P7) or omitting the primary antibody (not shown). These procedures abolished staining of the 53- and 27-kDa bands. Under the same denaturing conditions, the heavy (H) and light (L) chains of IgG migrated at 55 and 27 kDa, respectively (Fig. 1A, rat IgG). Although the 27-kDa band in cortex and IgG light chain from serum had the same mobility, there was a slight difference between the 53-kDa cortical band and the 55-kDa IgG heavy chain. The minor difference in their mobilities may be due to differences in the degree of glycosylation. Additionally, a band that migrated at 35 kDa was also seen in cytosol fractions. This band may be a degradation product of IgG, because it was also detected in serum IgG when probed with anti-IgG whole molecule antibodies (not shown). Cortical membrane fractions also contained Ig-reactive polypeptides, although to a lesser extent (not shown).

Purification and amino acid sequencing of the 53-kDa polypeptide

To determine if the 53-kDa cortical antigen was IgG heavy chain, the polypeptide was affinity purified and the amino acid sequence was determined. The sequential stages of purification of the antigen are shown in Figure 1B. Crude cytosolic fractions from P2 rat cortex contained multiple polypeptide bands (Fig. 1B, Cytosol). The DEAE column resulted in selective enrichment of the 53-kDa

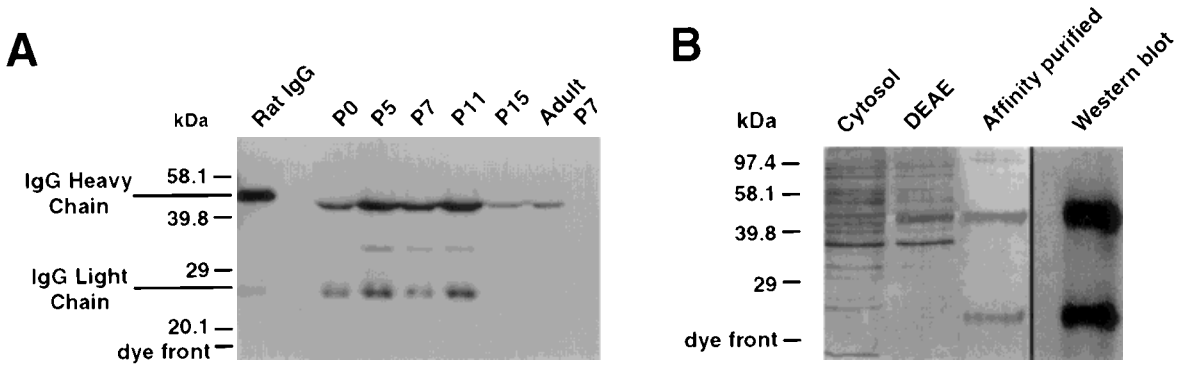

Fig. 1. **A:** Western blots of IgG-reactive proteins in postnatal rat cortical fractions show two polypeptides of 53 and 27 kDa. Rat serum IgG (heavy and light chains) was loaded into the first lane. Postnatal day (P) is designated at the top. At all ages examined, cortical cytosol fractions exhibit two IgG immunoreactive bands of 53 and 27 kDa. The last lane (P7) is a control in which the anti-rat IgG was preadsorbed with purified IgG. **B:** Affinity purification enriches both 53- and 27-kDa polypeptides from P2 rat brain. Silver-stained SDS-PAGE analysis shows polypeptides in unpurified cytosol (Cytosol, 10 µg). Fewer polypeptides remain after DEAE column chromatography (DEAE, 10 µg). Only two polypeptides (53 and 27 kDa) are enriched after immuno-affinity purification (affinity purified, 1 µg). A Western blot of the purified cortical antigen (1 µg) probed with anti-rat IgG shows that both purified polypeptides are IgG immunoreactive. Biotinylated reference proteins (SDS-6B, Sigma) indicated on the left of (A) and (B) correspond to: phosphorylase b (97.4 kDa), catalase (58.1 kDa), alcohol dehydrogenase (39.8 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa).

TABLE 1. Amino Acid Sequences and Putative Identity of the 53-kDa Cortical Band

Peptide	Amino acid sequence obtained[1]	Closest matching amino acid sequence in database	Alignment of peptide with database sequence	Putative identity
1	EEQFgstF	172 EEQFNSTF 179	EEQF_STF	IgG1 Cregion-rat
		179 EEQYNSTF 186	EEQ+_STF	IgG2bCregion-rat
2	VLAPPQEELSK	226 MAPPKEEMTQ 235	+APP+EE+++	IgG2aCregion-rat

[1]Upper case symbols represent sequence of amino acids obtained by Edman degradation; lower case symbols are tentatively assigned amino acids; +, conservative amino acid substitutions; underlined spaces, positions of nonaligning amino acids. The Bioscan Network Server, University of North Carolina, Chapel Hill, North Carolina, was used to search PIR database for amino acid homology and alignment (Singh et al., 1996).

band and slight enrichment of the 27-kDa band (Fig. 1B, DEAE). After anti-IgG chromatography, only these two bands were purified (Fig. 1B, affinity purified). Both bands remained strongly IgG immunoreactive following these purification procedures, as shown by Western blotting of the purified fraction (Fig. 1B, Western blot).

