Immunohistochemical Localization of GABA-Containing Neurons during Postnatal Development of the Rat Retina

Claudine Versaux-Botteri,* Roland Pocher,† and Jeanine Nguyen-Legros*

The localization of neurons containing gamma-amino-butyric acid (GABA)-immunoreactivity has been studied in the rat retina during postnatal development. Two populations of GABA-positive cells were observed. The first was located in the inner layers of the retina, with the number of cells and their immunoreactivity increasing during development until adulthood. Previous studies in adult rat enabled identification of these cells as a subpopulation of amacrine cells. The second was located in the outer layers of the retina. These cells displayed a transient GABA labelling, with no immunoreactivity detectable after postnatal day 15. Their localization and morphology corresponded to calbindin D-27kDa-positive horizontal cells. It was concluded that the transient GABA-positive cells were horizontal cells. Invest Ophthalmol Vis Sci 30:652-659, 1989

For several decades, gamma-aminobutyric (GABA) has been considered as a putative inhibitory neurotransmitter in the vertebrate central nervous system, including the retina. Two techniques have been used for the localization of GABA-containing neurons in the retina: the first is the uptake of tritiated GABA or GABA agonists like muscimol or isoguvacine, associated with autoradiography; the second is immunohistochemistry using antibodies against either L-glutamate decarboxylase (GAD, the rate-limiting enzyme in GABA biosynthesis) or, more recently, GABA itself. Two main types of GABA-containing neurons have been observed according to the species under study. In all vertebrates, a number of amacrine cells (and possibly ganglion cells) are labelled, while in lower vertebrates, horizontal cells are consistently labelled in addition to amacrine cells.

The development of the GABAergic system has been studied in a variety of vertebrate retinas. As observed in the majority of neurotransmitter systems during development, uptake and storage mechanisms are established before transmitter synthesis, so that GABA neurons can be visualized earlier by autoradiography than by immunohistochemistry. However, GAD immunohistochemistry has been used in developmental studies in mouse and rabbit. Two types of GAD-immunoreactive (GAD-I) neurons were observed: first, one population whose number and intensity of staining increased during postnatal maturation and remained prominent in the adult and which was located at the inner margin of the neuroblastic layer; and a second population whose immunoreactivity was transitory and which was located more sclerally within the neuroblastic layer. Although these latter neurons were in the position of future horizontal cells, clear identification of them still remains to be determined because their immunoactivity disappears in adult animals. Moreover, in rat, no GAD immunoreactivity was observed in the scleral part of the neuroblastic layer during development, whereas GABA-I and GAD-I horizontal cells were recently reported in adult primates. On the other hand, GAD and GABA immunohistochemistry have proven to give slightly different labelling patterns in the mammalian retina.

The aim of this work was threefold: (1) to describe the morphology and localization of GABA-containing neurons during the postnatal development of the rat retina, using a highly specific antibody directed against GABA; (2) to ascertain the existence of a class of transiently GABA-I expressing neurons in the rat; and (3) to identify these neurons, using an antibody to calbindin D-27kDa which has been reported to be a marker for horizontal cells.
Materials and Methods

All the animals were treated in a manner that conforms with the ARVO Resolution on the Use of Animals in Research.

Antisera

The antiserum to GABA was provided by Immunootech (Marseille, France). It was raised in rabbits against GABA coupled to protein carriers with glutaraldehyde. Its specificity has been already tested. It did not cross-react with glycine, glutamate, taurine, aspartate or beta-alanine.

Chick intestinal calbindin D-27kDa antibody was generously donated by D. E. M. Lawson (Cambridge, UK). It was prepared as described previously.

GABA Immunohistochemistry

Twenty-two albino Wistar rats were used, from birth to adulthood. Under deep ketamine anaesthesia, they were sacrificed by transcardiac perfusion of a fixative consisting of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Following perfusion, the eyeballs were dissected out and postfixed for 1 hr at 4°C in fresh fixative. The retinas were then removed and rinsed for 2 hr at 4°C in sodium cacodylate buffer containing 5% sucrose. The specimens were quickly frozen in isopentane cooled by liquid nitrogen and sectioned at −20°C with a cryostat microtome. The sections, 10 μm thick, were mounted on gelatin-coated slides. They were incubated for 24 hr at 4°C, in anti-GABA diluted (1/2000-1/4000) in tris buffer saline (TBS), 50 mM, containing 10% normal sheep serum and 0.3% Triton X-100. The second incubation was in goat anti-rabbit antiserum (1/10), followed by rabbit-PAP-complex (1/50), using diaminobenzidine as a chromogen. The sections were dehydrated and mounted with DPX.

