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Reductions in N-Acetylaspartylglutamate and the 67 kDa Form of Glutamic Acid Decarboxylase Immunoreactivities in the Visual System of Albino and Pigmented Rats after Optic Nerve Transections

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ABSTRACT

This study compares the immunohistochemical distributions of N-acetylaspartylglutamate (NAAG) and the large isoform of the 7-aminobutyric acid (GABA)-synthesizing enzyme glutamic acid decarboxylase (GAD_{67}) in the visual system of albino and pigmented rats. Most retinal ganglion cells and their axons were strongly immunoreactive for NAAG, whereas GAD_{67} immunoreactivity was very sparse in these cells and projections. In retinorecipient zones, NAAG and GAD_{67} immunoreactivities occurred in distinct populations of neurons and in dense networks of strongly immunoreactive fibers and synapses. Dual-labeling immunohistochemistry indicated that principal neurons were stained for NAAG, whereas local interneurons were stained for GAD_{67}. In contrast to the distribution observed in retinorecipient zones, most or all neurons were doubly stained for NAAG and GAD_{67} in the thalamic reticular nucleus. Ten days after unilateral optic nerve transection, NAAG-immunoreactive fibers and synapses were substantially reduced in all contralateral retinal terminal zones. The posttransection pattern of NAAG-immunoreactive synaptic loss demarcated the contralateral and ipsilateral divisions of the retinal projections. In addition, an apparent transynaptic reduction in GAD_{67} immunoreactivity was observed in some deafferented areas, such as the lateral geniculate. These findings suggest a complicated picture in which NAAG and GABA are segregated in distinct neuronal populations in primary visual targets, yet they are colocalized in neurons of the thalamic reticular nucleus. This is consistent with NAAG acting as a neurotransmitter release modulator that is coreleased with a variety of classical transmitters in specific neural pathways. J. Comp. Neurol. 458:221-239, 2003.

Indexing terms: NAAG; immunohistochemistry; glutamic acid decarboxylase; GABA; lateral geniculate

The retinal projection pathways in mammals utilize excitatory amino acid neurotransmission to initiate postsynaptic activity in neurons of the primary visual target areas. Electrophysiological and pharmacological studies have implicated both N-methyl-D-aspartate (NMDA) and non-NMDA-type glutamate receptors in this transmission process (Kemp and Sillito, 1982; Crunelli et al., 1987; Sillito et al., 1990a,b; Van den Pol, 1991; Funke et al., 1991). Ultrastructural immunocytochemical studies have demonstrated high levels of glutamate immunoreactivity in synaptic terminals of retinal origin (Montero and Wenthold, 1989; Montero, 1990; Cardozo et al., 1991). A nervous system-specific dipeptide, N-acetylaspartylglutamate (NAAG), also is found in very high concentrations in the visual system of mammals, e.g., 3.86 *μmol/g* weight in the optic tract of monkeys (Molinar-Rode and Pasik, 1992). It

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is localized in most retinal ganglion cells; in the optic nerves, chiasm, and tracts; and in the neuropil of all retinal target areas (Tieman et al., 1987, 1988, 1991a; Anderson et al., 1987; Moffett et al., 1990). Optic nerve transection and eye removal experiments have demonstrated that NAAG is localized in the synaptic terminals of retinal ganglion cells (Moffett et al., 1991b; Tieman et al., 1991b), and release experiments have demonstrated the calcium dependence of NAAG release (Williamson and Neale, 1988; Tsai et al., 1988; Zollinger et al., 1988; Williamson et al., 1991). Extracellular peptidase activities hydrolyze the dipeptide into glutamate and another nervous system-specific acetylated compound, N-acetylaspartate (Serval et al., 1990; Thomas et al., 2000).

