

Development of Inhibitory Circuitry in Visual and Auditory Cortex of Postnatal Ferrets: Immunocytochemical Localization of GABAergic Neurons

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ABSTRACT

The goal of this study was to describe the development of gamma-aminobutyric acid (GABA)-containing neurons in visual and auditory cortex of ferrets. The laminar and tangential distribution of neurons containing excitatory, inhibitory, and neuromodulatory substances constrain the potential circuits which can form during development. Ferrets are born at an early stage of brain development, allowing examination of inhibitory circuit formation in cerebral cortex prior to thalamocortical ingrowth and cortical plate differentiation.

Immunocytochemically labelled nonpyramidal GABA neurons were present from postnatal day 1 throughout development, in all cortical layers, and generally followed the inside-out pattern of neuronal migration into the cortical plate. Prior to postnatal day 14, pyramidal neurons with transient GABA immunoreactivity were also observed. The density of Nissl-stained and GABA-immunoreactive neurons was high early in development, declined markedly by postnatal day 20, then remained relatively constant until adulthood. However, examination of the proportion of GABA neurons revealed an unexpected late peak at postnatal day 60, then a decrease in adulthood. Visual and auditory cortex were similar in most respects, but the peak at postnatal day 60 and the final proportion of GABA neurons was higher in auditory cortex. The late peak suggests that inhibitory circuitry is stabilized relatively late in sensory cortical development, and thus that GABA neurons could provide an important substrate for experience-dependent plasticity at late stages of development. *J. Comp. Neurol.* 409:261-273, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: postnatal development; cortical development; GABA; critical period; immunocytochemistry; Cajal-Retzius cell

Studies of the neurochemical architecture of visual system pathways have provided important clues about their functional organization, development, and plasticity (Hendry and Calkins, 1998). Gamma-aminobutyric acid (GABA) is a ubiquitous inhibitory neurotransmitter in the central nervous system of adult mammals, and as such plays an important functional role. Neurons containing the neurotransmitter GABA are involved in the regulation of excitability and the shaping of receptive field properties in sensory neocortex (Sillito, 1975a,b; Berman et al., 1992; Crook and Eysel, 1992; Sato et al., 1995, 1996; Crook et al., 1996, 1997, 1998). There is evidence that GABA may also act either directly or indirectly as a neurotrophic factor during prenatal development, as it is present very early in

cerebral cortex in macaques (Shaw et al., 1991; Schwartz et al., 1992), affects the expression of other neurotrophic factors (Berninger et al., 1995), and influences neuronal proliferation (Antonopoulos et al., 1997) and migration (Behar et al., 1998). Postnatally, the importance of GABA

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is supported by its role in ocular dominance plasticity (Reiter and Stryker, 1988; Hensch et al., 1998), by observations that activity blockade causes a decrease in GABA-immunoreactive (-ir) neuron number in vitro through the action of brain-derived neurotrophic factor (BDNF; Rutherford et al., 1997), and by the demonstration that loss of visual input to visual cortex in adult monkeys reduces the number and density of GABA-ir neurons and puncta (Benson et al., 1991; Hendry and Carder, 1992; Hendry et al., 1994). These studies suggest that regulation of GABA may be important for activity-dependent plasticity throughout all stages of life. It is thus important to describe the pattern of GABAergic neurons and GABA receptor expression in model systems which can profitably be exploited in studies of postnatal development and plasticity.

Ferrets have become popular subjects for research on cortical development. Unlike monkeys, cats, rats, and mice, ferrets are born before thalamocortical inputs reach the subplate and well before development of the cortical plate is complete (Luskin and Shatz, 1985a; Jackson et al., 1989; Herrmann et al., 1994). Thus, examination of the postnatal development of GABA-ir neurons in ferret cortex is of general interest. Although the development of GABA expression has been examined in mouse neocortex (Del Rio et al., 1992), rat somatosensory cortex (Micheva and Beaulieu, 1995), and cat (Hogan et al., 1992) and monkey visual cortex (Schwartz et al., 1992), studies extending throughout postnatal development in ferrets have not been done, nor has there been a directed comparison of the development of inhibitory circuitry between sensory cortices representing two different modalities.

This study, in addition to providing information about the normal postnatal development of inhibitory circuitry in sensory neocortex, was primarily intended to provide a baseline for our studies on cross-modal plasticity. We are interested in the specification of cerebral cortex from a developmental and evolutionary perspective, and the role of sensory experience in this process (Pallas, 1990). To this end, we have been examining the laminar and areal distribution of GABA-ir neurons and GABA receptors in animals with early cross-modal rewiring manipulations that reroute visual inputs into the auditory pathway. In this paper, we describe the pattern of GABA-ir neurons from the beginning of ferret postnatal development in the two sensory cortical areas which are relevant in this context, primary visual (V1) and primary auditory cortex (AI). Our results suggest that GABA-ir neurons may be playing a role even in very late stages of cortical circuit construction, supporting our hypothesis that inhibitory circuits are a likely substrate for experience-dependent cross-modal plasticity.

These results have been presented previously in preliminary form (Pallas et al., 1994; Newman et al., 1996).

MATERIALS AND METHODS

Animals

A total of 64 ferrets was used in this experiment. Timed pregnant ferrets were obtained from Marshall Farms (North Rose, NY), kept on a 14/10 light/dark cycle, and fed ferret chow and fresh water ad lib. Kits at postnatal days (P) 1, 7, 14, 20, 40, and 60 were used in the developmental series, and the normal adults (120 days or greater) were jills which had finished rearing their litters. The latter three time points correspond to the start, peak, and end of the critical period for ocular dominance plasticity in ferret visual cortex (Chapman et al., 1996; Chapman and Stryker, personal communication). Three time points prior to P20 were chosen to provide information about early cortical development. Developmental events prior to P20 in the ferret occur prenatally in cats. All animals were treated in accordance with institutional and NIH guidelines for the Care and Use of Laboratory Animals.

Tissue preparation

Animals were overdosed with sodium pentobarbital (65 mg/kg i.p.) and perfused through the heart with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer (PB, pH 7.4). The brains were removed from the skull and postfixed with 4% paraformaldehyde/30% sucrose in PB for 24 to 48 hours at 4°C for cryoprotection. After rinsing with 30% sucrose in PB, brains were sectioned frozen in the coronal plane, alternating at 50 and 30µm in thickness, and collected in 0.1 M PB. One set of 50-µm sections at 160-µm intervals was mounted onto gelatin-subbed slides and stained for Nissl substance with cresyl violet for visualization of laminar and areal borders and for quantitative comparison of cell counts.

Immunocytochemistry

Coronal 30 or 50µm sections were used for immunocytochemistry. We found that the 30µm sections had better penetration of the antibodies than the 50µm sections. Thus, the thinner sections were used for immunocytochemistry in all but two adult brains used for V1 data and four adult brains used for AI data. Even in a 30-µm section, the antibodies did not penetrate to the center of the section, and thus we assumed that the extent of penetration of the antibody, and thus the number of GABA-ir cells per section, was comparable in 50 and 30µm sections. In fact, analysis of section thickness as a factor in quantitative measures of labelled cell numbers did not reveal any differences, and thus our quantitative data for 30 and 50µm sections were combined for analysis.

Sections were processed free-floating using the avidin-biotin method for localization of antigens with peroxidase. To reduce free aldehydes, the sections were first rinsed in PBS with 0.02% sodium azide and then treated for 1 hour in 0.34% L-Lysine and 0.05% sodium periodate (NaIO₄). Sections were then incubated in 3% normal goat serum (NGS) for 1 hour at room temperature to block nonspecific staining. Incubation in a commercially available primary antibody (mouse anti-GABA from ICN, Costa Mesa, CA, diluted at 1:1,000) with 3% NGS was done under constant agitation for 48 to 72 hours at 4°C. After rinsing, sections were incubated in secondary antibody (biotinylated goat anti-mouse, Vector Labs, Burlingame, CA) with 3% NGS at a dilution of 1:200 for 2 hours, washed in PBS, and then

Abbreviations

AI	primary auditory cortex
CP	cortical plate
GABA	gamma-aminobutyric acid
GABAergic	GABA-containing
GABA-ir	GABA-immunoreactive
IZ	intermediate zone
P	postnatal day
SP	subplate
V1	primary visual cortex

incubated in ABC solution (Vectastain Elite ABC kit, Vector, Burlingame, CA) at 1:500 for 1–1.5 hours. Sodium azide was left out of the buffer after incubation in the secondary antibody. The peroxidase reaction was performed with 0.01% diaminobenzidine and 0.004% hydrogen peroxide and intensified by adding 1% nickel ammonium sulfate and 0.34% imidazole. As a control for secondary antibody specificity, the primary antibodies were omitted from the staining procedure for some sections. No detectable labelling was observed without the primary antibody. Sections were mounted from buffer, dehydrated, and coverslipped with Permount. The data were not corrected for shrinkage due to histological processing.

