Effect of optic nerve transection on N-acetylaspartylglutamate immunoreactivity in the primary and accessory optic projection systems in the rat

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Evidence has been presented in recent years that support the hypothesis that N-acetylaspartylglutamate (NAAG) may be involved in synaptic transmission in the optic tract of mammals. Using a modified fixation protocol, we have determined the detailed distribution of NAAG immunoreactivity (NAAG-IR) in retinal ganglion cells and optic projections of the rat. Following optic nerve transection, dramatic losses of NAAG-IR were observed in the neuropil of all retinal target zones including the lateral geniculate nucleus, superior colliculus, nucleus of the optic tract, and the terminal nuclei of the accessory optic tract. Brain regions were microdissected and NAAG levels measured by a radioimmunoassay (RIA) (IC50: NAAG = 2.5 nM, NAA = 100 µM; smallest detectable amount = 1–2 pg/assay). Large decreases (50–60%) in NAAG levels were detected in the lateral geniculate, superior colliculus and suprachiasmatic nucleus. Moderate losses (25–45%) were noted in the pretectal nucleus and the nucleus of the optic tract. Smaller changes (15–20%) were detected in the paraventricular nucleus and the pretectal area. These results are consistent with a synaptic communication role for NAAG in the visual system.

INTRODUCTION

The optic projection system of mammals originates in the ganglion cells of the retina and terminates in several areas of the brain including the lateral geniculate nucleus, the superior colliculus, the nucleus of the optic tract, and the terminal nuclei of the accessory optic tract. There is also a well-documented retinohypothalamic projection in mammals that terminates in the suprachiasmatic nucleus of the hypothalamus. Although these pathways are thought to function through excitatory amino acid neurotransmission, the neurotransmitter released at ganglion cell terminals in these retinorecipient zones is not clear.

A substantial amount of evidence supports the hypothesis that excitatory amino acid transmission operates in the visual pathways of mammals. The electrophysiological data are the most suggestive of a role for amino acids in the early stages of visual processing. Kemp and Sillito17 offered the first convincing evidence that excitatory amino acid neurotransmission, rather than cholinergic transmission, was operating in the retinogeniculate pathway of cats. Later, in vitro studies in the rat lateral geniculate nucleus specifically implicated the non-N-methyl-D-aspartate (non-NMDA) type glutamate receptors in optic nerve activation of geniculate relay neurons9. Koch18 then proposed that of the 3 known types of excitatory amino acid receptors (kainate, quisqualate, and NMDA), the NMDA class were primarily involved in the projection from visual cortex back to the lateral geniculate nucleus. This corticogeniculate pathway is thought to be involved in the activity of geniculate neurons. Recently, more thorough investigations have been done on the comparative efficacies of various excitatory amino acid transmitter antagonists in blocking the effects of visual stimulation and agonist application in the lateral geniculate. One study confirmed the involvement of non-NMDA (i.e. kainate and quisqualate) receptors in retinogeniculate transmission33. A second study clearly demonstrated the dependence of the visual response in anesthetized cats on NMDA receptor activation in the lateral geniculate34. Thus, it appears as though multiple glutamate receptor systems are involved in the activation of lateral geniculate cells in response to visual and cortical input. Additional electrophysiological evidence supporting the role of excitatory amino acid transmission in retinofugal pathways comes from recent work on the retinal projections to the hypothalamus. The responses of suprachiasmatic nucleus neurons to optic
nerve stimulation and applied agonists both are blocked by amino acid receptor antagonists in this retinal target zone\textsuperscript{4,32}. Other evidence in support of acidic amino acid transmission in the visual system includes the demonstration in vivo of the release of both glutamate and aspartate in the superior colliculus following optic nerve stimulation in rabbit\textsuperscript{30} and pigeon\textsuperscript{6}. Release of glutamate and aspartate has also been demonstrated in the suprachiasmatic nucleus of the rat in response to optic nerve stimulation\textsuperscript{19}. Cells of the lateral geniculate nucleus\textsuperscript{15,44} and superior colliculus\textsuperscript{12} have been found to be highly sensitive to the excitotoxic effects of kainate, which appears to act most effectively on neurons with intact acidic amino acid receptor systems\textsuperscript{35}.