NAAG itself is a weak but selective agonist at NMDA receptors (Westbrook et al., 1986; Trombley and Westbrook, 1990; Sekiguchi et al., 1992; Valivullah et al., 1994). However, it does not produce consistent NMDA-mediated postsynaptic responses under several electrophysiological paradigms (Riveros and Orrego, 1984; Henderson and Salt, 1988; Whittemore and Koerner, 1989; Radhakrishnan and Henry, 1993). NAAG application, in the lateral geniculate nucleus (LG) of the cat, is reported to elicit slow, weak excitatory action on fewer than 50% of neurons, while having a weak inhibitory effect on approximately 10% of neurons tested (Jones and Sillito, 1992). For the suprachiasmatic nucleus, NAAG is reported to depolarize neurons and to potentiate the action of glutamate (Bos and Mirmiran, 1993). NAAG also has been implicated in the perception of chronic and neuropathic pain (Yamamoto et al., 2001).

NAAG inhibits forskolin-stimulated increases in neuronal cAMP through metabotropic glutamate receptors (Wroblewska et al., 1993). Current evidence indicates that NAAG acts specifically through the mGluR3 subtype of metabotropic glutamate receptor (mGluR) to reduce intracellular cAMP formation (Wroblewska et al., 1997, 1998). For example, in hippocampal slices, NAAG inhibits long-term potentiation at the perforant-granule cell synapse via mGluR3 receptor-mediated reductions in cAMP formation (Lea et al., 2001). NAAG is also neuroprotective, probably via mGluR activation (Bruno et al., 1998a; Lu et al., 2000; Thomas et al., 2000).

NAAG affects the release of classical transmitters, including stimulation of dopamine release in the striatum and substantia nigra (Galli et al., 1991) and inhibition of norepinephrine release in the hippocampus (Puttfarcken et al., 1993). Furthermore, NAAG inhibits KC1-stimulated [^{3}H]γ-aminobutyric acid (GABA) release from cortical interneurons (Zhao et al., 2001), and this inhibition was correlated with mGluR3-mediated reductions in cAMP and subsequent reductions in protein kinase A activity and L-type calcium channel conductance. These findings have led Neale et al. (2000) to hypothesize that NAAG acts predominantly at presynaptic mGluR3 receptors to modulate further transmitter release. Taken together, the mounting evidence on NAAG's actions in the nervous system strongly implicate this molecule in presynaptic neurotransmitter release modulation by reducing cAMP formation through the mGluR3 receptor. Low-level activation of some glutamatergic systems may also be one of NAAG's actions in the nervous system, but this action is less well established in vivo.

By utilizing a significantly improved fixation procedure for NAAG immunohistochemistry (Moffett et al., 1993, 1994) and highly purified antibodies (Moffett and Namboodiri, 1995), a detailed analysis was made of the distribution of this dipeptide throughout the visual system of both pigmented and albino rats 10 days after unilateral or bilateral optic nerve transections. The results obtained using the new methods were improved in several ways compared with previous studies of NAAG localization in the visual system. The newer methods provided dramatically greater detail in the staining of cellular and neuropil elements; comprehensive axonal labeling, which was not possible using previous methods lacking the dimethylsulfoxide (DMSO) penetrant; greatly reduced background staining; and uniform NAAG labeling throughout the brain. With these new methods, the first detailed comparison was made in the rat visual system between the distribution of NAAG and that of the 67 kDa form of glutamic acid decarboxylase (GAD_{67}), a marker for inhibitory neurons that utilize GABA as a neurotransmitter. This study also provides the first comparison of the visual projections in albino and pigmented rats using antibodies to both NAAG and GAD.

MATERIALS AND METHODS

Chemicals were from Sigma (St. Louis, MO), except where otherwise noted. Solutions were prepared with purified water. Normal goat serum (NGS), horseradish peroxidase (HRP)-labeled avidin-biotin complex, biotinylated secondary antibody, and HRP-labeled secondary antibody reagents were from Vector (Burlingame, CA). Polyclonal rabbit antibodies to GAD_{67} were from Chemicon (Temecula, CA; K2 antibody), and additional HRP-labeled goat anti-rabbit secondary antibodies were from Kirkegaard and Perry (Gaithersburg, MD). Animals were from, and surgery and postoperative care were performed by, Zivic Miller (Zelienople, PA). Research was conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23) and the Georgetown University Animal Care and Use Committee. Polyclonal antibodies to NAAG were produced in rabbits and were affinity purified and negative-affinity adsorbed as previously described (Moffett et al., 1994). Nitrocellulose-immobilized protein conjugates of N-acetylaspartate, glutamate, aspartate, N-acetylglutamate, and GABA as well as carbodiimide-treated whole brain proteins were used as adsorbants. The specificity of the NAAG antibodies has been described previously (Moffett and Namboodiri, 1995).