Calbindin D-27kDa Immunohistochemistry

Seven rats were used from postnatal day 4 to adulthood. The eyeballs were removed from animals under deep anaesthesia and fixed by immersion in Carnoy fixative for 3 hr at room temperature. After a rapid rinse in absolute ethanol and rehydration in 90% and 70% ethanol, the retinas were dissected out and rinsed for 24 hr at 4°C in TBS, 50 mM, containing 10% normal sheep serum and 0.3% Triton X-100. The retinas were removed, rinsed in phosphate buffer, dehydrated in graded ethanol and embedded in araldite–epoxy mixture (Durcupan, Fluka), as described earlier. Two micron semi-thin sections were cut, mounted on gelatin-coated slides and deplasticized in sodium ethylate. Alternate sections were treated with anti-calbindin, using the same immunohistochemical procedure as for sections.

Controls

Controls were made in two ways: (1) by omitting the primary antiserum or replacing it by nonimmune serum; and (2) by using antigen-absorbed primary antisera. The preabsorbed anti-GABA antiserum was provided by the group who had raised the antiserum and already tested its specificity. The preabsorbed anti-calbindin D-27kDa was prepared by one of us, exactly as described earlier. GABA and calbindin immunoreactivities were completely abolished in all cases of control experiments.

Double Label Experiment

The eyes of a 7-day-old rat were rapidly dissected out under deep anaesthesia and fixed for 2 hr in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The retinas were removed, rinsed in phosphate buffer, dehydrated in graded ethanol and embedded in araldite–epoxy mixture (Durcupan, Fluka), as described earlier. Two micron semi-thin sections were serially cut, mounted on gelatin-coated slides and deplasticized in sodium ethylate. Alternate sections were treated with anti-GABA and anti-calbindin D-27kDa as described above.

Results

GABA-Immunoreactive (GABA-I) Cells

At birth, two regions could be distinguished in the rat retina. In the inner one-third, some ganglion cells and a narrow inner plexiform layer (IPL) were present. The outer two-thirds were occupied by a thick neuroblastic layer, at the inner margin of which some cells were faintly labelled. Additionally, large immunoreactive cell bodies were also observed in the mid-regions of the neuroblastic layer (Fig. 1a, b).

At postnatal (PN) day 4, GABA-I cell bodies were observed in both the amacrine and ganglion cell layers, as well as some in the IPL, where up to three sublayers could be distinguished by the intense immunoreactive product. In the middle of the neuroblastic layer, the number of GABA-I cells increased (Fig. 2).

At PN day 6, the number of GABA-I cells greatly increased in the ganglion and amacrine cell layers. Some retinal whole-mounts were reacted with anti-calbindin, using the same immunohistochemical procedure as for sections.
Fig. 1. GABA-I in 10 μm thick vertical section from newborn rat retina. (a) Some cell bodies and their processes are labelled (arrow) in the outer part of the NL. (b) Some amacrine cell precursors are GABA-positive in the inner part of the NL (arrows) adjacent to the IPL. Bar: 20 μm (×640).

Fig. 2. Immunohistochemical localization of GABA in 10 μm thick vertical section from rat retina at PN day 4. Some cell bodies are labelled (large black arrow) in the outer part of the NL. Some amacrine cell precursors are also immunoreactive: small black arrows indicate the amacrine cells located in the NL adjacent to the IPL and small white arrows show the putative amacrine cells displaced to the IPL and to the GCL. Bar: 20 μm (×512).

Fig. 3. Immunohistochemical localization of GABA in 10 μm thick vertical section from rat retina at PN day 6. The OPL has appeared and separates the ONL from the INL. GABA-I cell bodies (arrows) are visible at the outer margin of the INL and their processes are lying in the newly formed OPL. Many cell bodies are stained in the ACL. Bar: 20 μm (×448).

Fig. 4. GABA-I in 10 μm thick vertical section from rat retina at PN day 12. GABA-I cells can be observed at the outer margin of the INL (large arrows). Their processes lie in the OPL. Blood vessels are clearly distinguished (small arrow). GABA-I amacrine cells are observed in the ACL and in the GCL. Their processes form a dense plexus in the IPL. Bar: 20 μm (×512).
middle of the neuroblastic layer were now located in the outermost row of the INL and their processes were lying in the newly formed OPL (Fig. 3). Occasionally, some processes arising from these cells extended in a vitreal direction.

