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N-Acetylaspartylglutamate: A transmitter candidate for the retinohypothalamic tract

(suprachiasmatic nucleus/supraoptic nucleus/peptide immunohistochemistry/circadian rhythms)

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ABSTRACT **The retinohypothalamic tract is the neural pathway mediating the photic entrainment of circadian rhythms in mammals. Important targets for these retinal fibers are the suprachiasmatic nuclei (SCN) of the hypothalamus, which are thought to be primary sites for the biological clock. The neurotransmitters that operate in this projection system have not yet been determined. Immunohistochemistry and radioimmunoassay performed with affinity-purified antibodies to *N*-acetylaspartylglutamate (NAAG) demonstrate that this neuron-specific dipeptide, which may act as an excitatory neurotransmitter, is localized extensively in the retinohypothalamic tract and its target zones, including the SCN. Optic nerve transections resulted in significant reductions in NAAG immunoreactivity in the optic chiasm and SCN. Analysis of NAAG concentrations in micropunches of SCN, by means of radioimmunoassay, showed approximately 50% reductions in NAAG levels. These results suggest that this peptide may act as one of the neurotransmitters involved in retinohypothalamic communication and circadian rhythm entrainment.**

The mammalian retinohypothalamic tract (RHT) is the neural projection involved in entraining circadian rhythms to the light-dark cycles of the environment (1, 2). This pathway acts to convey information about external light conditions from the retina to several areas of the hypothalamus, including the suprachiasmatic nucleus (SCN), a primary site of the biological clock or central timing system in the brain. The RHT in the rat originates in a selected group of ganglion cells in the retina and terminates primarily in the ventrolateral aspect of the SCN (3). The RHT alone appears sufficient for the entrainment of circadian rhythms, since rodents retain the ability to synchronize their endogenous rhythms with laboratory light–dark cycles when all other retinofugal projections are destroyed (4, 5). Even though it is known that the RHT projects to other areas of the hypothalamus (6–8) and that the SCN receives inputs from other visual areas of the brain (9), the retinal projection to the SCN via the RHT seems to be the primary pathway for photoperiod entrainment in mammals (10). This neural pathway is also directly involved in the suppression of melatonin production in the pineal gland in response to light (11). With regard to chemical neurotransmission in the RHT, data have been presented which indicate that excitatory amino acid receptors may be involved in the function of the RHT at the level of the SCN (12, 13). However, the identity of the excitatory transmitter released at the SCN by RHT nerve endings remains unclear.

Previously, we have found that retinal ganglion cells and their primary projection targets in the brain contain an acidic dipeptide, *N*-acetylaspartylglutamate (NAAG) (14). This acetylated dipeptide is found exclusively in the nervous system, and it has been implicated in excitatory neurotransmission in a number of sensory and motor systems in the brain (15). Therefore, it was of interest to determine if NAAG could be identified in the terminal fields of the RHT within the hypothalamus. Here we present immunohistochemical and radioimmunoassay data indicating extensive NAAG immunoreactivity (NAAG-IR) in the SCN and other target zones of the RHT in the rat. Further, the NAAG-IR in the optic chiasm and SCN decreased substantially following unilateral or bilateral optic nerve transections. This observation raises the possibility that NAAG may act as a transmitter mediating the effects of light in the retinohypothalamic system.

MATERIALS AND METHODS

Immunohistochemistry. Five sets of three male albino Sprague–Dawley rats (≈200 g) were prepared for immunohistochemistry as previously described, utilizing affinity-purified antibodies to NAAG (16). The procedure was modified slightly to prevent any potential differences in staining conditions between control and experimental animals. Immunohistochemistry was performed 10 days postoperative in sets of three animals; one sham operated, one with unilateral optic nerve transection, and one with bilateral transections (surgery performed by Zivic–Miller). The tissue sections were processed in specially designed polypropylene incubation trays which permitted solutions to flow between chambers through fine-mesh screening, while retaining the sections from each animal in separate compartments. Tissue sections that were to be compared were prepared simultaneously in these trays to ensure uniform treatment at all stages of processing.