Sprague-Dawley albino and Long-Evans pigmented (hooded) male rats, weighing between 130 and 180 g, were used for this study. They included six albino rats with preretinal (intraorbital) unilateral optic nerve transections, two albino rats with intracranial unilateral optic nerve transections, five hooded rats with preretinal unilateral transections, and six hooded rats with preretinal bilateral optic nerve transections. Three albino and three hooded rats without operations were also examined. Ten days after optic nerve transections, animals were deeply anesthetized with pentobarbitol (300 mg/kg) according to approved animal care and use protocols and were perfused transcardially with 500 ml of an aqueous solution of 6% l-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) containing 6% (n = 21) or 10% (n = 4) DMSO and 1 mM N-hydroxysuccinimide, as previously described (Moffett et al., 1993, 1994). Brains and eyes

were removed to a solution of 100 mM sodium phosphate in physiological saline at pH 8 containing 4% formaldehyde for 24-48 hr. The tissue was serially saturated with 10%, 20%, and 30% sucrose solutions in phosphate-buffered saline (PBS; pH 7.2) prior to freezing. Brains were sectioned in the frontal plane from the anterior preoptic area to the anterior pons at a thickness of 20 |jim, and retinas were cut in cross section at a thickness of 30 jjim.

Tissue sections were incubated with 2% NGS in PBS (pH 7.2, containing 0.1% sodium azide) for 30 minutes or longer before applying antibody. Brain sections were processed freely floating in 30 mm tissue culture dishes, whereas retinal sections were mounted on slides before staining. Purified anti-NAAG antibodies were diluted 1:200 to 1:400 (relative to the volume of crude serum) in PBS with 2% NGS and incubated with sections for 48 hr or longer at room temperature under constant rotary agitation in a humid chamber. Antibodies to GAD_{67} were diluted from 1:14,000 to 1:16,000 and incubated with adjacent tissue sections under the same conditions. Bound antibodies were visualized by the avidin-biotin complex method with peroxidase as the marker enzyme. The biotinylated secondary antibodies and avidin-HRP complex solutions were each incubated with tissue sections for 80-90 minutes, with four washes of PBS between. After final washing, the sections were developed with an Ni and Co enhanced diaminobenzidene/peroxide reaction (Pierce Chemical Co., Rockford, IL). The sections were mounted on treated slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA), dried, dehydrated in an ethanol series, and covered with xylene and resin.

Dual-labeling immunohistochemistry was performed sequentially for the two antigens. NAAG antibodies (1:200 to 1:400) were incubated with the sections for 48 hr and processed as described above. The chromogen employed in the first stage of staining was diaminobenzidene with catechol (HistoMark Orange; Kirkegaard and Perry), which produces an orange reaction product. The HRP marker enzyme used to visualize the first antigen was then inhibited by incubating sections for 30 minutes in 50:50 methanol/water containing 1% H_20_2 and 10 mM dithiothreitol. Sections were washed thoroughly with PBS and incubated for 30 minutes in 2% NGS in PBS. The sections were then incubated with GAD_{67} antibodies overnight (1:6,000 to 1:7,000 in 2% NGS), with constant agitation. The antibodies to GAD_{67} were detected with HRP-labeled goat anti-rabbit secondary antibodies (Kirkegaard and Perry) at a concentration of 5 µg/ml in PBS containing 0.2% bovine serum albumin (90 minute incubation). Sections were washed thoroughly in PBS and developed with a purple chromogen (VIP; Vector).

Control experiments were carried out to ensure that no primary (rabbit) anti-NAAG antibodies from the first incubation were detected by the labeled secondary (goat anti-rabbit) antibodies used in the localization of GAD_{67}. Sections were incubated for 48 hr in NAAG antibodies (1:200 in 2% NGS) and processed as usual for dual labeling, but they were not developed with chromogen. The peroxidase marker was inhibited as noted above, and then the sections were processed for a second antigen as described above, with the exception that the GAD_{67} antibodies were excluded, and, instead, the sections were incubated for 24 hr with 2% NGS. The sections were then washed and incubated for 90 minutes with 5 µg/ml HRP-labeled goat anti-rabbit secondary antibodies and developed with the Ni/Co enhanced chromogen. Other standard control tests for antibody specificity were also performed as previously described (Moffett and Namboodiri, 1995).