Two sequences were obtained (total of 19 amino acids) from two internal peptides generated from the 53-kDa band (Table 1). Upper case symbols indicate clear amino acid assignments and lower case symbols represent tentative assignments. Homology and alignment analyses showed a match between the purified antigen and the heavy chain of the rat IgG molecule. The sequence obtained from the first peptide corresponded to a portion of the CH1 domain of either IgG1 or IgG2b. The gamma subclass determination of this peptide was ambiguous due to the tentative assignment of three amino acids. The sequence obtained for Peptide 2 was located in the hinge region of the Ig gamma-2b chain. These data strongly suggest that the antigen present in postnatal cortex is in fact IgG.

Quantitative analysis of the postnatal decline in cortical Ig (H&L)

To estimate the quantity of IgG present in the developing cerebral cortex, ELISA assays were performed. The results revealed a dramatic decline in the amount of IgG per total cytosolic protein during the first postnatal week (Fig. 2). An average of approximately 130 ng/mg of total protein was detected at birth, which subsequently decreased to less than 80 ng/mg by P7 and remained low thereafter. Statistical analyses revealed the most significant loss of IgG occurred between birth and postnatal day 7. These data correspond closely with the loss of IgG reactivity seen in the cortical subplate (below).

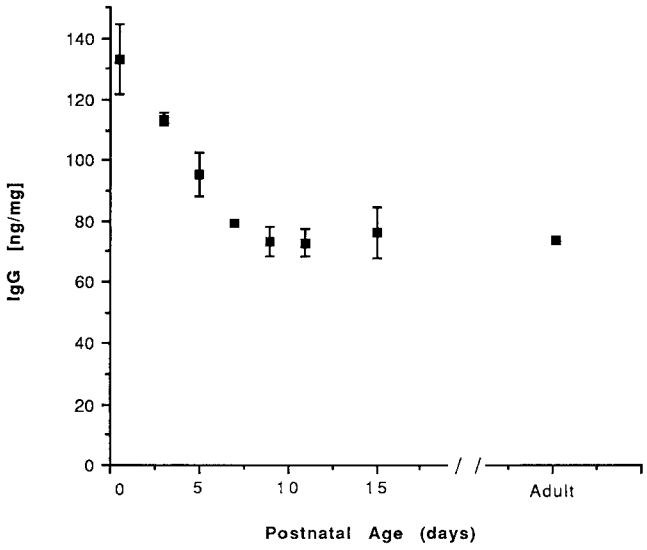

Fig. 2. Postnatal decline of cortical IgG. Amount of IgG in total cytosolic protein (ng/mg) is plotted against age. There is a high level of IgG at birth, which steadily declines until P7 and remains at low levels thereafter. Results from pooled and individual cytosol preparations follow the same pattern of decline. Each square represents the average of measurements from 3 animals and two separate ELISA assays. Bars show standard error.

Loss of IgG in the marginal zone and subplate

To determine the spatial and temporal distribution of IgG in the postnatal cortex, immunocytochemical studies were conducted. Changes in the cytoarchitecture of the developing cerebral cortex were compared in alternate sections with corresponding changes in IgG immunostaining. As shown in Figure 3A, cortical layers 2–6 were not yet differentiated from the cortical plate by P1 (CP, Boulder Committee, 1970). The subplate zone (SP) was a compact layer separated from the overlying CP by a small gap. In adjacent sections, prominent IgG immunoreactivity was observed in nearly every neuron within the subplate region on P1 and P6 but not P15 (Fig. 3B). In P1, but not older animals, IgG staining was also observed in the extracellular matrix of the marginal zone and radially oriented processes spanning the cortical plate (Fig. 3B). Although remnants of the subplate neuron population were clearly present at P15, these neurons were not IgG immunoreactive (Fig. 3B, P15 SP). Occasional endothelial cell staining was also observed at all ages (Fig. 3B, arrowheads). No staining was seen in control immunostaining experiments, when anti-rat IgG antibodies were preadsorbed with pure rat serum IgG (data not shown). These results suggest that, although IgG immunoreactivity is lost from subplate neurons after the first postnatal week, slight IgG immunoreactivity persists in endothelial cells. Thus, the small amount of IgG detected by ELISA assays of P15 or adult tissue most likely reflects IgG associated with endothelial cells.

E13 but not E17 generated neurons show IgG immunoreactivity

The times of neurogenesis of IgG-immunoreactive cortical neurons were determined by BrdU birthdating. BrdU incorporation in E12–E14 fetuses labeled subplate and marginal zone neurons at postnatal ages 2, 7, and 15, in agreement with previous studies (Bayer and Altman, 1990, data not shown). BrdU incorporation in E16–E17 fetuses resulted in upper cortical plate labeling. Double-labeling experiments were carried out in P7 pups to verify that the IgG-immunoreactive cortical neurons had birthdates typical for subplate neurons (Fig. 4). IgG-immunoreactive subplate neurons were BrdU immunoreactive after injections on E13 (Fig. 4A,B). BrdU was not observed in the IgG-immunoreactive subplate neurons when the injections were made on E17. Conversely, when upper cortical plate neurons were labeled by E17 BrdU injections (Fig. 4C), they were not IgG immunoreactive (Fig. 4D). When the P7 labeling was quantified, it was found that IgG/BrdU double-labeling occurred in 70% of the subplate neurons (90 of 127 IgG+/BrdU+). However, when littermates were allowed to survive until P15, none of the remaining BrdU+ subplate neurons were IgG immunoreactive (0 of 42 IgG+/BrdU+). These results suggest that levels of IgG in subplate neurons decline with maturation. Alternatively, a majority of subplate neurons may initially contain IgG, and these neurons might be preferentially eliminated by cell death. If so, then IgG immunoreactivity may be a marker for subplate neurons destined to undergo developmentally regulated cell death.