Antibody specificity controls were performed with the affinity-purified antisera to test for possible antibody binding to related molecules including aspartylglutamate, *N*-acetylaspartate, and glutamate. The affinity-purified antisera (dilutions from 1:150 to 1:300) were preincubated with the amino acids or peptides coupled to bovine serum albumin via a carbodiimide reaction. The *N*-acetylaspartate, aspartylglutamate, and glutamate conjugates were incubated with working dilutions of primary antibody at 5 μg/ml overnight before application to tissue sections. In the case of the NAAG conjugate, a 1 μg/ml dilution was used.

Radioimmunoassay (RIA). Concentrations of NAAG were measured from micropunches of SCN tissue by using an RIA. The microdissections of the SCN were performed in fresh frozen brains by the micropunch technique (17). The brains were sectioned in the coronal plane at a thickness of 300 μm in a cryostat set at −10°C. While still frozen, the nuclei were dissected under a dissection microscope with 300- to 1000-

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Abbreviations: NAAG, *N*-acetylaspartylglutamate; NAAG-IR, NAAG immunoreactivity; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus.

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μm-diameter needles, and the micropunches were kept frozen until processed. The micropunch samples (≈50 μg) were sonicated in 250 μl of sodium phosphate buffer (1 mM, pH 7.2) and NAAG was estimated in 25-μl aliquots. The RIA was done using the same affinity-purified antibody employed in immunohistochemistry. Protein A-bearing *Staphylococcus aureus* cells (Pansorbin cells; Calbiochem) were washed and incubated with affinity-purified NAAG antisera for 18 hr 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 [^{3}H]NAAG (≈50,000 cpm/100 μl, 50.7 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq). The mixture was incubated for 24 hr at 4°C on an orbital shaker and microcentrifuged at 7000 × *g* for 5 min, and the pellets were rinsed twice before scintillation counting. All assays were done in duplicate and included a NAAG standard curve. Protein concentrations were determined by using the bicinchoninic acid method (BCA; Pierce) with bovine serum albumin as standard. Validation of the results was accomplished by 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 (L.W., D. A. Eagles, M. J. Brady, J.R.M., M.A.A.N., and J. H. Neale, unpublished data).

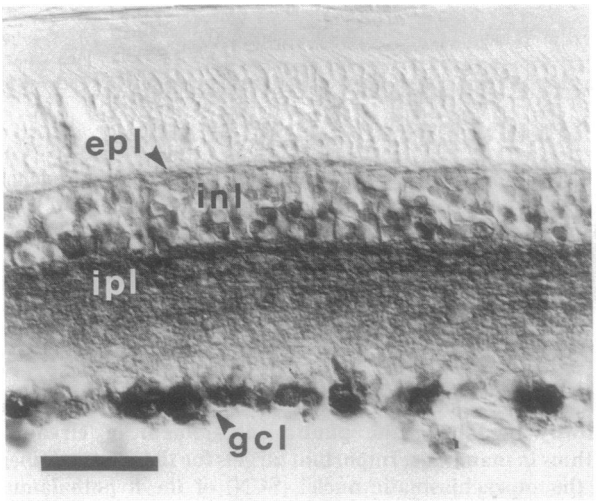

FIG. 1. NAAG-IR in the retina of the rat. Affinity-purified polyclonal sera directed against protein-coupled NAAG were applied to tissue sections that had been fixed to retain N-blocked peptides. NAAG-IR appears most intense in the ganglion cell layer (gcl), while being present in more moderate amounts in the inner plexiform layer (ipl), external plexiform layer (epl), and the inner nuclear layer (inl). Note the absence of staining in the photoreceptor cell bodies and outer segments. (Differential interference contrast; bar = 50 μm.)