The neuroanatomical nomenclature used in this study was taken from Swanson (1992). The photomicrographs were taken on 35 mm Kodak color Kodachrome ASA 25 film or Tmax ASA 100 black-and-white film, with a Zeiss Axiophot microscope, and digitally scanned using a Nikon Coolscan II film scanner (1,350 dpi). Black-and-white digitized images were not corrected for contrast or brightness, but color images were color corrected when necessary using PC-based imaging software.

RESULTS

Enhanced carbodiimide fixation (Moffett et al., 1993) provided a significant improvement in the immunohistochemical localization of NAAG in the rat visual system compared with previous studies. Greater cellular and neuropil detail and dramatically improved axonal labeling permitted a detailed analysis of the localization of this dipeptide throughout the retinal projections. Two concentrations of DMSO were used to increase fixative penetration into tissues. The higher concentration of DMSO (10%), was found to produce fixation artifacts and excessive tissue shrinkage but did not alter the overall pattern of immunoreactivity. The lower concentration (6%) yielded excellent results both in terms of uniform staining for NAAG throughout the tissue and in terms of good tissue preservation. Tissue shrinkage was still apparent but was not excessive. The use of nickel and cobalt to enhance the opacity of the diaminobenzidene reaction product provided additional sensitivity for fine processes and puncta.

Highly specific antibodies to NAAG were made possible by several purification steps. Antibodies were first incubated with nitrocellulose-bound protein (bovine serum albumin) conjugates of several molecules, including glutamate, aspartate, N-acetylglutamate, N-acetylaspartate, and GABA, as well as with immobilized carbodiimide-treated rat brain proteins. This initial "negative-affinity adsorption" step was followed by affinity purification (Moffett et al., 1994). After affinity purification, anti-NAAG antibodies were tested for specificity against serial dilutions of the conjugates listed above. If any remaining cross reactivities were observed, the antibodies were again incubated overnight with the nitrocellulose-bound protein conjugates until assays showed no remaining cross reactivity (Moffett and Namboodiri, 1995).

The results obtained with the combined use of these new methods were improved over results from previous studies in several ways, including 1) greater cytological detail, 2) comprehensive axonal labeling, 3) reduced background staining, and 4) uniform labeling across areas. The lack of penetrant in previous studies of the visual system limited the ability to demonstrate clearly the loss of staining in fiber pathways after optic nerve section. In addition to the improvements in NAAG immunolabeling, this study is the first detailed analysis of the differences in NAAG and GAD staining in the visual projections of albino and pigmented rats.

In general, NAAG immunoreactivity (NAAG-IR) was punctate in nature, often appearing as densely packed puncta throughout the somata and proximal dendrites of neurons. NAAG staining was never seen in the distal dendritic branches of immunoreactive neurons. Intense