Qualitative observation and quantitative analysis

Qualitative analysis included examination of the morphology, size, and the areal and laminar distribution of labeled neurons in V1 and AI. For quantitative analyses, immunostained sections and adjacent Nissl-stained sections were compared in order to determine the distribution, density, size, and number of GABA-ir neurons compared to Nissl-stained neurons. Sections were chosen to be at a comparable anteroposterior level, and only well-stained sections were included in the data set. In a few cases, more than one section was used from each brain and the results were averaged. Normal cells were counted if they contained a bounded, smooth, round border. Small, darkly stained cells likely to be glia were excluded. Neurons were counted in $50 \times 500\mu\text{m}$ wide vertical strips across all layers of cortex using a $63\times$ or $100\times$ oil immersion objective with a camera lucida and Neurolucida software (MicroBrightfield, Burlington, VT). Care was taken to employ procedures which eliminated double-counting of neurons (Pallas et al., 1988). Our interest was in relative comparisons between reference populations rather than absolute density or absolute cell number, and thus cell counts were corrected using the Abercrombie method (Abercrombie, 1946, cf. Guillery and Herrup, 1997). Measures of strip area and neuronal number within the strip were obtained with Neuromorph software (MicroBrightfield). Density of immuno- and Nissl-stained neurons and percentage of immunostained neurons were then calculated. Measures of cell size were made by drawing the outlines of somata in a $50 \mu\text{m}$ diameter strip throughout the cortical layers on a bitpad and calculating an average diameter through the use of Neuromorph. The data are presented below as means \pm standard error.

RESULTS

For analysis of Nissl-stained neurons, 70 sections were examined from 52 animals for the primary visual cortex (V1) data and 67 sections from 54 animals were analyzed to provide the primary auditory cortex (AI) data. For analysis of GABA-ir neurons, 40 sections from 35 animals in V1 and 37 sections from 33 animals in AI were examined (Table 1). Many of the 64 brains used in this study were also used for other studies, in order to reduce the number of animals used.

Development of the cortical plate

Sensory cortex appeared very immature in newborn ferrets. Photomicrographs of Nissl-stained coronal sec-

TABLE 1. List of Experimental Groups^{1,2}

Age	Number of animals used per group			
	Neuron counts		GABA-ir neuron counts	
	V1	AI	V1	AI
P1	7	7	4	4
P7	6	6	5	4
P14	8	8	5	5
P20	8	10	6	7
P40	9	9	6	5
P60	6	6	5	4
>P120	8	8	4	4

¹Each value indicates the number of animals that were used for each type of data.

²For abbreviations, see list.

tions of ferret visual and auditory cortex at each developmental stage are presented in Figures 1 and 2. Figure 1 presents developmental stages from P1 to P14, which is approximately equivalent to embryonic day (E)42 to E56 in a prenatal cat. At P1, only the deep layers of cerebral cortex were present in V1 and AI, but they could not easily be distinguished from the dense cortical plate (CP, Fig. 1A, B). By P7, layers 5 and 6 were visible. The neurons which would comprise layer 4 were migrating into position but could not be distinguished from the deep layers (Fig. 1C, D). By P14, layer 4 was visible as a separate layer (Fig. 1E, F).

Ferrets are born 3 weeks earlier in gestation than cats, and thus P21 in ferrets is equivalent to a P1 cat. Figure 2 presents stages from P20 to adulthood. By P20 all of the cortical layers were present (Fig. 2A, B), and by P40 the cortical layers had become thicker (Fig. 2C, D). In P60 animals, the cortex looked much as it does in the adult (Fig. 2E–H). These results on development of the cortical layers were consistent with those reported previously (Jackson et al., 1989). As expected from the general anteroposterior sequence of brain development (Bayer and Altman, 1991; Noctor et al., 1997), the development of auditory cortex was advanced slightly compared to the development of visual cortex (e.g., compare Fig. 1E to 1F where layers 5 and 6 were more clearly separable).

Laminar distribution of GABAergic neurons

Qualitative observations revealed that the distribution of GABA-ir neurons was bilaminar at early stages and widespread during late postnatal development. In adult ferrets, GABA-ir neurons were distributed uniformly across all layers of cortex in V1 and AI, except for a reduced density of labeled neurons in layer 5 (Figs. 3, 4). Layer 5 also contained fewer total neurons (Fig. 1). At P1 and P7, there were two bands of GABA-ir neurons, one in the CP in presumptive layer 5, and one in the subplate (SP) directly beneath the cortical plate. There were also neurons located in the marginal zone, but labelled neurons were sparse in layer 6 (Fig. 3). In addition, there were some immunoreactive cells resembling migrating neurons which were found in the cortical plate and in the subventricular and intermediate zones. As the cortical plate developed, GABA-ir cells were seen in layer 6, and labelling became progressively more widespread throughout the cortical layers, with a slightly reduced density of GABA-ir neurons in layer 5. By P14, layer 4 had appeared and contained GABA-ir neurons. At P20, migrating neurons could still be seen occasionally, and GABA-ir neurons remained distributed across the cortical layers, with a reduced density in layer 5 (Fig. 4A, B), reflecting the reduced density of layer 5 neurons in