RESULTS

Antibody-blocking studies indicated that the affinity-purified antisera were highly specific for NAAG compared with *N*-acetylaspartate, glutamate, and aspartylglutamate. When the affinity-purified primary antisera were preincubated with

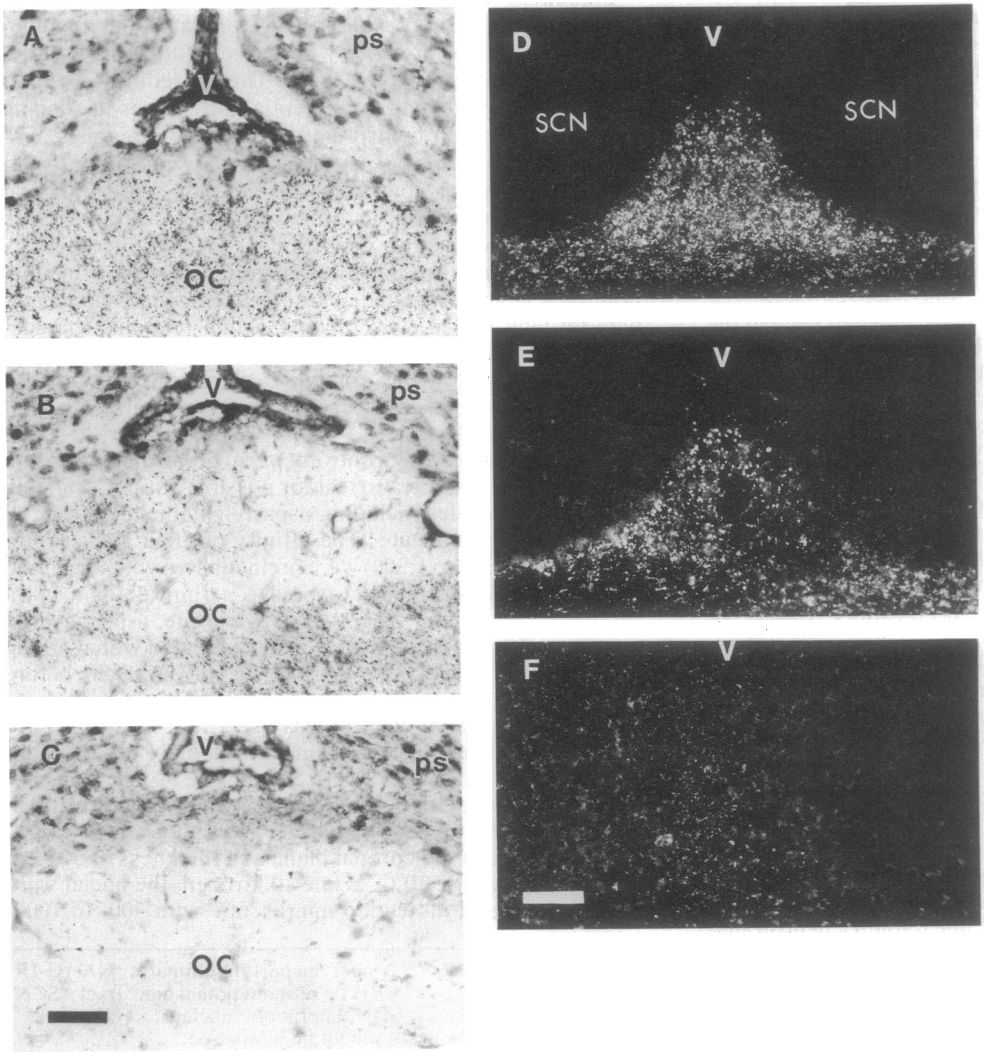

FIG. 2. NAAG-IR in the axons of the mid portion of the optic chiasm (OC) in the rat (coronal sections). The rostral portion of the chiasm at the level of bregma is shown in the bright-field photomicrographs (*A*, *B*, and *C*). The optic chiasm at the level of the SCN (approximately 900 μm caudal to bregma) is shown in the dark-field photomicrographs (*D*, *E*, and *F*). Axons of retinal origin, cut in cross section, are visible as densely stained puncta in *A–C* and as bright spots in the dorsomedial extension of the chiasm between the SCN in *D–F*. Ten days after unilateral optic nerve transection, the loss of NAAG-IR fibers in the dorsomedial extension of the chiasm occurs ipsilateral to the cut nerve, while in the main body of the chiasm the loss occurs on the contralateral side at the levels shown in *B* and *E*. The almost complete loss of immunoreactive fibers after bilateral transection is shown in *C* and *F*. V, third ventricle; ps, preoptic suprachiasmatic nucleus. (Bar = 50 μm.)

the control conjugates at 5 μg/ml, no detectable decreases were noted with NAA or glutamate coupled to bovine serum albumin. A slight reduction in staining intensity was observed with the aspartylglutamate-albumin conjugate at 5 μg/ml, while the NAAG conjugate inhibited antibody binding to tissue sections completely at a concentration of 1 μg/ml.