Retinal ganglion cells exhibit transient IgG immunoreactivity

Because birthdating studies suggested that only early-generated cortical neurons might be IgG positive, we next asked whether early-generated retinal neurons were also IgG positive. Previous studies showed that retinal ganglion cells are generated between E14 and E20 (Reese and Colello, 1992). Double-labeling studies were performed with sections of rat retina, by using anti-IgG sera in combination with an anti-neurofilament monoclonal antibody (NF-200) specific for phosphorylated and nonphosphorylated forms of the heavy subunit. From previous studies, it was known that this subunit was detectable in a small fraction of ganglion cells of the rodent retina (Drager et al., 1984). As shown in Figure 5, NF-200 and IgG colocalized in some neurons in the ganglion cell layer. Although IgG immunoreactivity was present in a majority of ganglion cells at P9, the heavy subunit of neurofilament protein was present in only a subset. Although NF-200 labeling was present in most, if not all, retinal ganglion cells of the adult retina (Shaw and Weber, 1982), IgG immunoreactivity was absent (not shown).

Other IgG-immunoreactive populations in the developing CNS

To gain further insight into possible sources and functions of intraneuronal IgG, other regions were examined from developing rat brain (Fig. 6). One distinctive population of IgG-immunoreactive neurons was in the anterior olfactory nucleus (Fig. 6A). In the cerebellum, transient neuronal staining was noted in both the cerebellar medulla (Fig. 6B) and deep cerebellar nuclei (Fig. 6C). Patches of stained neurons were also observed in the superior and inferior colliculi and brainstem regions (data not shown), as well as neurons in the ganglion cell layer of the retina (described above). Although these regions exhibited strong staining within the first postnatal week, IgG immunostaining was undetectable by the end of the second week. Thus, the pattern of transient neuronal staining found in the cerebral cortex was replicated in a number of other CNS regions.

DISCUSSION

This is the first study to show by amino acid sequencing that one of the transient IgG-immunoreactive bands in developing brain corresponds to the heavy chain of the antibody molecule. Additionally, we report that the levels of antibody decrease rapidly from the brain during maturation. This study establishes a link between the time of neurogenesis and the presence of antibody molecules in subsets of cortical and retinal neurons. These results extend previous findings that determined that cortical subplate neurons in cats and rodents contain antigens recognized by monoclonal or polyclonal anti-IgG antibodies (Naegele et al., 1991; Fairen et al., 1992; Henschel and Wahle, 1994; Dunn et al., 1995).

Antibody molecules comprise four peptide subunits that include two identical heavy and light chains held together by disulphide bonds (Roitt, 1994). Immunoglobulin G, the most abundant class of antibody, contains an extensive cysteine and proline-rich sequence called the hinge region. In this study, one of the sequenced polypeptides ("peptide 2") was contained within the hinge region of IgG2b. This region becomes split during proteolysis to yield two Fab