While the evidence for excitatory amino acid neurotransmission in the retinal projections is compelling, it has still not been demonstrated that glutamate or aspartate is the endogenous transmitter in the mammalian visual system. For example, no convincing immunohistochemical evidence has been presented which demonstrates high levels of glutamate in the retinal ganglion cells of mammals and their axons and terminals in the brain. Alternatively, a number of neuropeptides, including somatostatin\textsuperscript{28} and substance P\textsuperscript{41}, have been identified in subpopulations of retinal ganglion cells in recent years. It is not known what role these compounds play in neurotransmission in the mammalian visual system.

In preliminary studies, it was shown that the neuropeptide N-acetylaspartylglutamate (NAAG) is localized in retinal ganglion cell bodies and a number of target zones including lateral geniculate and superior colliculus\textsuperscript{1}. Further, Tsai et al. have used microdialysis to show calcium-dependent release of exogenous [\textsuperscript{3}H]NAAG from rat superior colliculus in response to depolarization\textsuperscript{39} as well as the release of endogenous NAAG from the colliculus, as measured by RIA, in response to optic nerve stimulation\textsuperscript{40}. These data suggest that NAAG may contribute to synaptic neurotransmission in the optic projection system of mammals. In the present study, we have extended our observations on the immunolocalization of NAAG to the entire visual system of the rat, using a recently improved method for fixing N-blocked peptides, such as NAAG, in brain tissue\textsuperscript{20}. This modified fixation has permitted the identification of NAAG containing optic nerve terminals throughout the primary and accessory optic systems of the rat.

**MATERIALS AND METHODS**

**Immunohistochemistry**

The procedures employed in NAAG immunohistochemistry were identical to the methods described previously\textsuperscript{20,27}. Five sets of 3 male albino Sprague–Dawley rats (approximately 200 g) were

**TABLE 1**

RIAl analysis of NAAG in the rat optic projection system

Analysis of NAAG by RIA was done as described in the Methods section. The values represent the mean ± the standard deviation from 6 animals.

<table>
<thead>
<tr>
<th>Area</th>
<th>Control</th>
<th>Transected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suprachiasmatic nucleus</td>
<td>14.8 ± 0.5</td>
<td>6.7 ± 0.5*</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.3**</td>
</tr>
<tr>
<td>Lateral geniculate</td>
<td>10.3 ± 1.2</td>
<td>5.5 ± 0.5*</td>
</tr>
<tr>
<td>Nucleus of the optic tract</td>
<td>10.9 ± 0.9</td>
<td>8.2 ± 1.6**</td>
</tr>
<tr>
<td>Pretectal nucleus</td>
<td>13.9 ± 0.3</td>
<td>8.0 ± 0.4</td>
</tr>
<tr>
<td>Pretectal area</td>
<td>13.2 ± 0.6</td>
<td>11.1 ± 1.6***</td>
</tr>
<tr>
<td>Superior colliculi</td>
<td>8.6 ± 0.4</td>
<td>4.4 ± 0.6*</td>
</tr>
</tbody>
</table>

* Significantly different from control, \( * P < 0.001, ** P < 0.01, *** P < 0.05 \).