Retina. When the affinity-purified antisera were used, most of the ganglion cells in the retina of the rat displayed high-intensity NAAG staining (Fig. 1). All ganglion cell classes were immunoreactive for NAAG. A small number of cells in the ganglion cell layer showed little or no NAAG-IR. Light to moderate NAAG staining was present in a subpopulation of cells observed along the inner margin of the inner nuclear layer of the retina. Additionally, the inner plexiform layer displayed moderate to heavy staining for NAAG which appeared punctate at higher magnification. In some areas of the retina, the inner plexiform labeling exhibited a layered or striated appearance, containing between two and four longitudinal bands of increased NAAG staining within the neuropil. Light irregular staining was also observed in the outer plexiform layer of the retina. No immunoreactivity was observed in the cell bodies or outer segments of the photoreceptor cells.

Optic Chiasm and RHT Fibers. The axons of retinal ganglion cells making up the optic nerves, chiasm, and tracts were highly immunoreactive for NAAG (Fig. 2*A*). In the rat, the main optic projections are predominantly crossed, but the RHT projection to the SCN is only partially crossed (18). Ten days after optic nerve transection, NAAG-IR is completely eliminated in the fibers originating in retina. After unilateral optic nerve transection, the great majority of fibers retaining NAAG-IR cross in the rostral portion of the optic chiasm, but a segment of dorsomedially placed NAAG-IR axons appeared to diverge from the main crossed component. Thus, in coronal sections, from the preoptic area to the mid-SCN level, the distribution of NAAG-IR fibers in the dorsomedial chiasm was reversed with respect to the main portion of the chiasm (Fig. 2 *B* and *E*). In sections stained for NAAG, axons in this dorsomedial segment of the chiasm appeared to cross farther caudally than the main optic projections. These latter crossing NAAG-positive fibers may constitute the middle division of the RHT in the rat, and they were observed both in the dorsomedial extension of the chiasm between the SCN and coursing immediately ventral to the SCN in the chiasm. Some of these fibers could be traced as far caudal as the medial retrochiasmatic area. In the animals with bilateral nerve transections, immunoreactivity was lost in most of the axons of the optic chiasm (Fig. 2 *C* and *F*), but a very small number of NAAG-positive fibers, presumably of central origin, were observed throughout the chiasm.

Suprachiasmatic Nucleus. Many NAAG-positive fibers in the dorsomedial segment of the optic chiasm could be seen penetrating the ventral SCN (Fig. 3*A*). Numerous heavily stained puncta were visible on cells and in neuropil throughout this region in the control animals (Fig. 3*B*). The density of NAAG-IR puncta in the ventral SCN was substantially lower in the anterior SCN than in the middle and posterior portions of the nucleus.

Significant reductions in NAAG staining were noted bilaterally within the SCN 10 days after bilateral optic nerve transections, particularly in the ventral region of the nuclei (Fig. 4). The NAAG-IR puncta observed in the neuropil and on perikarya in the ventral SCN were greatly reduced in number after bilateral transections (Fig. 3*C*). These observations suggest that the loss of NAAG-IR within the SCN occurred in the neuropil constituting the terminal fields of the RHT. It can be seen in Fig. 4*B* that NAAG-IR was not eliminated in the SCN after bilateral transections, where some staining is still evident in perikarya and the surrounding neuropil. The incomplete reduction of NAAG-IR in the neuropil of this nucleus suggests that other NAAG-containing axon terminals, not of retinal origin, may also synapse in the SCN.