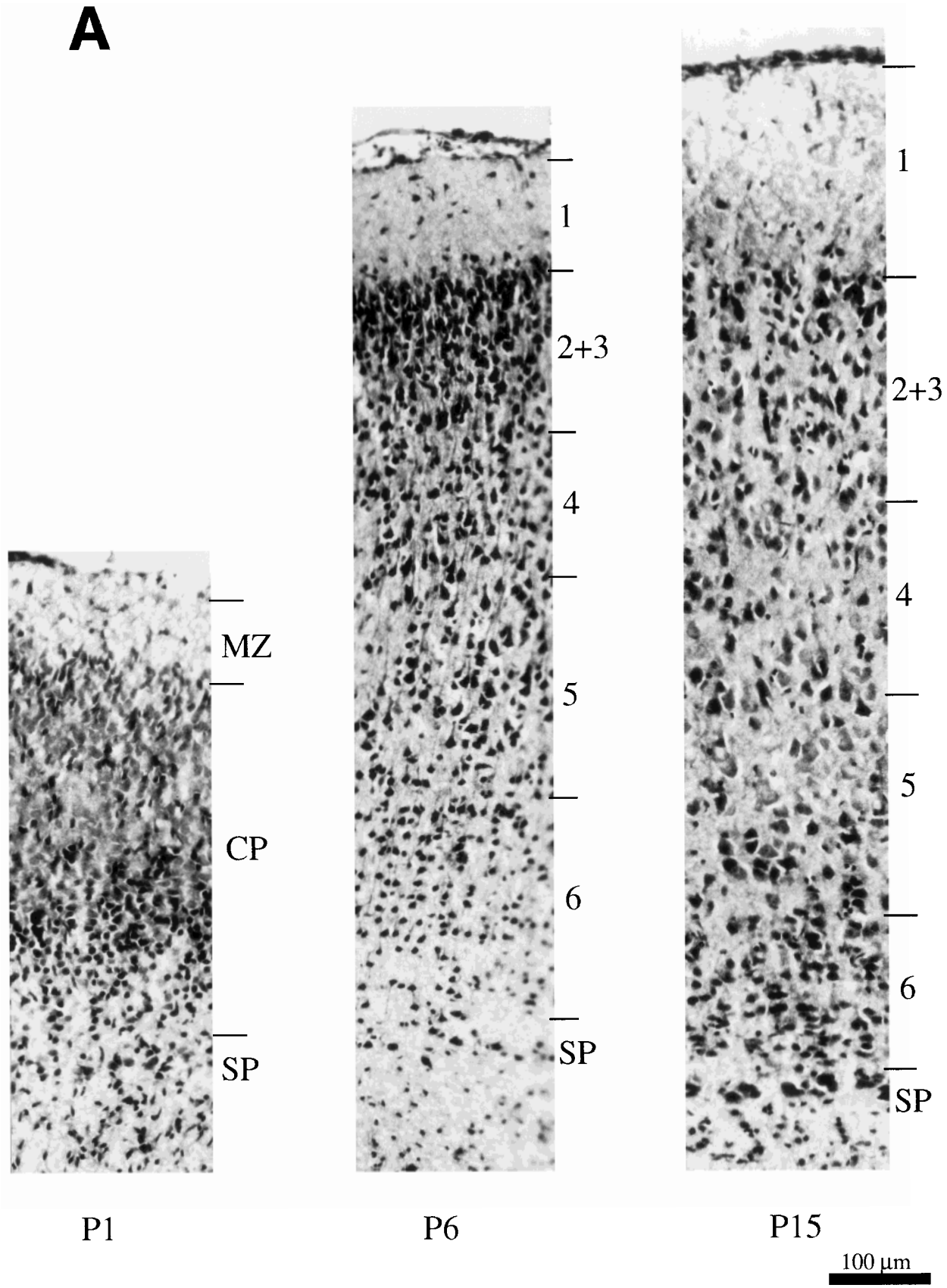

Fig. 3. **A:** Postnatal growth and laminar organization of the cerebral cortex. Sections of rat cortex were stained with azure blue to define the changes in cytoarchitecture during postnatal development. At P1, the cortical layers are not yet defined and are designated as comprising the cortical plate (CP). The subplate (SP) and marginal zone (MZ) are distinct layers below and above the cortical plate. By P7, the thickness of the cortex has almost doubled, and cortical layers are quite distinct (1–6). At P15, a slight increase in size is evident and the subplate layer appears much thinner and less dense. Scale bar = 100 µm.