Fig. 1. NAAG-IR in the axons of the optic chiasm (OC) in the rat. Axons of retinal origin, cut in cross section, are visible as densely stained puncta in this highly magnified photomicrograph of the chiasm (A). Ten days following bilateral optic nerve transection a dramatic loss of immunoreactive fibers was observed throughout the optic chiasm (B) [bar = 15 \( \mu \)m].
prepared for immunohistochemistry utilizing affinity-purified antibodies to NAAG. Immunohistochemistry was performed 10 days postoperative in sets of 3 animals; one sham-operated, one with unilateral optic nerve transection, and one with bilateral transections (surgery performed by Zivic Miller, Allison Park, PA). The tissue sections were processed in specially designed polypropylene incubation trays which permitted solutions to flow between chambers through Teflon screening, while retaining the sections from each animal in separate compartments. Tissue sections, which were to be compared directly, were incubated simultaneously with each immunohistochemical reagent in these trays to insure uniform treatment during each stage of processing.

Antibody specificity controls were performed with the affinity-purified antibody preparations to test for possible antibody binding to related molecules including aspartylglutamate (AG), N-acetylaspartate (NAA), and glutamate. The affinity-purified antibody preparations (dilutions from 1:150 to 1:300) were preincubated with the amino acids or peptides coupled to bovine serum albumin via a carbodiimide-mediated reaction. Working dilutions of the primary antibody were incubated overnight at 4°C with the NAA, AG and glutamate conjugates, at a concentration of 5 µg/ml, before applying to tissue sections. In the case of the NAAG conjugate, a 1 µg/ml dilution was preincubated with the primary antibody solutions. Under these conditions, no detectable decreases were noted with the NAA or glutamate conjugates, a slight reduction was observed with the AG conjugate, while the NAAG conjugate inhibited antibody binding to tissue sections completely.

Fig. 2. NAAG-IR in the lateral geniculate nucleus. Immunoreactivity was reduced dramatically in the lateral geniculate nucleus (LGN) 10 days following optic nerve transections. Shown here are the LGN from a control animal (A) and from an animal with bilateral optic nerve transections 10 days following surgery (B) [bar = 200 µm]. The reduction in NAAG-IR can be seen throughout the LGN, and in the fibers of the optic tract (OT) running along the lateral edge of the nucleus (arrows A and B). The loss of NAAG immunoreactivity in the OT and neuropil of the ventral magnocellular portion of the nucleus is shown at higher magnification in (C) and (D) [magnified areas designated by boxes in A and B] [bar = 50 µm].
Radioimmunoassay

Concentrations of NAAG were measured from micropunches of brain tissue using a radioimmunoassay (RIA). The microdissections were performed as previously described. Fresh frozen brains were sectioned at 300 μm in the coronal plane in a cryostat at -10 °C. The brain regions of interest were dissected with 300-1000 μm diameter needles, and the micropunches were kept frozen until processed. The micropunch samples (~ 50 μg tissue weight) were sonicated in 250 μl of sodium phosphate buffer (1 mM, pH 7.2) and NAAG was estimated in 25 μl aliquots. The RIA utilized the same affinity-purified antibodies as were employed in immunohistochemistry. Protein A PANSORBIN cells (Calbiochem, San Diego, CA) were washed and incubated with affinity-purified NAAG antibody for 18 h at 4 °C with gentle agitation. The mixture was sedimented at 7000 g for 5 min and the pellets were resuspended in 50 μl of tissue extract and 100 μl of [3H]NAAG (approx. 50,000 cpm/100 μl, S.A. = 50.7 Ci/mmol, New England Nuclear, Boston, MA). The mixture then was incubated for 24 h at 4 °C on an orbital shaker, microcentrifuged at 7000 g for 5 min, and the pellets were rinsed twice before determining the radioactivity by liquid scintillation spectroscopy. All samples were assayed in duplicate. Protein concentrations were determined using the biuretichinic acid method (BCA; Pierce Chemical, Rockford, IL) with BSA as standard. Data analyses were performed using the IBM PC Data Reduction System RIA computer program (M.L. Jaffe & Assoc., Silver Spring, MD). Validation of the results was accomplished via HPLC analysis of NAAG. Specificity of the RIA was determined by comparing competitive displacement curves for NAAG and several related molecules including N-acetylaspartate, glutamate and aspartate.