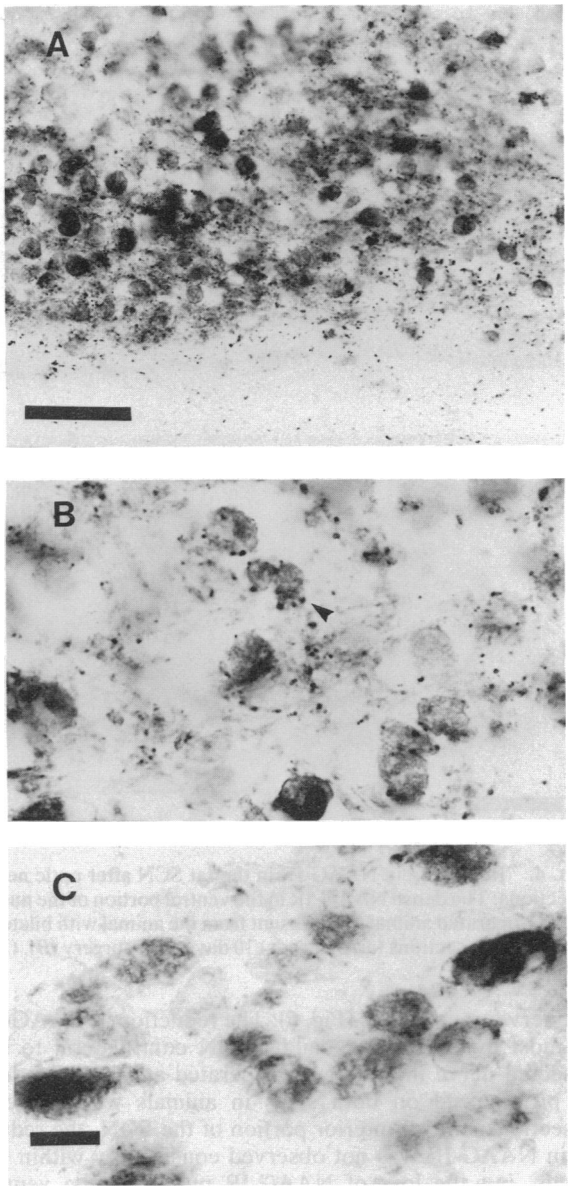

FIG. 3. NAAG-IR fibers and puncta in the ventral SCN. Axons in the optic chiasm stained for NAAG can be seen entering the ventromedial aspect of the SCN (*A*). Highly immunoreactive puncta are visible on the cell bodies (arrowhead) and in the neuropil of the ventral SCN (*B*). Ten days after bilateral optic nerve transection the punctate immunoreactivity in this region is greatly reduced (*C*). (Bar = 40 μm in *A* and 15 μm in *C*; *B* and *C* are the same magnification.)

RIA. The RIA performed with affinity-purified NAAG antibodies was found to be highly specific for NAAG in soluble extracts of brain tissue. The IC_{50} values for NAAG and *N*-acetylaspartate were 2.5 nM and 100 μM, respectively, while the values for aspartate and glutamate were in excess of 1 mM each. Analysis of NAAG levels in the SCN by RIA indicated a decrease of about 55% over controls in animals with bilateral transections (14.8 ± 0.27 and 6.6 ± 0.31 nmol/mg of protein, respectively; mean ± SD, $n = 6$).

Supraoptic Nucleus (SON). The neurons of the SON were highly immunoreactive for NAAG (Fig. 5*A*). The ventral dendritic zone of the SON, and the ventral glial lamina in which the SON dendrites are invested (19), were also very immunoreactive. Significant reductions in NAAG-IR were noted in the SON, and their ventral dendritic zones, after