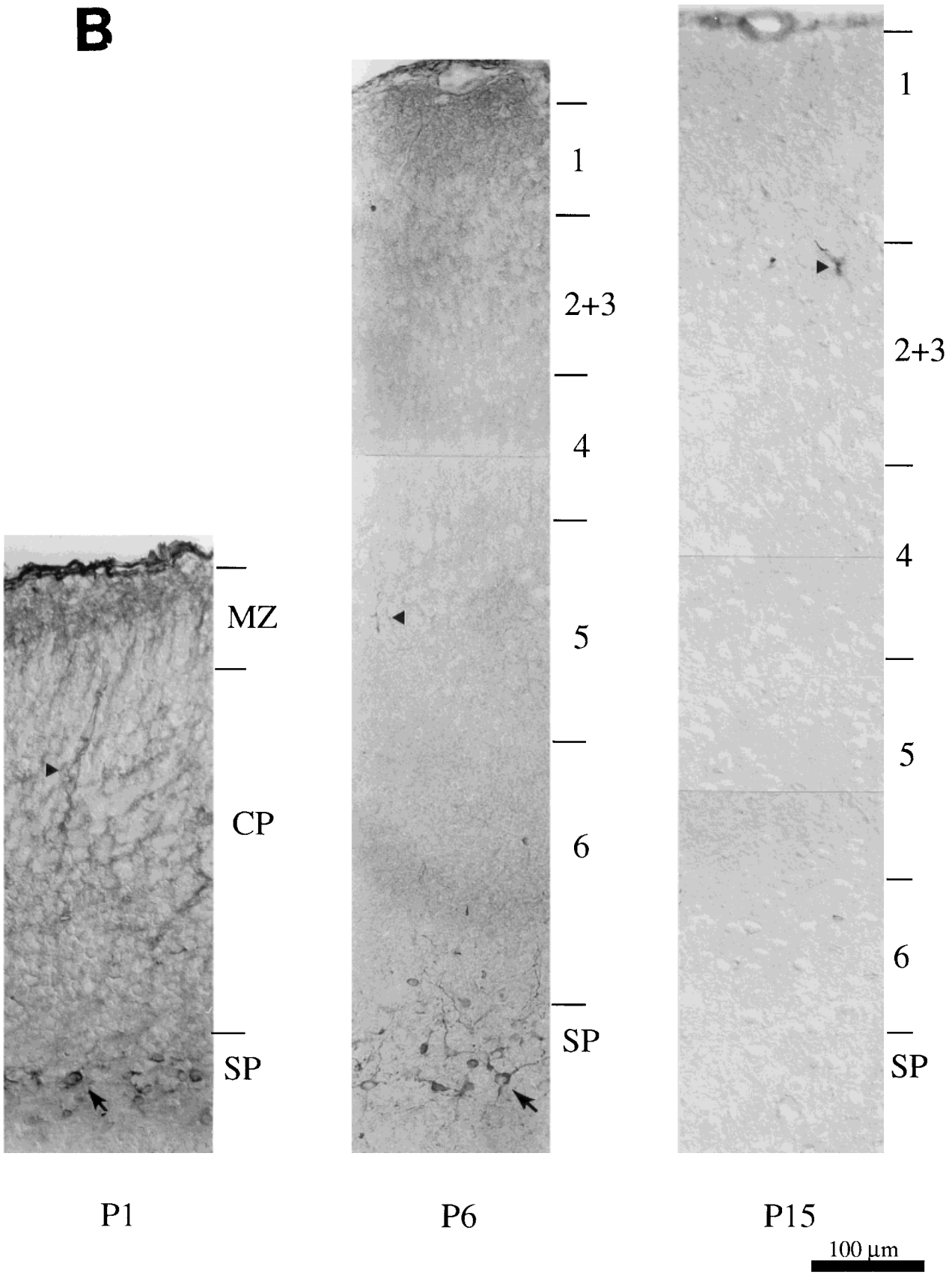

Fig. 3 (Continued). **B:** IgG is transiently present in deep layers of the cortical plate and subplate of the occipital cortex but disappears by postnatal day 15. Cortical sections from three ages were stained with rat IgG antisera. At P1, immunoreactive neurons (arrow) were located in a broad layer below the cortical plate. Heavy neuropil staining was present at this age in the cortical plate and the marginal zone. At P6, IgG-like staining was localized to a more restricted band of neurons (arrow) in the subplate zone. By P15, IgG immunoreactivity was absent, except for occasional endothelial cell staining (arrowhead). Arrowheads at P1 and P6 point to IgG-immunoreactive endothelial cells.

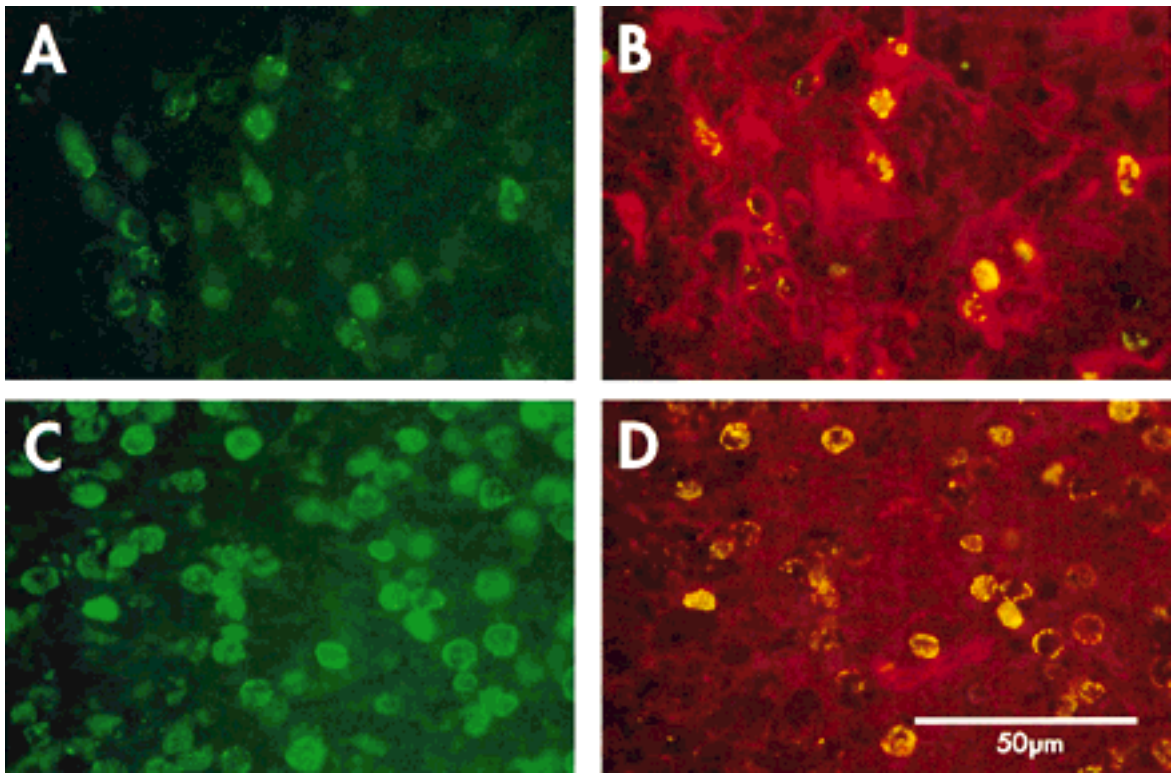

Fig. 4. IgG-immunoreactive neurons are derived from the subplate but not the cortical plate. Photomicrographs of double labeled sections from P6 and P7 cerebral cortex show BrdU labeling viewed with FITC (**A,C**) and double-labeling of BrdU and IgG, viewed with dual Texas Red and FITC filters (**B,D**). Although IgG immunoreactivity colocalizes with E13 BrdU labeling (B), colocalization of IgG and BrdU immunoreactivity in subplate neurons was not observed following E17 BrdU injections. IgG immunoreactivity was also not detected in cortical plate neurons labelled by E17 BrdU injections (C,D). Scale bar = 50 μm.