RESULTS

Marked decreases in NAAG-IR were detected in optic nerve axons and all retinorecipient zones following optic nerve transections. Quantification of the loss of NAAG
Fig. 4. NAAG-IR in the superior colliculus. NAAG immunoreactivity in the superior colliculus of a rat, 10 days following unilateral optic nerve transection (A) [bar = 500 μm]. Dense neuropil labeling can be seen in the superficial layers of the superior colliculus. The midline of the superior colliculus indicated by the box in (A) is shown at higher magnification in (B) [bar = 50 μm], with the densely staining neuropil visible ipsilateral to the cut nerve (right), but absent contralateral to the cut nerve (left).

by RIA confirmed the results of the immunohistochemical analyses (see Table I for RIA results).

Optic chiasm and tract

Axons of retinal ganglion cells comprising the optic nerves, chiasm and tracts were highly immunoreactive for NAAG (Fig. 1A). Ten days following bilateral nerve transections, immunoreactivity was lost in almost all of the axons of the optic chiasm (Fig. 1B), but a very small number of NAAG-positive fibers, presumably of central origin, were observed throughout the chiasm. The loss of NAAG immunoreactive fibers was also evident in the main optic tracts at the border of the lateral geniculate (Fig. 2) and the basal optic tracts at the edge of the cerebral peduncles (Figs. 3A and 5).

Lateral geniculate nucleus

Extensive immunoreactivity for NAAG was observed in both the neuropil and cell bodies in all subdivisions of the lateral geniculate nucleus. NAAG immunoreactivity was reduced dramatically in the lateral geniculate nucleus 10 days following optic nerve transections. The reduction in NAAG-IR was observed in the optic nerves, chiasm, and the fibers of the optic tract running along the lateral edge of the nucleus. Dense NAAG-IR was observed in the neuropil within both the dorsal and ventral divisions of the nucleus, and in the intergeniculate leaflet (Fig. 2A, C). The loss of NAAG-IR was observed mainly in the neuropil of all nuclear subdivisions (Fig. 2B, D), but a loss of immunoreactivity was also observed in cell bodies. Analysis of NAAG levels in micropunches of lateral geniculate nucleus by RIA indicated approximately a 50% loss 10 days following bilateral optic nerve transections.

Nucleus of the optic tract

The nucleus of the optic tract displayed a high degree of NAAG-IR in the neuropil and cell bodies (Fig. 3A, D). Ten days after optic nerve transection, the loss of NAAG-IR in the neuropil was almost complete (Fig. 3C). In animals with a unilateral transection, the loss was observed only in the contralateral nucleus of the optic tract. RIA analysis of micropunch samples indicated a loss of approximately 25% following transections.

Superior colliculus

NAAG immunoreactivity was reduced dramatically in the retinal recipient zone of the superior colliculus 10 days following unilateral optic nerve transection. Dense neuropil labeling was observed in the superficial layers of the superior colliculus in control animals and ipsilateral to a transection in animals with a unilateral optic nerve cut (Fig. 4). The densely staining neuropil visible ipsilateral to the cut nerve was virtually absent contralateral to the cut nerve (Fig. 4B). RIA analysis of micropunches of the superior colliculus 10 days after bilateral optic nerve transections indicated a 50% loss of NAAG as compared with sham-operated controls.

Medial terminal nucleus

The medial terminal nucleus of the accessory optic tract displayed the densest neuropil NAAG-IR of all the visual target zones. This neuropil immunolabeling obscured all details within the ventral portion of the nucleus, but was more diffuse in the dorsal subdivision. Cells within the medial terminal nucleus were also NAAG positive, as were neighboring cells in the sub-
Fig. 5. NAAG immunoreactivity in the medial terminal nucleus. Dense immunoreactivity for NAAG was observed in the fibers of the accessory optic tract (AOT) and in the neuropil of the medial terminal nucleus (MTN) of control animals as shown in (A). A substantial decrease in NAAG-IR is seen in the MTN and the AOT 10 days following bilateral optic nerve transections as shown in (B) [bar = 50 \mu m].