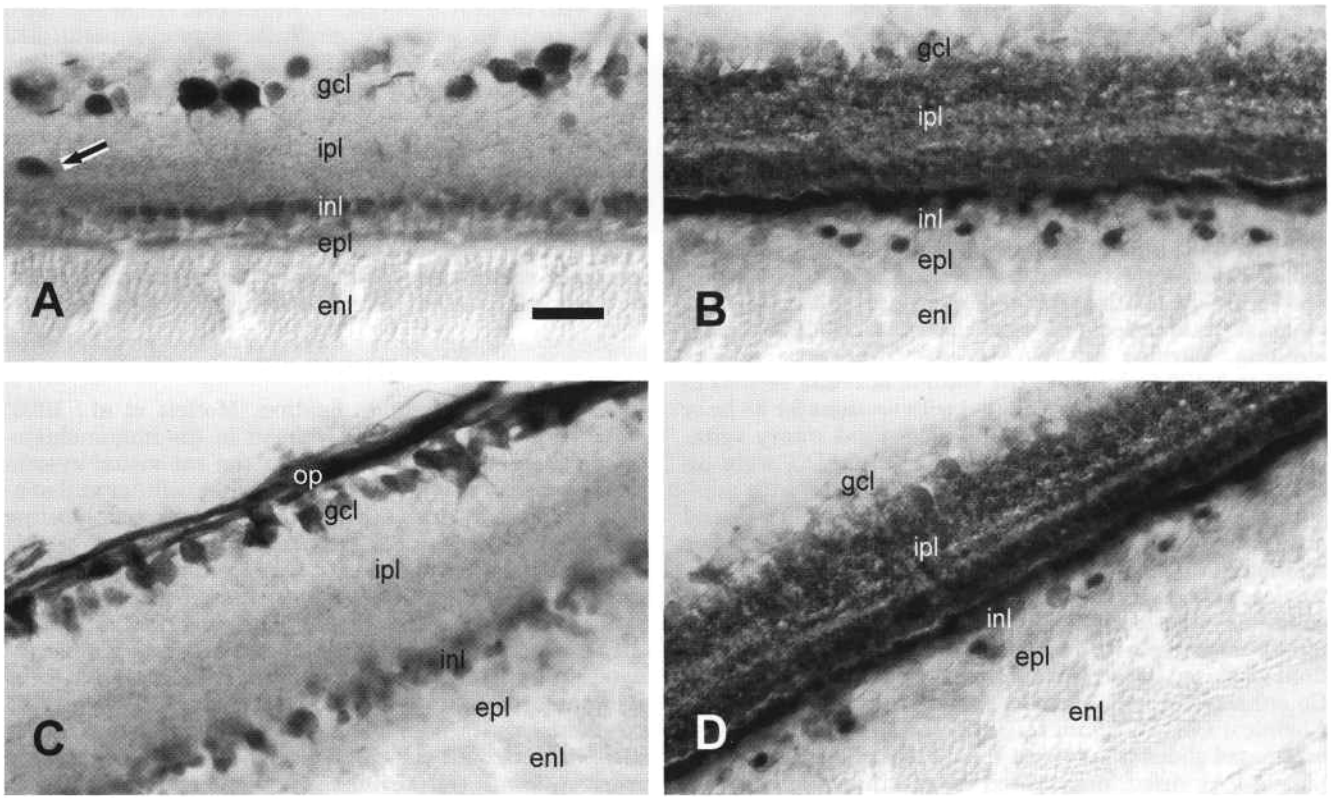

Fig. 1. NAAG-IR and GAD_{67}-IR in the albino rat retina. NAAG-IR (A,C) was observed in many, but not all, neurons of the ganglion cell layer (gcl). Occasional neurons in the inner plexiform layer (ipl) were also stained for NAAG (arrow in A). Neurons concentrated on the inner margin of the inner nuclear layer (inl) were moderately stained for NAAG, whereas photoreceptor cells of the external nuclear layer (enl) were not stained. Patchy, light NAAG-IR was present in the external plexiform layer (epl in A). Light to moderate punctate NAAG-IR was observed in the ipl neuropil, with higher levels in the more superficial layers bordering the inl. Most or all axons within the optic fiber layer (op in C) were intensely stained for NAAG. GAD_{67}-IR in the rat retina was observed in a population of neuronal cell bodies in the inl and in a dense network of strongly stained puncta concentrated in several longitudinal bands within the neuropil of the ipl (B,D). GAD_{67}-immunoreactive neurons in the inl were scattered throughout the layer, unlike NAAG-IR neurons, which were selectively localized to the border with the ipl. Many neurons in the gcl exhibited light, punctate GAD_{67}-IR on their surfaces, suggestive of synaptic contacts. Scale bar = 20 μm.

axonal staining occurred in specific neural pathways, such as the retinal ganglion cell projections, but not all NAAG-positive cell groups had immunopositive efferent fiber tracts. For example, the somata of neurons in the thalamic reticular nucleus were all strongly positive for NAAG and GAD, but their GAD-positive axons in the internal capsule were never stained for NAAG. The new fixation method employing a penetrant ensured that NAAG could be detected throughout fiber pathways, so the lack of immunoreactivity in certain projections was not due to incomplete fixation of NAAG in white matter.