fragments that retain a site for antigen binding and one Fc fragment, unable to bind antigen. The other polypeptide sequenced ("peptide 1") was contained within the Fc region of IgG1. Previous studies in cat using mAb SP-1 and polyclonal anti-cat IgG antisera identified brain, liver, and plasma antigens that were IgG immunoreactive (Naegele et al., 1991; Henschell and Wahle, 1994; Dunn et al., 1995). Both the monoclonal and polyclonal antibodies against cat IgG gave specific but transient staining of subplate neurons in the developing feline cortex. Purification and sequence analysis of Ig-reactive antigens from serum yielded 20 amino acids that were 94% homologous to a sequence within the cat IgG heavy chain in the Fab region (Henschell and Wahle, 1994; Dunn et al., 1995). These previous results provided evidence that the cat subplate antigen might be immunoglobulin or share epitopes with IgG molecules. The new brain antigen sequence found in the present study, supports and extends the previous work. Because epitope sharing between different proteins is common, one idea articulated by several labs has been that the transient cortical antigen is a new member of the IgG superfamily. Thus, the identity of the transient brain antigen has been an open question until now. The major new findings in our study address this deficiency by Western blots, immunocytochemistry, ELISA assays, and amino acid sequencing. Our study is the first to show that the transient cortical antigen is IgG.

There are some differences between our results and those reported in a previous study published by Fairen et al. (1992). This group presented Western blot data from newborn rat and mouse neocortical fractions probed with anti-rat or anti-mouse IgG (H&L chain specific). They detected only a 93-kDa band, whereas we find bands at 53 and 27 kDa in rat. Preliminary work in our lab has also shown two immunoreactive bands of approximately 53 and 27 kDa in developing mouse cortex (Dunn et al., 1996). Several methodological differences between Fairen's study and ours could account for the detection of different bands. Although there were no apparent differences in the Western blotting techniques or the sensitivity of the detection system used for the blots, the brain samples were detergent extracted and centrifuged differently. Furthermore, the bands we identified as having mobilities similar to heavy and light IgG chains would have been below the lowest molecular weight protein shown on the blot presented in Fairen et al. (1992). We tentatively conclude that technical differences in both tissue processing and gel electrophoresis may have led to their failure to detect the IgG bands we now report.

One major question raised by our findings is the source of antibody in the young brain. In situ hybridization studies have suggested that the plasma proteins albumin, α-fetoprotein (AFP), fetuin, and transferrin may be synthesized in the embryonic brain (Dziegielewska et al., 1984, 1993; Mollgard et al., 1988). Thus, one source of IgG could be synthesis by neurons or other cell types. By contrast, it has also been shown that some other plasma proteins are transported across the blood-brain barrier (Habgood et al.,