stantia nigra and paranigral nucleus. Ten days following unilateral optic nerve transection, all NAAG-IR in the contralateral accessory optic tract and medial terminal nucleus was lost (Fig. 5). The loss of immunoreactivity in the medial terminal nucleus occurred in fibers and puncta within the neuropil, as well as in nerve cell bodies. The loss of NAAG-IR was pronounced on both sides in animals with bilateral transections.

Other areas

Other NAAG losses of approximately 50% were measured by RIA in micropunch samples from the suprachiasmatic nucleus of the hypothalamus and the pretectal nucleus. A smaller change was noted in the paraventricular nucleus of the hypothalamus (20%).

Loss of NAAG-IR in visual cortex

Optic nerve transection resulted in a loss of NAAG immunoreactivity in visual cortex (Fig. 6). NAAG was generally absent from large pyramidal neurons in cortex, but was observed in some small and medium sized neurons in layers II through VI. NAAG-IR was also observed in an apparent synaptic zone in layer IV. The major loss of NAAG-IR was in the synaptic zone in layer IV of area 17, although a loss of cellular staining throughout area 17 was also observed. The staining in adjacent areas of cortex was unaffected. These results indicate that the thalamocortical fibers originating in the lateral geniculate nucleus also contain NAAG, and that the levels of NAAG in lateral geniculate neurons, and their terminals, decrease after optic nerve transection, as do the levels in neurons of primary visual cortex.

DISCUSSION

The results presented here clearly demonstrate that NAAG is localized in the terminal fields of both the primary and accessory optic systems of the rat. Optic nerve transection resulted in substantial decreases in the NAAG-IR in all major optic nerve terminal fields. The decrease in NAAG-IR appeared to occur primarily in the neuropil of these target zones. Analysis of NAAG in micropunch samples of several visual system areas by RIA confirmed that the predominant loss of NAAG occurred in retinorecipient zones.

NAAG is known to be present in the nervous system in high concentrations and RIA data have indicated higher concentrations in the optic chiasm and tract than in any other brain region examined. Several lines of evidence indicate that NAAG may act as a transmitter or modulator in certain sensory and motor pathways. First, it has been localized by immunohistochemistry in well-defined systems in the CNS, including the motor horn of the spinal cord, primary spinal afferents, the optic
NAAG is hydrolyzed rapidly in broken-cell and intact cell preparations to N-acetylaspartate and glutamate via dipeptidase activity which is thought to act extracellularly\(^7\)\(^{27}\)\(^{31}\). Although NAAG is known to be released from neurons, its synaptic fate and mode of action are still in question. As such, at least two possible postsynaptic roles can be envisioned for NAAG. It is possible that the peptide may act on one type of presynaptic or postsynaptic receptor in some cases, while in others the released NAAG may be cleaved in the synaptic cleft to form glutamate as the neuroactive agent. A precedent for this dual synaptic fate and postsynaptic action can be found in the case of purinergic neurotransmission wherein ATP released from presynaptic endings can act directly on one type of purine receptor, or can be cleaved to yield adenosine which acts on another type of purine receptor\(^{36}\). Evidence in spinal cord cultures argues for the former case\(^{42}\), wherein high concentrations of NAAG act at the NMDA type of glutamate receptor, eliciting a response identical to NMDA. However, the high levels of dipeptidase activity in brain suggest that NAAG may be a potent source of glutamate in neurotransmission. It is interesting to note that the relay cells of the lateral geniculate which receive retinal and cortical input have been proposed to respond via both the NMDA and non-NMDA classes of glutamate receptor\(^{33}\)\(^{34}\).