The immunoreactive pattern remained unchanged until PN day 12, while the inner layers of the retina appeared morphologically mature. The vitreal half of the INL was heavily GABA-I. Numerous cell bodies were observed in both the ganglion and amacrine cell layers (Fig. 4). Some GABA-I processes could be observed in the optic nerve fiber layer. Many cells were also labelled in the outermost row of the INL. Their intermingled processes were particularly visible in tangential sections (Fig. 5). In these sections, GABA-I processes could be observed crossing the INL, but it was not possible to trace them to their parent cell bodies, so that it could not be shown whether they came from GABA-I cells located at the inner or outer edge of the INL. The processes of the outer GABA-I cells could be observed in close vicinity with fully developed blood vessels (Fig. 4).

From this stage, GABA-I did not change in the inner retina, while in the outer retina it began to decrease and disappeared almost completely by PN day 15. In the adult, the pattern of GABA-I was very similar to that observed at PN day 15 (Fig. 6).

Calbindin D-27kDa-Immunoreactive (CaBP-I) Cells

At PN day 4, CaBP-I cells were observed in the outer part of the retina, in the middle of the neuroblastic layer. Their somata were large and their processes emerged in stellate fashion (Fig. 7). Some scattered cells were also faintly labelled in the innermost row of the neuroblastic layer.

At PN day 6, the number of CaBP-I cells increased in the outer part of the retina. With the appearance of the OPL, the CaBP-I cells were distributed in a regular mosaic, as seen in whole-mounted retina (Fig. 8). In sections, the processes of the CaBP-I cells became intermingled in the OPL and their somata were located at the outer margin of the INL.

By PN day 11, many CaBP-I processes forming a continuous plexus could be observed in the OPL. Their parent cell bodies, located in the outermost row of the INL, were strongly positive (Figs. 9, 10). In the inner retina, some scattered CaBP-I cells were observed in the ganglion and amacrine cell layers. The pattern of labelling was unchanged until adulthood.

GABA-CaBP Labelling on Serial Sections

Due to the transient nature of GABA immunoreactivity in the outer retina (present only between PN days 4 and 15), PN day 7 retina was selected for serial section labelling. At this stage serial sections clearly indicated that cells from the outer retina displaying a positive immunoreaction for GABA were also always positive for Calbindin (Fig. 11). However, the number of GABA-I cells was slightly lower than that of CaBP-I cells.

Discussion

Two classes of GABA-I cells could be observed in the developing rat retina. The cells of the first class were located in the inner layers of the retina, on both sides of the IPL. They became increasingly numerous and heavily labelled from birth to PN day 15 and remained unchanged until adulthood.

These results are in agreement with previous reports which have demonstrated the presence of GABA in the inner layers of the rat retina, within the amacrine cells. As early as the day of birth, some weakly GABA-I cells could be observed in the innermost row of the neuroblastic layer and can be regarded as amacrine cell progenitors. During the first postnatal week, the number of GABA-I cell bodies and sublayers of processes in the IPL increased rapidly. The number of GABA-I sublayers in the IPL confirmed the existence of numerous morphological subtypes of GABA-I amacrine cells sending processes to the different synaptic levels of the IPL. The analysis of these subtypes is not possible because of the high density of intermingled processes. As in other species, a large number of GABA-I cells are located in the amacrine cell layer as well as in the ganglion cell layer. Some cells, located in the middle of the IPL, can be compared to the interstitial amacrines. It is likely that the majority of those located in the ganglion cell layer represent a population of displaced amacrine cells, in agreement with biochemical data about the absence of GABA in the optic nerve and the persistence of these cells after optic nerve destruction. However, occasional GABA-I fibers could be observed in the nerve fiber layer. Although it was not possible to trace them to their parent cell bodies, they are reminiscent of a special type of ganglion cell which has a recurrent axon in the mammalian retina.

The presence of GABA-I interplexiform cells can be confirmed. Although GABA-I processes crossing the INL could not be reliably traced to cell bodies located either at the inner or outer margin of the INL during early stages, the persistence of these processes after the complete disappearance of GABA-I from cells in the outer INL strongly indicates their outgrowth from interplexiform cells, whose cell bodies are located among the amacrine cells.

The second type of GABA-I cells was located pri-
Fig. 5. GABA-I in 10 μm thick semitangential section from rat retina at PN day 12. GABA-I cells can be observed in the outer part of the INL with their intermingled processes in the OPL. Some immuno-reactive processes crossing the INL are visible (arrows). GABA-I amacrine cells are stained in the ACL. Bar: 20 μm (×410).

Fig. 6. GABA-I in 10 μm thick vertical section from rat retina at PN day 15. GABA-I has disappeared from the cells located at the outer margin of the INL and from the OPL. The inner layers: ACL, IPL and GCL remain intensely GABA positive. Bar: 20 μm (×410).