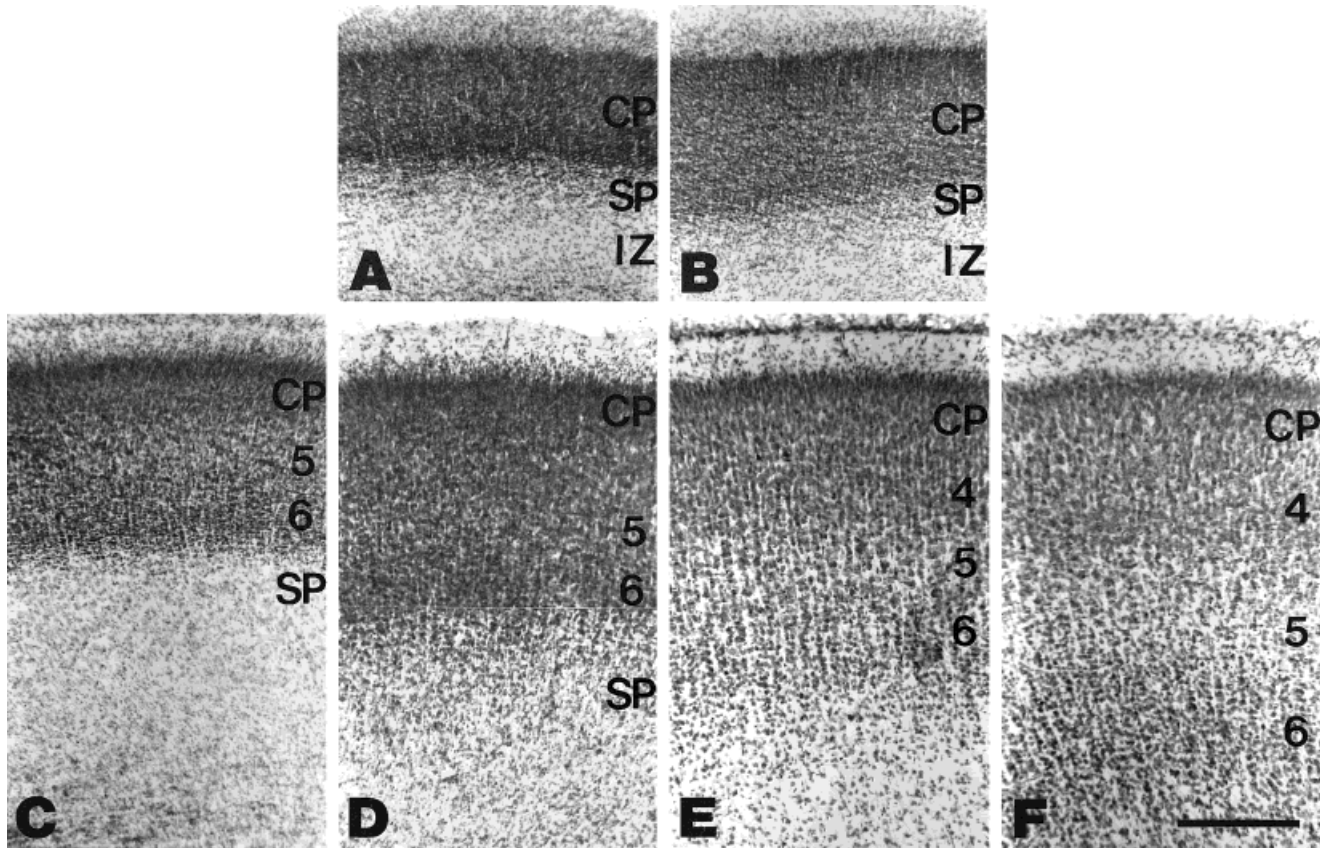


Fig. 1. Early development of the cortical plate in ferrets. In this and the following figures, sections from primary visual cortex (V1) and primary auditory cortex (AI) are arranged as left-right pairs at each age. Here are shown Nissl-stained neurons at early postnatal ages, with a clear distinction between the cortical plate (CP) and the intermediate zone (IZ). The subplate (SP) lies at the border of these

two strata, and the marginal zone is the cell-sparse layer just below the pial surface. At the time of birth, layers could not easily be distinguished in V1 (A) or AI (B). By postnatal day (P) 7, layers 5 and 6 were obvious in V1 (C) and AI (D). At P14, layer 4 neurons were migrating into position (E: V1, F: AI). Scale bar = 250 μ m.

general (Fig. 2). The dense band of small GABA-ir neurons in the subplate was no longer apparent by this age. By P40 (Fig. 4C, D), it was clear that there was a decline in the number of GABA-ir neurons, although the distribution was still uniform across the cortical layers. But by P60 there was a marked increase in density and number of GABA-ir neurons and terminals, with some neurons staining much more darkly than others (Fig. 4E, F). In addition, many of the GABA-ir neurons at this stage were quite large and more mature in their morphological development. In adulthood, there was a reduction in GABA-ir neurons and terminals, and the dendritic processes did not stain as well with the GABA antibody (Fig. 4G, H). The development of GABA immunoreactivity was similar between visual and auditory cortex, with no obvious differences either in the laminar distribution of GABA-ir neurons or the pattern of change. Thus, genesis of GABA-ir neurons generally followed an inside-out gradient, as reported previously (Pezuzzi, 1988).

Morphology of GABAergic neurons

GABA-containing neurons are of many different morphological types, and this heterogeneity is even more pronounced during development, because some types of cells are present only transiently or contain GABA only at early

developmental stages. We observed that horizontally oriented neurons with thick dendrites (probably Cajal-Retzius cells) were common in the marginal zone at P1 (Fig. 5A, B). At P7 they were still present but had shorter dendrites, and at P14 and subsequent ages, GABA-ir cell bodies were observed in the marginal zone, but dendrites were not labeled (not shown). As the brain grew, these layer 1 GABA-ir cells became increasingly sparse. Bipolar neurons with fusiform somata were seen in the cortical plate at P1 and P7 (Fig. 5A, B, E, F). These are likely to have been migrating neurons, and they were not seen in older cortex. In the SP at P1, and extending slightly over the border of the CP into layer 6, there were many randomly oriented, small, spherical cells, each with one trailing dendrite (tadpole-shaped), as well as occasional horizontally oriented, bipolar cells (Fig. 5C, D). There were a few of these cells in the white matter at P1 also (not shown), but they did not appear at later ages. At P1 and P7, there were neurons with pyramidal morphology (thick apical dendrites and pyramid-shaped somata) in the upper part of the CP which were not seen at subsequent stages (Fig. 5E, F). These were most numerous at P7, and constituted approximately one-third of the GABA-ir neurons in that layer. Pyramidal neurons were never labelled with the GABA antibody at any stage later than P7. By

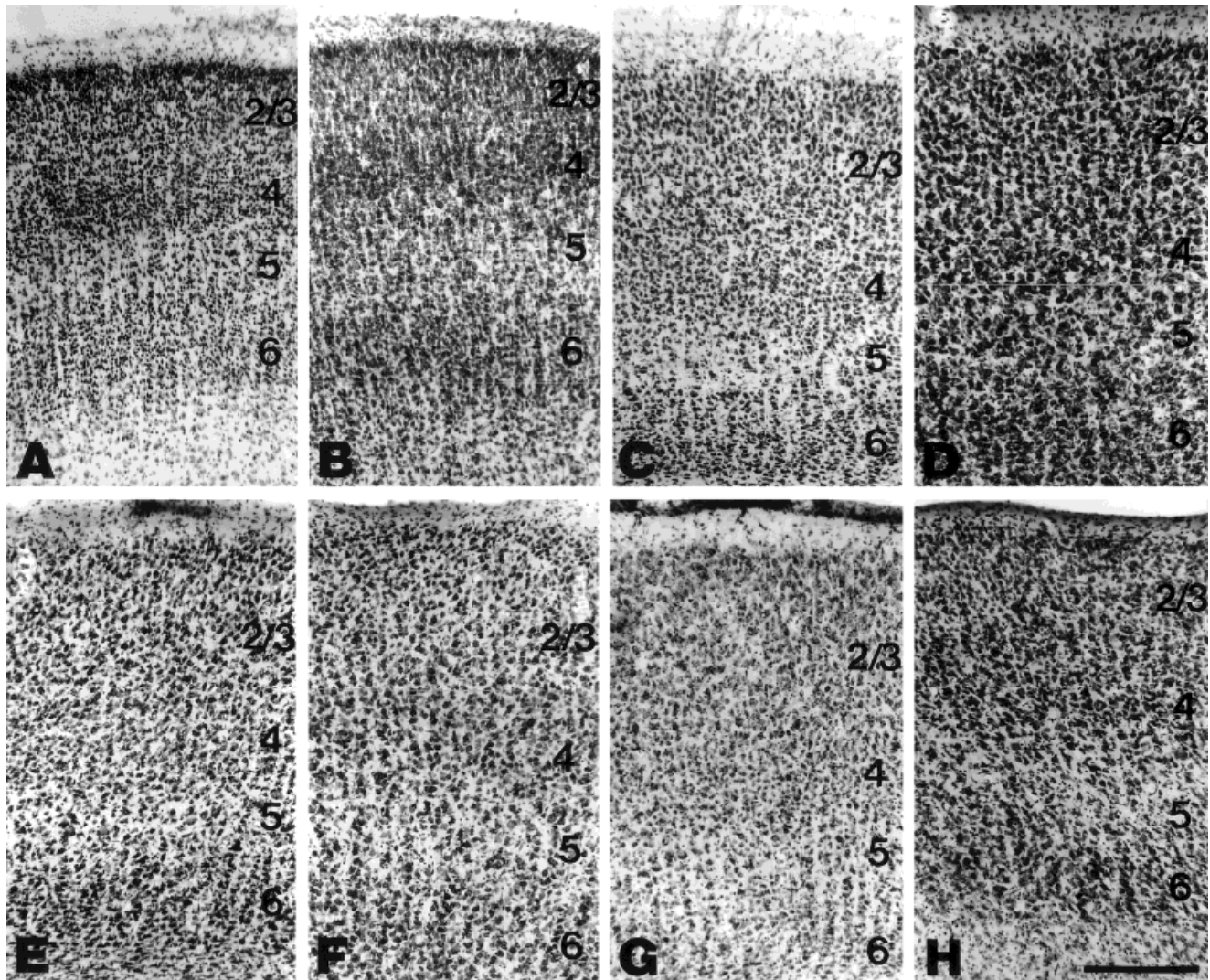


Fig. 2. Late development of the cortical plate in ferrets. These micrographs show Nissl staining of ferret cerebral cortex at later stages of postnatal development in primary visual cortex (V1; A, C, E, G) and primary auditory cortex (AI; B, D, F, H) from postnatal day

(P)20 to adulthood. By P20, all of the cortical layers were present (A, B), and by P40 (C, D), the layers were becoming thicker and more easily distinguishable. At P60 (E, F), the cortical layers were generally similar in appearance to those of adults (G, H). Scale bar = 250 μ m.