Retina

As observed in previous studies (Anderson et al., 1987; Moffett et al, 1991b; Williamson et al, 1991), NAAG-IR in the retina was most prominent in retinal ganglion cells (Fig. 1A,C). No differences in immunoreactivity were observed between albino and hooded rat retinas. Most of the cells in the ganglion cell layer were immunoreactive for NAAG, with cells ranging from lightly to intensely stained. All size classes of ganglion cells were stained for NAAG, and no relationship was observed between staining intensity and the size of the neuronal somata. The proximal dendrites of some ganglion cells could be seen entering multiple sublayers of the inner plexiform layer. Most ganglion cell axons within the optic fiber layer were strongly stained for NAAG (Fig. 1C). NAAG-immunoreactive puncta occurred throughout the inner plexiform layer, being more concentrated near the inner nuclear layer. NAAG-immunoreactive neuronal somata were present in the inner nuclear layer, particularly along the border with the inner plexiform layer (Fig. 1A,C). Very light punctate NAAG-IR was observed in the external plexiform layer of the retina. Photoreceptor cells with cell bodies in the external nuclear layer were the only cell type in the retina that never stained for NAAG.

GAD_{67}-IR in the rat retina was intense in scattered neuronal cell bodies in the inner nuclear layer and within the neuropil of the inner plexiform layer, where staining was concentrated in several longitudinal bands (Fig. 1B,D). GAD_{67}-IR was also associated with the surface of neurons in the ganglion cell layer, suggestive of GAD-containing synaptic contacts. A very small number of neurons in the ganglion cell layer contained moderate immunoreactivity for GAD_{67} throughout their cytoplasm, possibly representing displaced amacrine cells. However,

no GAD_{67}-IR occurred in axons in the optic fiber layer or in photoreceptor cells in the external nuclear layer.

Optic chiasm and suprachiasmatic nucleus

Most or all of the axons of the optic nerves were immunoreactive for NAAG in both albino and pigmented rats (Fig. 2A). The optic chiasm from a pigmented rat is shown in Figure 2B stained for NAAG 10 days after unilateral transection at several rostrocaudal levels. In the animals with unilateral optic nerve transections, the loss of NAAG-IR in the optic chiasm produced a staining pattern very similar to that observed following injection of tracer compounds into one eye (Fig. 2B; Kita and Oomura, 1982; Johnson et al., 1988). When bilateral optic nerve transections were performed, all immunoreactivity was lost in the optic nerves (data not shown). However, a belt of NAAG-immunoreactive fibers remained below the optic chiasm, as can be seen in Figure 3B. These fibers made up the supraoptic commissure and were not directly associated with the retinal projections.

NAAG-IR in the suprachiasmatic nucleus (SCN) was observed in both neurons and neuropil (Fig. 3A,C,E). Neurons with NAAG-IR occurred throughout the SCN, but those with the highest degree of immunoreactivity were located in the dorsomedial aspect of the caudal portion of each nucleus. Immunoreactivity for NAAG in the neuropil was also observed throughout the SCN, but the density of NAAG-immunoreactive puncta was notably greater in a relatively restricted zone in the ventrolateral quadrant of the posterior half of the nucleus (Fig. 3A,C). The NAAG-IR in this neuropil was present in very fine puncta that could be observed only at high magnification (Fig. 3E). Much of the punctate NAAG-IR in the ventrolateral neuropil was lost in both SCN after bilateral optic nerve transections (Fig. 3B,D,F).

GAD_{67}-immunoreactive fibers were not observed in the optic nerves and chiasm. However, GAD_{67}-IR was very strong in the SCN neuropil, as previously reported (Moore and Speh, 1993), but was difficult to detect in cell bodies. After unilateral and bilateral optic nerve transections, changes in GAD_{67}-IR in the SCN were not apparent (data not shown).