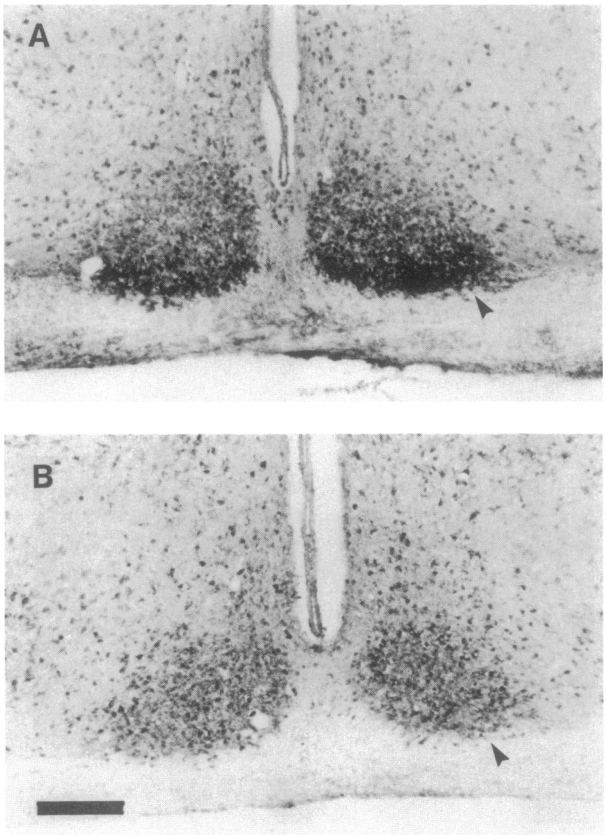

FIG. 4. Reduction in NAAG-IR in the rat SCN after optic nerve transections. The dense NAAG-IR in the ventral portion of the nuclei of a sham-operated animal (*A*) is absent from the animal with bilateral optic nerve transections (arrowheads) 10 days after surgery (*B*). (Bar = 200 μm.)

optic nerve transections (Fig. 5). The reduction in NAAG-IR was most evident in the caudal SON contralateral to the transected nerve in unilaterally operated animals. The loss was pronounced on both sides in animals with bilateral transections. In the anterior portion of the SON, the reduction in NAAG-IR was not observed consistently within the nucleus, but the loss of NAAG-IR puncta in the ventral dendritic zone was obvious throughout the rostrocaudal extent of the nucleus. It was not possible to determine whether the NAAG-IR loss in the dendritic zone beneath the SON resulted from the loss of immunoreactive optic fibers diverging from the chiasm into this region or from a loss of immunoreactivity in the supraoptic dendrites.

Other Hypothalamic Regions. The highest levels of NAAG immunoreactivity observed in the entire rat brain were in the optic chiasm and portions of the lateral hypothalamus. Medium and large multipolar neurons throughout much of the lateral hypothalamus were intensely immunoreactive within their cell bodies and larger dendrites, and dense punctate NAAG-IR was associated with their neuronal processes. NAAG immunoreactivity was also observed in the hypothalamic paraventricular nuclei, the periventricular nucleus surrounding the anterior third ventricle, the preoptic area, and the anterior hypothalamic nucleus. Little or no reduction in NAAG-IR was observed in these areas after optic nerve transections.

Sparse NAAG-positive fibers could be found in the medial retrochiasmatic area of control animals, and immunoreactive puncta were observed on perikarya and in the neuropil of this region. The immunoreactive fibers and puncta in the central portion of the retrochiasmatic area were reduced in number after optic nerve transections.

DISCUSSION

The results presented above indicate that NAAG is localized in the cells of origin, axons, and terminal fields of the RHT in the rat, and that optic nerve transection results in significant decreases in NAAG-IR in the optic chiasm and SCN. The termination zones of the RHT in the rat have recently been demonstrated by anterograde transport of labeled cholera toxin and include the SCN, the lateral hypothalamus, the anterior hypothalamic area, and the retrochiasmatic area (18). We did observe significant reductions in NAAG-IR in the SCN and retrochiasmatic area but not in the lateral hypothalamus or anterior hypothalamus. This may be due to several factors. First, the projections to these latter two areas via the RHT are relatively sparse when compared with those to the SCN (18). Additionally, the extensive perikaryal and neuropil NAAG-IR in the lateral hypothalamus and anterior hypothalamic area may have obscured any loss of immunoreactivity due to optic nerve transection.