P14, GABA neurons were beginning to acquire a more mature, complex morphology (Fig. 5G, H), and both small and large multipolar and bipolar cells were observed. In older animals, they became progressively larger and more differentiated (Fig. 6A–F), and looked increasingly like the GABA-ir neurons in adult animals (Fig. 6G, H). GABAergic neurons are a heterogeneous group and include basket cells, chandelier cells, and double bouquet cells (Fairén et al., 1984; Hendry and Carder, 1992; Prieto et al., 1994). There were no obvious differences in cellular morphology between GABA-ir cells in V1 and AI.

Soma diameter of Nissl-stained neurons increased steadily during postnatal development until P60, with a slight decline at adulthood, but the soma diameter of the population of GABA-ir neurons remained fairly steady (Table 2). These soma diameter values were used for Abercrombie correction of the quantitative data (see Materials and Methods).

In a comparison of Nissl-stained to GABA-ir neurons (Table 2), we found that, at P1 and P7, the soma sizes of GABA-ir neurons were significantly larger than those of the Nissl-stained neurons in both V1 and AI. At P14 they were of approximately equal size. After P14, this relationship inverted for both V1 and AI, and the soma diameters of Nissl-stained neurons were larger than those of GABA-ir neurons at all age groups from P20 to P60. In adult V1, the GABA-ir neurons were slightly larger than those of Nissl-stained cells, but not significantly, whereas in adult AI GABA-ir neurons were significantly smaller than Nissl-stained cells. The larger diameter of GABA-ir neurons at P1 and P7 may be due to the GABA-ir pyramidal neurons, which were not present at later stages.

Comparison of V1 to AI neuron sizes showed a closer match than for Nissl-stained and GABA-ir neurons, but there were still significant differences in some cases (Table 2). Interestingly, the Nissl-stained neurons in adults were

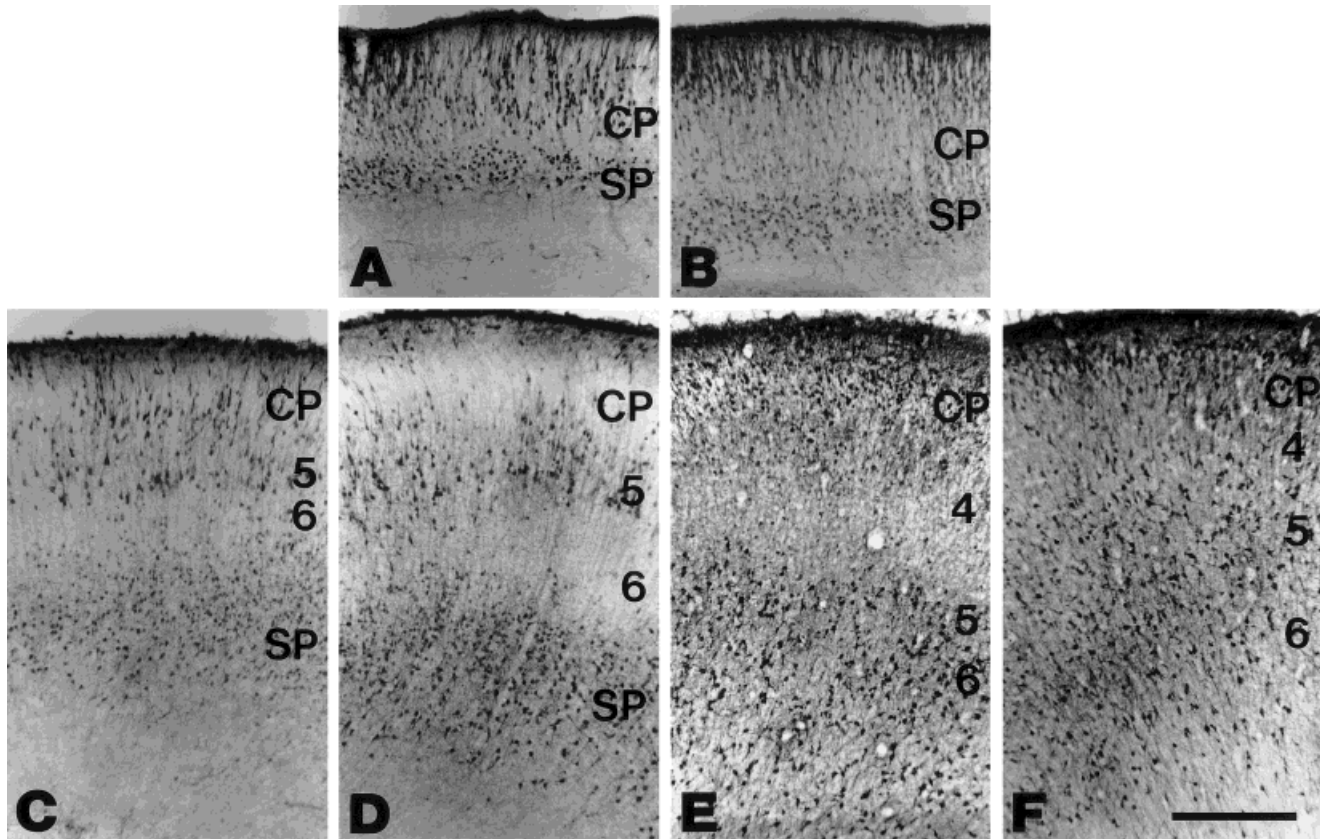


Fig. 3. Early postnatal development of gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons in ferret primary visual cortex (VI; **A, C, E**) and primary auditory cortex (AI; **B, D, F**). At postnatal day (P)1 (**A, B**), GABA-ir neurons were observed in the cortical plate (CP), subplate (SP), and marginal zone. There was a layer devoid of immunoreactivity between the small neurons in the subplate and the larger neurons in the cortical plate. By P7 (**C, D**), layers 5 and 6

became distinguishable, but few GABA-ir cells were found in layer 6. Numerous small GABA-ir neurons were located in the subplate, and larger neurons were found in layer 5, the CP, and the marginal zone. By P14 (**E, F**), GABA-ir neurons were more evenly distributed than during the first week of postnatal development but were still more sparse in layer 6. Scale bar = 250 μ m.

significantly larger in AI than V1, but the GABA-ir neurons exhibited a significant difference in the other direction in that V1 neurons were larger than AI neurons.

Neuronal density

Estimates of Nissl-stained neuron number in layers 1–6 were calculated for each developmental stage in each cortical area and divided by the area (area rather than volume was used due to the incomplete penetrance of the antibody in the 30 and 50 μ m sections). Somata were counted and not nucleoli, because a comparison of counts of Nissl-stained neurons with counts of GABA-ir neurons was the goal, and it usually was not possible to see nucleoli in the darkly stained GABA-ir neurons. Estimates of cell number were corrected using the Abercrombie method (see Materials and Methods). The reliability of the counting method was witnessed by the low variability in counts between animals at the same ages (see error bars in Fig. 7A).