LG and optic tract

The LG was conspicuous in NAAG-stained sections, containing substantially greater NAAG-IR than surrounding thalamic nuclei (see Fig. 4B). Immunoreactivity for NAAG in the optic tract of albino and pigmented rats was intense in the majority of axons, and these were observed entering the LG at all levels (Fig. 4B). NAAG-immunoreactive fibers diverged from the optic tracts to penetrate both the dorsal LG (LGd) and the ventral LG (LGv). The supraoptic commissure runs adjacent to the optic tracts at the level of the LG, and most of its fibers were stained moderately for NAAG. Scattered NAAG-immunoreactive neurons were intercalated within the supraoptic commissure.

NAAG-stained elements were extensive in the rat LGd and included axons, terminals, and somata of principal LGd neurons. NAAG-immunoreactive fibers formed numerous terminals of various sizes in the LGd and LGv, many terminals in the LGd being exceptionally large and concentrated in clusters. The principal neurons of the LGd were moderately immunoreactive for NAAG, but their dendrites and the axons forming the cortical projections were unstained. In the animals with unilateral optic nerve transections, NAAG-IR was reduced to only a few scattered fibers in the optic tract contralateral to the cut nerve. The loss of NAAG-IR in the neuropil of the contralateral LGd observed 10 days after unilateral optic nerve transection was extensive (Fig. 4A). A patch of NAAG-immunoreactive puncta remained in the core region of the contralateral LGd that receives input from the ipsilateral eye (Fig. 4C). The homotopic core region in the opposite LGd (ipsilateral to the cut nerve) contained only sparse NAAG-immunoreactive puncta (Fig. 4D), whereas the surrounding geniculate body retained its strong NAAG-immunoreactive afferentation (Fig. 4B,D).

The ventral division of the lateral geniculate (LGv) contained cells and neuropil that were immunoreactive for NAAG (Fig. 5F,H). The medial division of the LGv was characterized by low NAAG-IR in the neuropil and strong NAAG-IR in medium-sized to large neuronal somata. Less intense NAAG-IR was observed in neurons with smaller somata. A very dense plexus of NAAG-IR puncta was present in the lateral subdivision of the LGv. After unilateral optic nerve transections, large reductions in NAAG-IR were observed in the neuropil of the lateral portion of the contralateral LGv (Fig. 5E,G). A vertically oriented strip of NAAG-IR remained at the outer boarder of the LGv contralateral to the cut optic nerve. This remaining ipsilateral projection was more prominent in pigmented than in albino rats (cf. Fig. 5E and G). No noticeable changes in NAAG-IR occurred in the medial LGv after optic nerve transections.

GAD_{67}-IR was very limited in the optic tract of albino and pigmented rats (Fig. 4E,F). Sparse GAD_{67}-immunoreactive fibers and very small numbers of stained neurons were visible in the optic tracts. The supraoptic commissure, which courses adjacent to the optic chiasm and tracts, contained substantially more GAD_{67}-immunoreactive fibers and neuronal somata. In the LGd, many GAD_{67}-immunoreactive neurons were observed, with somata that were generally smaller than those with NAAG-IR in the same area. Ten days after unilateral optic nerve transections, a reduction in GAD_{67}-IR was observed in the LGd, with a pattern similar to that of the loss observed for NAAG-IR (Fig. 4E-H). In pigmented rats, a patch of higher GAD_{67}-IR was observed in the core of the LGd contralateral to the cut optic nerve, in the same position as was observed with the remaining NAAG-immunoreactive puncta (Fig. 4E). The loss of GAD_{67}-IR in the deafferented LGd appeared to be accompanied not by a reduction in the number df immunoreactive neuronal somata but rather by lower levels of immunoreactivity in the interneurons and associated neuropil in the denervated regions.

GAD_{67}-IR in the neuropil of the LGv was much more intense than that in the LGd. In the lateral portion of the LGv, a very dense network of GAD_{67}-immunoreactive puncta was present along with some strongly stained neurons with small cell bodies. The GAD_{67}-immunoreactive puncta were less dense in the medial LGv, and no immunoreactive somata were observed there. No observable reduction in GAD_{67}-IR occurred in the LGv after optic nerve transections (data not shown).

NAAG-IR in albino and pigmented retinogeniculate projections

After unilateral optic nerve transections, the distribution of NAAG-IR in the LGd of hooded rats exhibited the