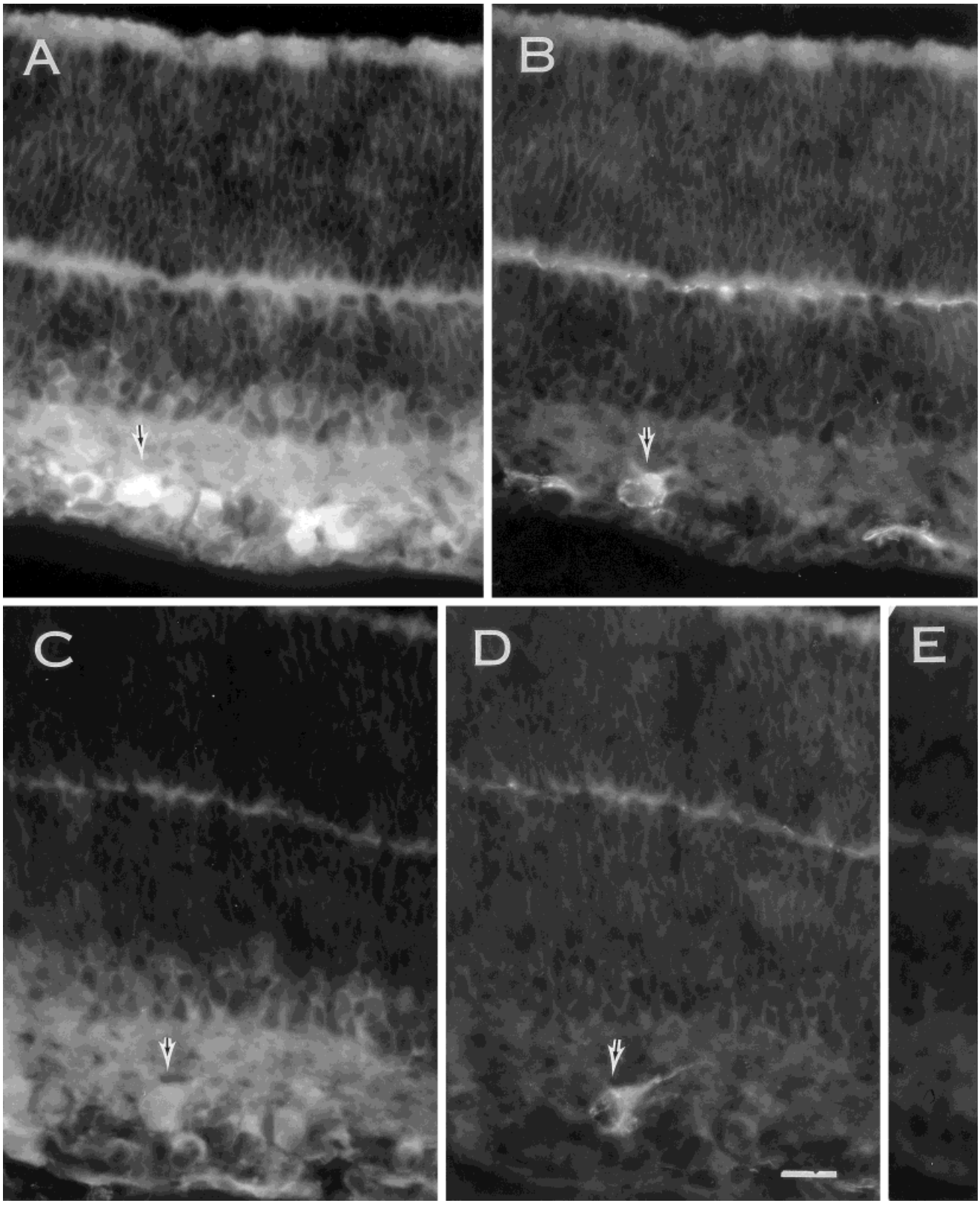

Fig. 5. Subsets of IgG-immunoreactive retinal neurons are also neurofilament positive. Sections of P9 retina were double labeled for rat IgG antisera and neurofilament. **A,C:** Majority of neurons in the ganglion cell layer are IgG immunoreactive (arrows). **B,D:** A subset of these IgG positive neurons also stain for neurofilament (arrows). **E:** Preadsorption of rat IgG antiserum with pure IgG abolished ganglion cell layer staining. Scale bar = 20 μm.