The distribution of NAAG-IR in the visual system of the rat is extensive. In the retina, NAAG was found to be absent in retinal photoreceptors, but present in moderate amounts in cells of the inner portion of the inner nuclear layer and high amounts in most of the retinal ganglion cells and their axons in the optic pathways\(^{21}\). In the present study, NAAG was found both in presumed synaptic terminals of the ganglion cells, and in their postsynaptic target cells of the lateral geniculate, the superior colliculus, the nucleus of the optic tract, the suprachiasmatic nucleus and the nuclei of the accessory optic tract. NAAG was absent from the majority of large
pyramidal cells in layers III, IV and V of occipital cortex, but a thick band of punctate NAAG-IR was evident in the neuropil of layer IV in this geniculocortical recipient zone. A subpopulation of small and medium sized neurons scattered throughout cortical layers II through VI were highly immunoreactive for NAAG.

These observations indicate that NAAG is found in several links in the visual pathways to cortex and brainstem, and it is therefore conceivable that NAAG performs some type of modulatory function in synaptic neurotransmission throughout the visual system. This possibility is consistent with findings that NAAG is colocalized with several classical neurotransmitters in the spinal cord and brain stem. In the extrapyramidal system, we have observed extensive NAAG immunoreactivity in cell groups which have been reported to be rich in GABA and dopamine, including the cells of origin for the nigrostriatal dopaminergic pathway.

More speculatively, the presence of NAAG in multiple links in a projection system might be related to the recent observations that hippocampal NMDA receptors are tonically active, a condition presumed to be due to the chronic presence of extracellular glutamate. This heightened level of channel activity was shown to strengthen the coupling between excitatory input and action potential output in hippocampal pyramidal neurons. It is possible that NAAG provides a source of this tonic activation of endogenous NMDA receptors, either by direct NAAG binding to the NMDA receptor, or via the production of extracellular glutamate through the enzymatic action of peptidases. In either case, the function of NAAG would be to increase the 'gain' of retinogeniculate and geniculocortical output in the form of enhanced coupling between synaptic input and action potential output. It should be mentioned that if NAAG acts primarily by providing substrate for a synaptic peptidase, resulting in the production of free glutamate in the cleft, then the enzyme activity level provides an additional control point in synaptic transmission at this type of glutamatergic synapse. In this regard, it will be important to control for the activity of synaptic peptidases in future electrophysiological experiments involving NAAG application.

Finally, in the present study it was observed that NAAG-IR decreased in occipital cortex area 17 following optic nerve transection, two synaptic links removed from retinal ganglion cell terminals. This suggests that postsynaptic NAAG concentrations may be changing dynamically based upon synaptic input and/or neuronal activity levels. While the potential transynaptic regulation of NAAG levels in functionally defined neural pathways suggests a dynamic communication function for this peptide, it is also possible that this change was due to a general transynaptic drop in neuronal activity in the visual system. This type of reduction in neuronal activity in the visual projections has been shown in visual cortex by [14C]-2-deoxyglucose autoradiography following orbital enucleation and after kainate lesions of the lateral geniculate, as measured by decreases in neuronal cytochrome oxidase staining. Nonetheless, whether this effect is due to a general drop in neuronal metabolism, or due to specific changes involving altered synthesis or degradation of the peptide, the transsynaptic regulation of NAAG levels in functionally defined neural pathways warrants further study.

In conclusion, the substantial loss of NAAG in the primary and accessory optic termination zones following optic nerve transection indicates that this peptide is localized in the optic nerve terminals in these areas. Excitatory amino acid neurotransmission is apparently involved in these ganglion cell projections to the brain. Immunohistochemistry suggests that NAAG, rather than glutamate, is localized extensively in retinal ganglion cell somas and in their terminals in the brain of mammals. NAAG is found in most or all retinal ganglion cells in the rat, cat and human. The highly specific localization of NAAG in the visual system suggests that it is likely to play a significant role in chemical neurotransmission in the mammalian optic nerve projections, and may be a major transmitter in this system.

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