Fig. 7. CaBP-I HCs in 10 μm thick section from rat retina at PN day 4. Bar: 20 μm (×512).

Fig. 8. CaBP-I HCs from whole mounted rat retina at PN day 6. Their characteristic distribution in regular mosaic can be observed. Bar: 50 μm (×240).

Fig. 9. CaBP-I cells in 10 μm thick section from rat retina at PN day 11. The HC and their processes are intensely labelled. Some amacrine cells in the ACL and some cell bodies in the GCL are also positive. Bar: 20 μm (×512).

Fig. 10. CaBP-I cells in 10 μm thick semitangential section from rat retina at PN day 11. HCs are clearly distinguished by their heavy labelling. Bar: 20 μm (×640).
Fig. 11. Serial 2 μm semithin sections from rat retina at PN day 7, showing the same HCs labelled (a) with the anti-CaBP antiserum and (b) with the anti-GABA antiserum. Bar: 10 μm (X1024).

The first problem concerning these cells is their identification. Their localization in the middle of the neuroblastic layer and the flat organization of their processes are typical of horizontal cells. However, the period of development in which the transient GABA-I could be observed in these cells is concomitant with the period of blood vessel growth in the OPL. The accompanying macrophages, whose morphology is very close to that of developing horizontal cells, could be transiently immunoreactive to GABA. Thus, in order to unequivocally identify these cells, we have used the anti-calbindin D-27kDa antiserum which has proved to be a marker of horizontal cells in the mammalian retina. This marker has other target cells in the retina, depending on the species examined, but in rat demonstrates an intense labelling of horizontal cells, with a complete mosaic visible in flat-mounted retinas. Moreover, the anti-CaBP recognizes horizontal cells early in development. Sections stained with anti-GABA and anti-CaBP antisera allowed us to observe immunoreactive cells exhibiting similar morphological characteristics and process formation in the OPL. Moreover, labeling of serial sections with anti-CaBP and anti-GABA respectively demonstrates the coexistence in the same horizontal cell of CaBP and GABA. It can therefore be concluded that some horizontal cells are transiently GABA-positive.

Two sorts of horizontal cells have been described in the rat retina, axon-bearing type B and axonless type A. Whether the GABA-I horizontal cells belong to one or the other category could not be determined, but a reduced number of horizontal cells were la-
belled with anti-GABA as compared with the number labelled with anti-CaBP. Moreover, GABA is a neurotransmitter candidate for cone horizontal cells, and thus the axonless ones, in the catfish retina.43

The second problem is the significance of the transient GABA immunoreactivity in horizontal cells. Contrary to the mouse, no GAD immunoreactivity was reported in horizontal cells of the developing rat retina.22 Although we have not attempted to confirm this observation, the origin of the GABA remains problematic. During the first two postnatal weeks, the horizontal cells can exhibit some other transient properties, like acetylcholinesterase activity,44 expression of catecholamine biosynthetic enzymes (unpublished observations) or abnormal membrane permeability.45

If GABA is not synthesized within the cells, it could be taken up from the extracellular pool since the release of GABA has been shown to start during the first postnatal week in rabbits.20 Since, on the basis of their intermediate filament immunoreactivity, the horizontal cells can be regarded as glia-like,46 they could exhibit some cell membrane properties which account for a glia-type GABA uptake during development. The disappearance of GABA immunoreactivity is concomitant with the moment of full maturation of horizontal cells, i.e., the acquisition of specific neuronal properties. If GABA is synthesized by quantities of GAD, undetectable by histochemistry, its presence in horizontal cells may be related to the neurotrophic role of this substance during retinal development.47,48 since GABA immunoreactivity is no longer observable after PN day 15 which follows closely eye opening and the beginning of the retinal activity in rats.45 Whether GABA immunoreactivity disappears from horizontal cells because of cell death or changes in their properties is not known, but pyknotic nuclei have not been observed in the PN day 15 retina. These observations provide further evidence for the special status of horizontal cells among the retinal neural cells.

**Abbreviations**

ACL: amacrine cell layer  
CaBP-I: calbindin D-27kDa immunoreactivity  
GABA-I: GABA immunoreactivity  
GCL: ganglion cell layer  
HC: horizontal cells  
INL: inner nuclear layer  
IPL: inner plexiform layer  
NL: neuroblastic layer  
ONL: outer nuclear layer  
OPL: outer plexiform layer  
PN day: postnatal day

**Key words:** anti-GABA antiserum, retina, development, calbindin D-27kDa, horizontal cells

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**References**