Figure 7A shows the results of counts of total neurons in Nissl-stained sections throughout cortical development. As seen in Figure 1, the cortical plate was very densely packed with neurons at the earliest stages of development. The density of neurons declined rapidly during the subse-

quent period of naturally occurring cell death and expansion of the cortical lobes. The decline in neuron density was sharpest from P7 to P20 and then was more gradual until P60. After P60, a peculiarity of ferret brain development became apparent. Ferrets undergo a dorsoventral flattening of the skull, which causes a compression of the cortical layers and thus an increase in neuronal density (Apfelbach and Kruska, 1979; Kruska, 1993).

Density of GABA-ir neurons

Density of GABA-ir neurons was calculated in the same way as density of Nissl-stained neurons, by counting a sample of GABA-ir neurons in a strip through the cortical layers, estimating the actual number, and dividing by the area sampled. The time course of changes in GABA-ir neuron density is shown in Figure 7B. The density of GABA-ir neurons was high early in postnatal development, as was the density of total neurons (Fig. 7A). Peak density occurred at P1 in both V1 and AI, and declined sharply thereafter. The density was initially somewhat higher in AI than in V1. There was a slight increase in GABA-ir neuron density at P60 in AI. In adulthood, density of GABA-ir cells was comparable in V1 and AI.

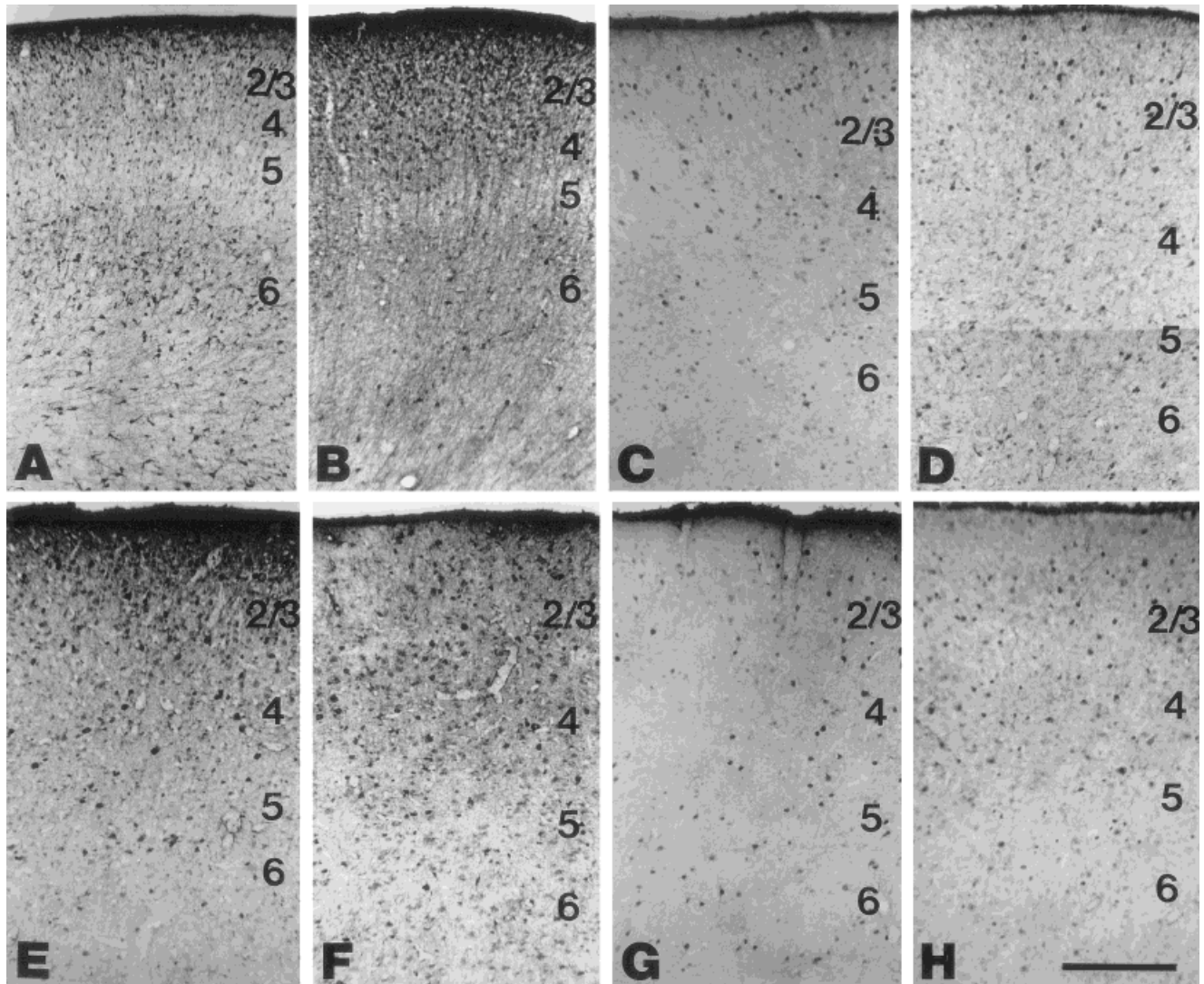


Fig. 4. Late postnatal development of gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons in both primary visual cortex (VI; A, C, E, G) and primary auditory cortex (AI; B, D, F, H). At postnatal day (P)20 (A, B), migrating neurons could still be observed in the superficial layers, but GABA-ir cells were distributed across all

cortical layers, with a lower density in the bottom half of layer 5. By P40 (C, D) the GABA-ir neurons were uniformly distributed in all cortical layers with relatively low density. At P60 (E, F) the GABA-ir neuronal density increased markedly and then it decreased by adulthood (G, H). Scale bar = 250 μ m.

Proportion of GABA-ir neurons

Comparisons of Nissl-stained and GABA-ir neuron density can be problematic due to the possibility of differential shrinkage from different tissue-processing protocols or even differential shrinkage of early versus late postnatal tissue. Measurement of the relative proportions of GABA-ir and Nissl-stained neurons is less subject to this problem, and also reveals interesting relationships between neuron populations. For quantitative analysis of the proportion (percentage) of GABA-ir neurons, counts of neurons in Nissl-stained sections and adjacent immunostained sections were compared, taking care to use the same cortical location in both sections. During postnatal development, neuronal density declines sharply (Fig. 7A) and thus examination of the percentage of GABA-ir neurons rather than their density filters out this decline. In both V1 and

AI, the proportion of GABA-ir neurons has a small peak from P1 to P14, declines slightly at P20, then exhibits a sharp increase from P20 to P60, especially in AI, after which it declines by adulthood (Fig. 8). This means that the proportion of GABA-ir neurons within the entire population of neurons is increasing late in postnatal development. This can also be readily observed in the sections pictured in Figures 3 and 4.

DISCUSSION

Proportion of GABAergic neurons during development

We found that GABA neuron density was high until P20 and then dropped considerably by adulthood. However, because the density of all neurons was dropping sharply