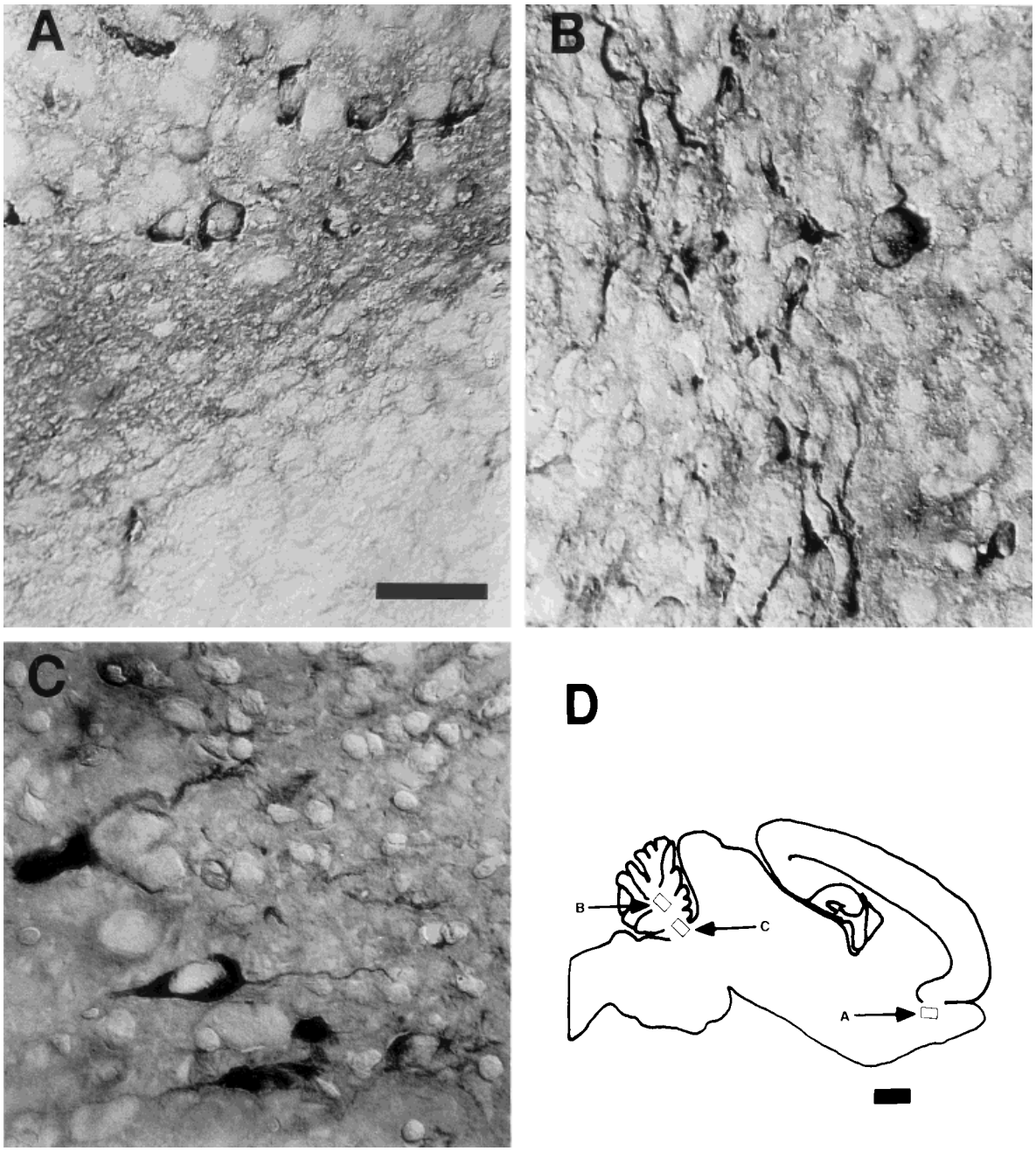

Fig. 6. Neuronal populations exhibit IgG-like immunoreactivity. Sections of postnatal rat cortex and cerebellum, stained with rat IgG antiserum, revealed several populations containing IgG immunoreactivity. Neuronal and neuropil staining were prominent in the P8 anterior olfactory nucleus (**A**). IgG immunoreactivity is present in neurons and fibers in P8 cerebellar medulla (**B**), as well as deep cerebellar nuclei (**C**). Camera lucida tracing of P8 rat brain section, (**D**). Boxes designate regions of photomicrographs shown in A–C. Scale bar = 20 μm in A, B, C and 2 mm in D.

1992). In the developing cerebral cortex, IgG appears to have a more specific distribution pattern than other serum proteins (Mollgard and Jacobsen, 1984). In rats, diffuse immunoreactivity has been reported for IgM, albumin, α-fetoprotein, α1-antitrypsin, transferrin (Cavanagh and Warren, 1985; Broadwell and Sofroniew, 1993), and the α-glycoprotein; α_2-HS (Sarantis and Saunders, 1986). At present, there is some controversy concerning the permeability of the fetal blood brain barrier, as well as the time at which it forms. It is thought that capillaries present in the brain prior to E15 have not yet formed a mature barrier (Risau and Wolburg, 1990). In the cerebral cortex, many of the IgG-containing neurons become postmitotic between E13 and E14, when they would be exposed to maternal antibodies leaking through perineural blood vessels (Yoshida et al., 1988). Because the only subclass of antibody molecules that passes through the placenta is IgG, uptake of maternal IgG is entirely consistent with our results.

The rapid postnatal decline observed for IgG in cortical and retinal neurons could be due to degradation, down-regulation, or cell death. Because our results suggest neurons generated by E17 or later do not contain IgG, uptake would occur before then or there would need to be a specific mechanism allowing early generated neurons to selectively take up serum IgG. Previous studies have shown that IgG1 and IgG2 have a half-life of approximately 23 days (Benjamini and Leskowitz, 1991). If IgG in subplate neurons was taken up at the time of mitosis on E13–E14 and then subjected to the normal rate of proteolysis, it would degrade by about postnatal day 12. Although this time course corresponds well with the observed loss of immunoreactive neurons from the cortex, the ELISA data indicate a more rapid decline of IgG occurring during the first few postnatal days. Thus, our ELISA results suggest that additional mechanisms may also be responsible for the rapid loss of IgG in the first postnatal week. One possibility is down-regulation, if the protein is synthesized in the brain.

A second possibility is that cell death might contribute to the loss of immunoreactive cells in the cortex, retina, and cerebellum. In rodents, each of these regions undergoes a postnatal period of cell death which peaks in the first postnatal week (Cunningham et al., 1982; Horsburgh and Sefton, 1987; Ashwell, 1990; Ferrer et al., 1992; Price et al., 1997). Thus, the IgG-containing neurons might be eliminated by programmed cell death. Preliminary work in our lab has shown that macrophages are often apposed to IgG-containing neurons in the cerebral cortex during the period of cell death (Upender et al., 1996). Additionally, Ferrer et al. (1992) found that macrophages are concentrated in the subplate and ventricular zone postnatally. In some structures, macrophages may also be necessary for cell death and tissue remodeling to occur. For example, in the embryonic mouse eye, in the absence of macrophages, hyaloid vessels and pupillary membranes persist up to 2 weeks after the time they are normally eliminated (Lang and Bishop, 1993).

To learn more about the actions of IgG at the cellular level, we are currently studying cerebral cortical development in mice with the *scid* mutation (severe combined immunodeficiency, Bosma et al., 1983). Homozygous scid/scid mice lack functional T and B cells and fail to produce detectable IgM, IgG1, and IgG2 (Bosma et al., 1983). In addition to lacking plasma IgG, our studies showed that these mice also lack intraneuronal IgG (Dunn et al., 1996). Therefore, this mutation may allow us to clarify whether IgG promotes, delays, or alters the development of the cerebral cortex.

In summary, these results identify IgG as an excellent molecular marker for early-generated neurons in the cerebral cortex and retina. The ease of identifying these neurons with this marker will facilitate analysis of their developmental roles and mechanisms of their elimination.

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