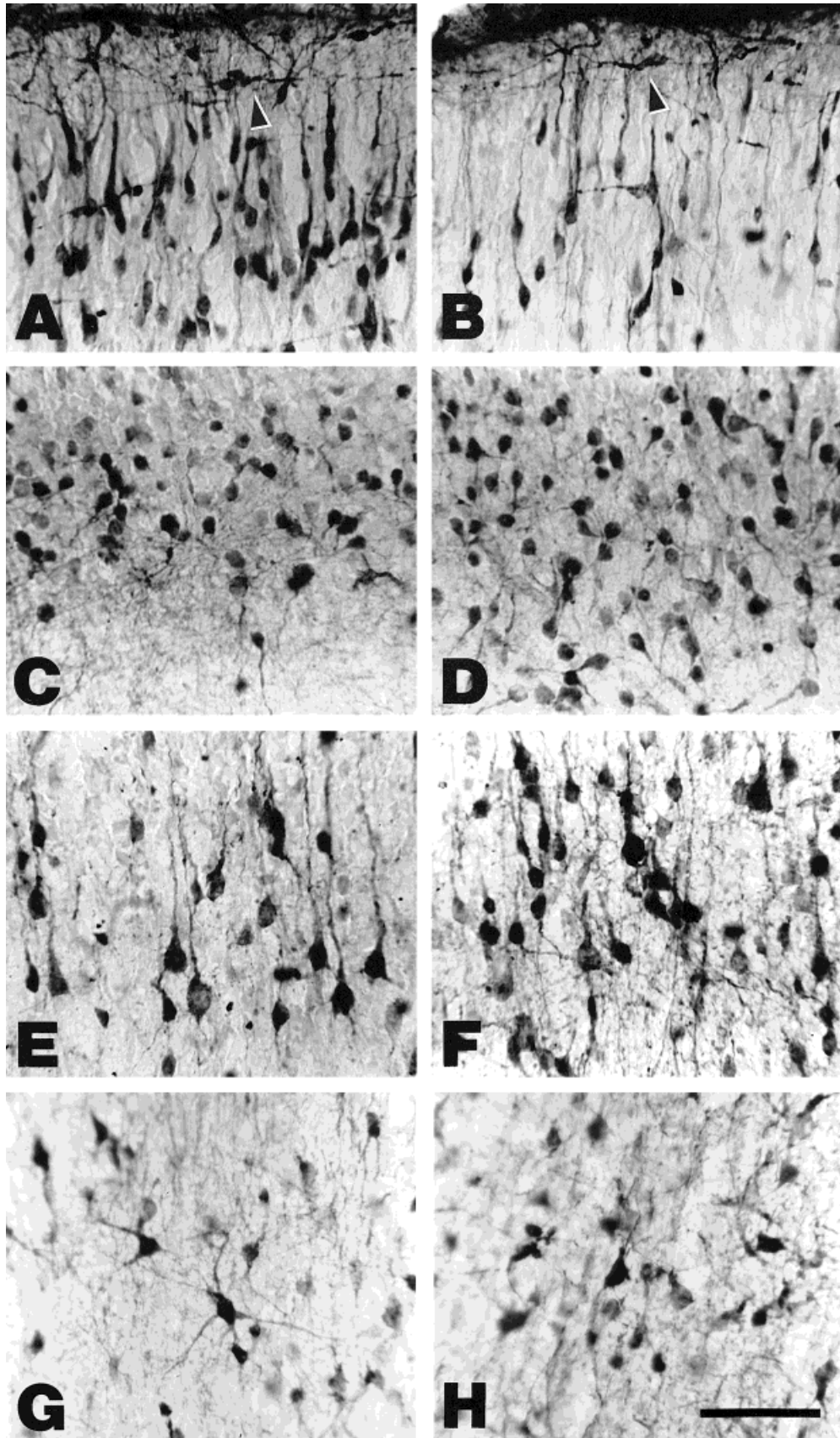


Fig. 5. Morphological development of gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons at early postnatal ages in both primary visual cortex (V1; **A, C, E, G**) and primary auditory cortex (AI; **B, D, F, H**). At postnatal day (P)1 (**A-D**), many GABA-ir neurons in the marginal zone could be identified as Cajal-Retzius cells (arrowheads). In the cortical plate (CP), numerous bipolar cells were observed which may have been neurons migrating into their laminar position (**A, B**).

Small GABA-ir, tadpole-shaped neurons were densely distributed in lower layer 6 and the subplate (**C, D**). Their shapes and sizes were quite different from that of the neurons in the upper CP. By P7 (**E, F**), some of the pyramidal neurons in the middle part of the CP also transiently expressed GABA. By P14 (**G, H**), the morphology of GABA-ir neurons was generally similar to that of neurons in later stages (Fig. 6), but they were smaller in size. Scale bar = 50 μ m.

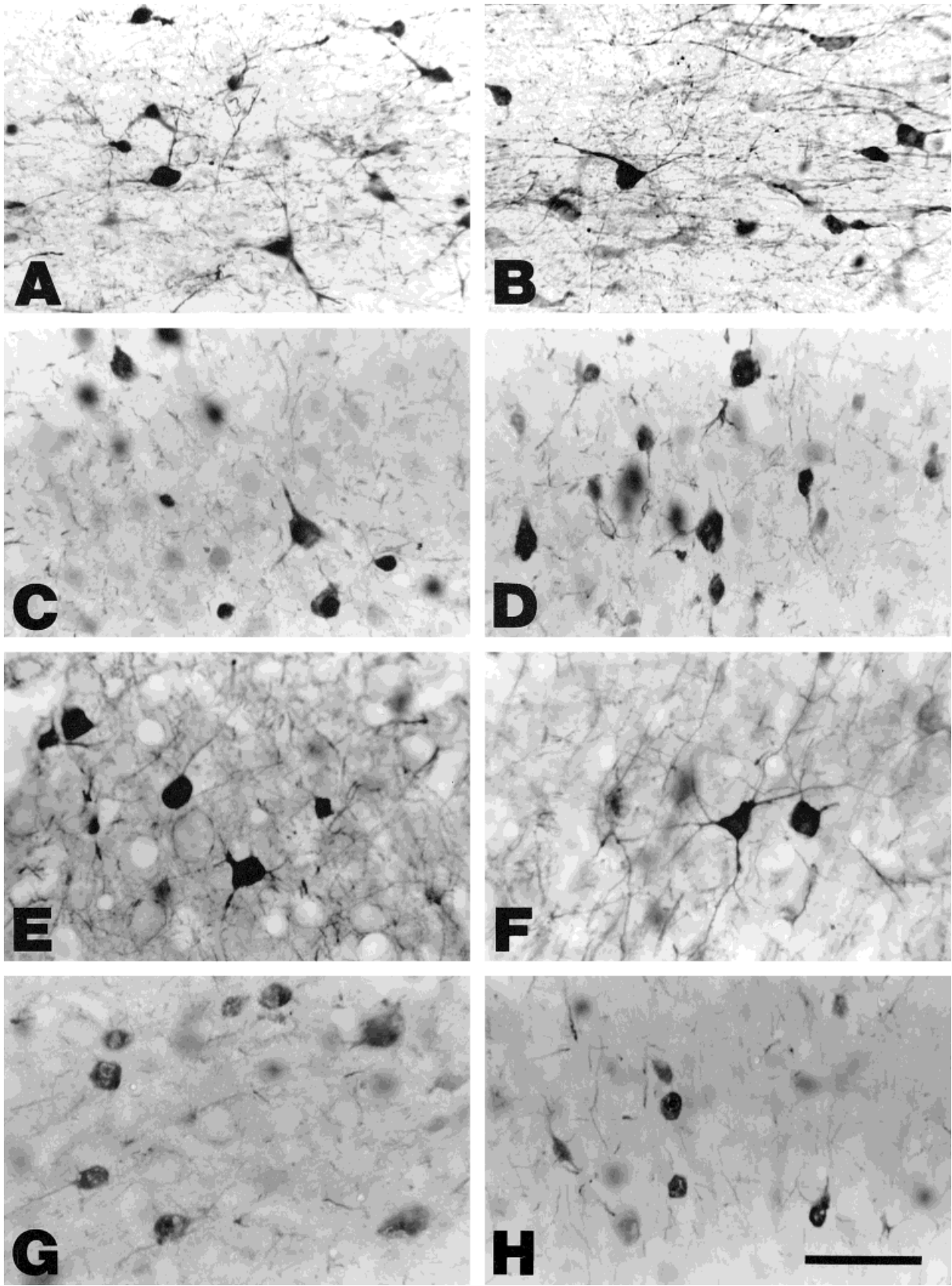


Fig. 6. Photomicrographs illustrating the morphology of gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons at later postnatal ages in both primary visual cortex (V1; **A, C, E, G**) and primary auditory cortex (AI; **B, D, F, H**). The morphology of GABA-ir neurons in the superficial layers at postnatal day (P)20 (**A, B**) looked similar in

both V1 and AI but their sizes were smaller than those of the later age groups. After P40 (**C, D**), the GABA-ir neuronal morphologies and size were generally adult-like (compare to P60 [**E, F**] and adult [**G, H**]). Scale bar = 50 μ m.

TABLE 2. Somatic Diameter (μm) of Nissl-Stained and GABA-ir Neurons at Different Postnatal Ages^{1,2,3}

Postnatal age	V1 Nissl	AI Nissl	V1 GABA-ir	AI GABA-ir
1	5.2 \pm 0.15	5.5 \pm 0.08	8.1 \pm 0.31	8.8 \pm 0.20
7	6.2 \pm 0.18	5.7 \pm 0.11	8.7 \pm 0.19	9.2 \pm 0.21
14	9.4 \pm 0.22	9.8 \pm 0.17	9.7 \pm 0.25	9.5 \pm 0.21
20	8.1 \pm 0.19	11.1 \pm 0.29	8.0 \pm 0.29	9.6 \pm 0.34
40	11.2 \pm 0.28	13.3 \pm 0.36	9.5 \pm 0.31	10.2 \pm 0.28
60	13.8 \pm 0.53	14.1 \pm 0.65	9.5 \pm 0.38	9.8 \pm 0.43
120	9.7 \pm 0.32	12.0 \pm 0.19	10.1 \pm 0.39	8.6 \pm 0.23

¹Probabilities that Nissl and GABA-ir neurons were of different sizes: (V1) P1 $P < 10^{-10}$; P7 $P < 10^{-10}$; P14 $P = 0.46$; P20 $P = 0.73$; P40 $P = 1.92 \times 10^{-5}$; P60 $P < 10^{-10}$; Adult $P = 0.426$; (AI) P1 $P < 10^{-10}$; P7 $P < 10^{-10}$; P14 $P = 0.33$; P20 $P = 0.004$; P40 $P = 2.33 \times 10^{-7}$; P60 $P = 4.7 \times 10^{-9}$; Adult $P < 10^{-10}$.

²Probabilities that V1 and AI neurons were different sizes: (Nissl) P1 $P = 0.61$; P7 $P = 0.001$; P14 $P = 0.20$; P20 $P < 10^{-10}$; P40 $P = 1.16 \times 10^{-4}$; P60 $P = 0.635$; Adult $P = 4.2 \times 10^{-9}$. (GABA) P1 $P = 0.012$; P7 $P = 0.026$; P14 $P = 0.53$; P20 $P = 9.54 \times 10^{-5}$; P40 $P = 0.12$; P60 $P = 0.66$; Adult $P = 4.34 \times 10^{-5}$.

³For abbreviations, see list.

during this time due to brain growth and cell death, the proportion of GABAergic neurons compared to Nissl-stained neurons was calculated. This analysis revealed an unexpected late peak in GABA-ir neurons. At birth, GABA-ir neurons were estimated at 6.7% of the total neurons in V1 and 9.4% in AI, dropping sharply by P20, but then increased about threefold at P60, followed by a decline back to lower levels by adulthood (7.1% in V1, 10.4% in AI; Fig. 8). Others have also reported high levels of GABA early in development, but have not reported a late peak (e.g., Hogan et al., 1992). The peak in the percentage of GABAergic neurons at P60 occurred well after the thalamocortical axons arrived in layer 4, and over a month after the cortical neurons have all migrated into position (Jackson et al., 1989).

The values reported for proportion of GABA-ir to total neurons in adult carnivore sensory cortex vary widely in the literature. A study in cat visual cortex found 20.6% GABA-ir neurons (Gabbott and Somogyi, 1986). An investigation of cat auditory cortex reported 24.6% GABA-ir neurons (Prieto et al., 1994). In the only other study in ferret V1, GABA-ir cells comprised 15% of the total (Pezuzzi, 1988). Other than differences in counting methods, one possible reason why the proportions of V1 GABA neurons were lower in ferrets relative to what has been reported for the cat may be the smaller layer 4 in ferrets (Rockland, 1985; Law et al., 1988). In monkeys, layer 4 of V1 is particularly high in GABA-ir neurons (Hendry et al., 1987).

Morphological development of GABAergic neurons

In adult ferrets as in other mammals, GABAergic neurons have predominantly bipolar or multipolar morphology. But early in development other morphological classes express GABA. At P1 and P7, we observed horizontally oriented GABA-ir neurons in the marginal zone with thick dendrites which resembled Cajal-Retzius cells (Marin-Padilla, 1988). At later ages, large somata in layer 1 expressed GABA, but because the dendrites were not immunoreactive, it was not possible to identify them as Cajal-Retzius cells. There is some disagreement in the literature about the fate of Cajal-Retzius cells during postnatal development. Pezuzzi noted horizontally oriented GABA-ir cells in the marginal zone of early postnatal but not adult ferret V1 (Pezuzzi, 1988). In adult cat visual and somatosensory cortex, a population of large

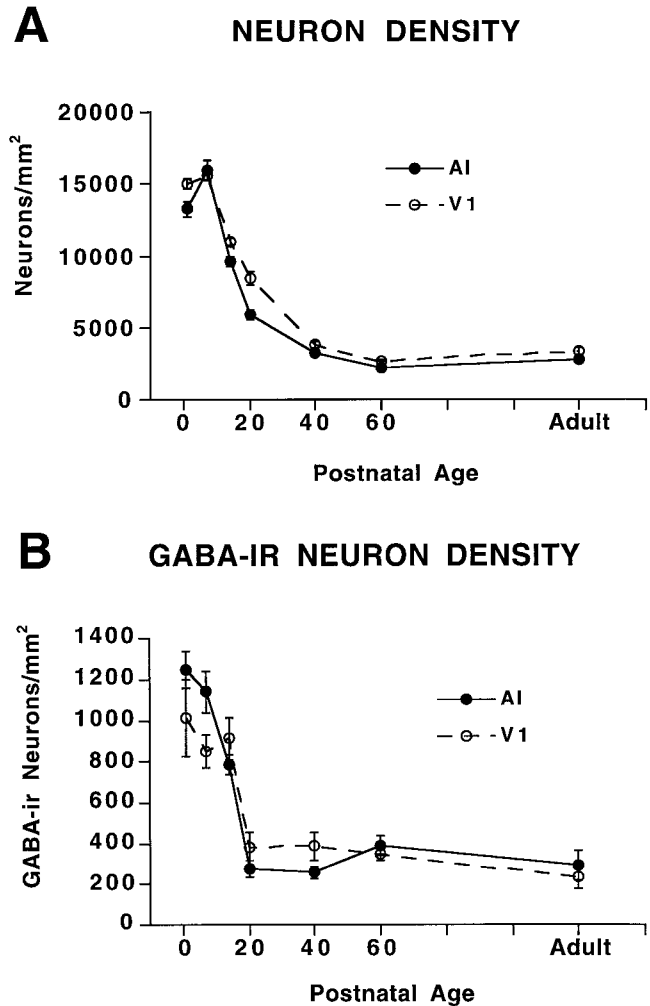


Fig. 7. The density of Nissl-stained and gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons. **A:** Developmental decline in neuronal density for Nissl-stained neurons. This graph shows neuronal density in cortical layers 1 through 6 in Nissl-stained primary visual cortex (V1) and primary auditory cortex (AI) during cortical development from postnatal day (P)1 to adulthood. The neuronal density was very high in both visual and auditory cortex at P1, as expected by the appearance of the densely filled cortical plate (Fig. 1). Due to the expansion of the brain with growth, the neuronal density declined sharply from P1 to P20 and then declined more gradually until P60. At adulthood, there was a slight increase due to the dorsoventral flattening of the skull. There were no marked differences in density between V1 and AI. **B:** Changes in GABA-ir neuron density during postnatal development. The GABA-ir neuron density was high at P1 in both V1 and AI, and declined dramatically thereafter until P40. The density increased slightly at later postnatal ages and decreased again by adulthood.

GABAergic horizontal neurons in layer 1 has been reported (Gabbott and Somogyi, 1986; Li and Schwark, 1994). Also, glutamic acid decarboxylase (GAD)-positive horizontal neurons resembling Cajal-Retzius cells have been found in layer 1 of adult cat AI (Prieto et al., 1994). Luskin and Shatz (1985b) reported that Cajal-Retzius cells disappear in adult cat visual cortex. Jackson et al. (1989) reported that there are two populations of layer I neurons produced during ferret cortical development which are both found in low numbers in adults. The early-generated

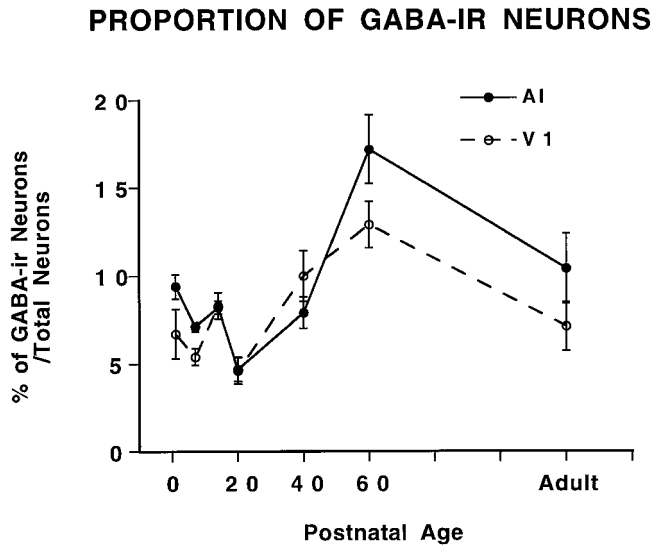


Fig. 8. Quantitative analysis of the proportion of gamma-aminobutyric acid-immunoreactive (GABA-ir) neurons compared with adjacent Nissl-stained sections. In both primary visual cortex (V1) and primary auditory cortex (AI), the percentage of GABA-ir neurons exhibited a small peak from postnatal day (P)1 to P14, declined slightly at P20 and then sharply increased from P20 to P60, especially in AI. Thereafter, the percentages declined by adulthood but remained higher in AI than V1.

ones express GABA, whereas the later ones do not (Peduzzi, 1988). More recent studies suggest that Cajal-Retzius cells may become so diluted by cortical growth that they are difficult to detect in adults (Marin-Padilla, 1988, 1998; Valverde et al., 1995), and our results are consistent with this interpretation.

We were surprised to find that GABA was transiently expressed by pyramidal neurons in ferret sensory cortex (Fig. 5E,F). When these neurons stopped expressing GABA after P7, there remained a GABA-ir sparse region in layer 5. Faint, transient parvalbumin and calbindin immunoreactivity have been reported in layer 5 pyramids in cat neocortex (Hogan et al., 1992; Hogan and Berman, 1993; Alcántara and Ferrer, 1994, 1995), but not in primary sensory cortex. The pyramidal cells that labeled transiently with calbindin were not GABAergic, and they were no longer present by P27 (Hogan and Berman, 1993). In E54 cat primary visual cortex, transient calbindin staining of pyramidal cells was reported (Stichel et al., 1987). The time course of ferret postnatal development is such that P21 ferret cortex is approximately equivalent to P1 cat cortex, and thus it is possible that cat V1 contains calbindin-positive or GABA-positive pyramidal cells prenatally. To our knowledge, ours is the first report of GABA-containing pyramidal neurons at any developmental stage in any animal. Glutamatergic pyramidal neurons are widespread at this age in ferrets (Herrmann, 1996). It would be interesting to characterize these GABAergic pyramidal neurons further, particularly with regard to whether they also contain glutamate.

Some of the bipolar cells which we observed below the cortical plate looked like migrating neurons and resembled those reported in other species (Del Rio et al., 1992; Yan et al., 1995). In addition, there were postmigratory bipolar cells resident in lower layer 6 and especially in the

subplate. In the lower cortical plate and below the subplate in the intermediate and subventricular zones are sparse neurons of various types including the tadpole-shaped cells reported by others (Del Rio et al., 1992; Yan et al., 1995), and these are probably migrating neurons (Roberts et al., 1993).

Methodological considerations

There are several concerns in any immunocytochemical study. For example, immunocytochemical methods have potential problems in the reliability of antibody labelling. Consequently, in this study, controls for reliability were included (see Materials and Methods). The antibody we chose (monoclonal mouse anti-GABA clone 5A9 from ICN Biomedicals) is commercially available and has been well-characterized (Szabat et al., 1992). Further confidence in the specificity of the staining comes from the fact that only nonpyramidal neurons were stained in older animals (although there was transient staining of pyramidal neurons at P1–7).

The goal of this study was to look at changes over time and between cortical areas, and the quantitative data should not be interpreted as absolute values (Guillery and Herrup, 1997). The numbers presented here may also be affected by incomplete penetrance of the antibody in the 30 or 50 μ m sections. Because the number of GABA-ir neurons was not larger in 50 μ m compared to 30 μ m sections, it must be assumed that the antibody penetrated incompletely, and therefore neuronal density values are presented as neurons per area rather than volume.

Comparison of visual and auditory cortex

Our estimated counts of GABA-ir neurons in V1 and AI were obtained with a technique that is useful for comparing reference populations. By comparing the reference population in V1 to that in AI, we found that the development of inhibitory circuitry was similar in these two primary sensory cortical areas, as was the morphology of individual neurons. There was a slightly higher density of GABAergic neurons in AI than V1 at P60, and the proportion of GABA neurons was also higher, perhaps due to the earlier development of AI (Bayer and Altman, 1991; Noctor et al., 1997). Lack of tangential analysis limited conclusions about patterns of GABAergic inputs to cortical circuits, but this would be an interesting avenue for further investigation. These data support the idea that different sensory cortical areas are similar in certain respects even though the response properties and topography of the two areas are quite different.

Growth of sensory cortex in ferrets

On P1 of ferret postnatal development, the cortical plate was densely occupied by neurons from the nascent layers 5 and 6 plus neurons destined for upper layers which are migrating toward the top of the cortical plate. As development proceeded, the cortical layers were formed as neurons migrated into their final positions, and the density of neurons was reduced both by cell death and by an increase in the volume of the brain with growth. This decline in neuronal density was exponential. There was a slight increase in neuron density between P60 and adulthood. This is probably a result of the flattening of the skull and resulting compression of the hemispheres (Apfelbach and Kruska, 1979; Kruska, 1993).

Time course of postnatal development

The main advantage of examining development of GABA-ir in ferrets is their relative immaturity at birth. Although ferret and cat cortical development follow a very similar time course (Luskin and Shatz, 1985a; Jackson et al., 1989), ferrets are born 21 days earlier. Thus, we were particularly interested in examining development of GABA-ir prior to P20 in ferrets.

The early high levels of GABA which we observed in ferret V1 and AI suggest that it may play a trophic role for developing neurons (Lauder, 1993). The composition of the GABA receptor is different at this stage (Laurie et al., 1992), and GABA causes excitation rather than inhibition of the postsynaptic cell early in development (Cherubini et al., 1991), allowing for the entry of calcium into the cell and the triggering of calcium-dependent signal transduction pathways. The late increase in GABA-ir coincides with an increase in GABA_A receptor levels (Pallas et al., 1994), suggesting that inhibitory synaptic transmission in general is upregulated, although GABA may operate extrasynaptically as well.

The increase in GABA immunoreactivity at P60 is difficult to integrate with what is known about postnatal developmental events in ferret sensory cortex. The critical period for ocular dominance plasticity begins after P20 and peaks from P35 to P43 (Chapman et al., 1996; Chapman and Stryker, personal communication). By P49 to P58, only a partial ocular dominance shift occurs with monocular deprivation, and by P68, ocular dominance shifts can no longer be induced at all. Thus, by P60 we would have expected ferret primary visual cortex to exhibit very little plasticity. Our results suggest that GABA may be playing a special role in plasticity in late postnatal development and may be operating through a different pathway than ocular dominance plasticity. These observations, coupled with numerous studies reporting activity-dependent regulation of GABA (Hendry and Carder, 1992; Rutherford et al., 1997; Hensch et al., 1998), suggest a role for GABA in cortical circuit construction and experience-dependent plasticity during late stages of development. In fact, our preliminary results suggest that the late increase in GABA can be stabilized by certain manipulations of sensory experience. We have found in ferret AI with early anomalous visual input that the proportion of a subpopulation of GABAergic neurons remains high after P60 in auditory cortex with early, anomalous visual inputs (Pallas et al., 1994).

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