Recent findings indicate that NAAG may be an excitatory transmitter in some systems, and that it may act through one or more of the known excitatory amino acid receptors (20–26). Further, other studies have indicated that excitatory amino acid receptors may be involved in the RHT afferents to the SCN (12, 13, 27). On the basis of this information and the evidence presented, it is reasonable to propose that NAAG may be the excitatory transmitter of the RHT. No compelling evidence has been afforded to date which suggests that any other potential excitatory neurotransmitter is present throughout the optic projections to the hypothalamus in mammals. Whether NAAG is acting as the primary neurotransmitter in the RHT or is acting in association with other transmitters is presently not clear.

It is now known that NAAG is degraded rapidly to *N*-acetylaspartate and glutamate by the action of a specific dipeptidase that is believed to act extracellularly to inactivate

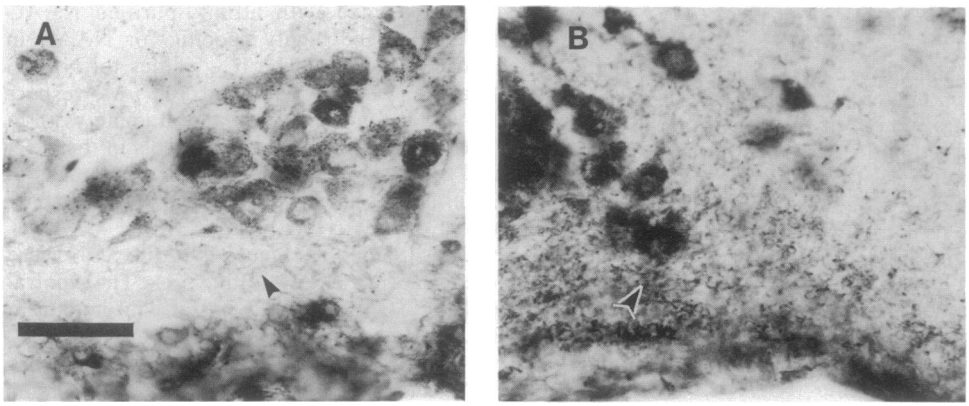

FIG. 5. Loss of NAAG-IR in the SON and its ventral dendritic zone after optic nerve transection. In an animal with a unilateral transection, the immunostaining ipsilateral to the cut nerve is fairly dense in the neuropil and perikarya (*B*). The NAAG-IR is noticeably reduced in the ventral neuropil (arrowheads) and perikarya 10 days after a bilateral transection (*A*). (Bar = 40 μm.)

NAAG (28, 29). Although NAAG is known to be released from neurons, its synaptic fate and mode of action are still in question. If NAAG acts as a neurotransmitter, two possible postsynaptic roles can be envisioned. It is possible that in some cases NAAG may act on specific postsynaptic receptors on target neurons, while in other cases the released NAAG may be cleaved extracellularly 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 in the peripheral nervous system. In this transmission scheme, ATP released from presynaptic endings can act directly on one type of purine receptor, or it can be cleaved to yield adenosine, which acts on another type of purine receptor (30).

Another possible function for NAAG in the retinohypothalamic projection system could be related to the unique behavior of neuronal *N*-methyl-D-aspartate (NMDA) receptors recently reported in the hippocampus (31). It has been demonstrated that this type of glutamate receptor may be tonically active in hippocampus due to the extracellular levels of glutamate and that the sensitivity of pyramidal neurons to depolarizing inputs may be altered by changing the glutamate concentrations in the extracellular space. Potential sources of extracellular glutamate in the brain are not certain, but since NAAG is present in very high concentrations in the central nervous system and is distributed widely throughout many neuronal systems, it represents a plausible candidate for tonic NMDA receptor activation. NAAG has been shown to act directly on NMDA receptors in spinal cord cultures, although requiring relatively high concentrations compared with NMDA and glutamate (20). Therefore, there are two possible mechanisms whereby NAAG could provide a source of tonic activation of NMDA receptors. NAAG might act by direct binding to the NMDA receptor or by the production of extracellular glutamate through enzymatic action. In either case, in this scenario, the function of NAAG in the RHT would be to increase the sensitivity of SCN neurons, receiving direct retinal input, to the action of other neurotransmitters.

An unexpected observation in the present study was that optic nerve transection resulted in an apparent decrease in perikaryal NAAG-IR in the SON 10 days after the operation (Fig. 5). A very sparse RHT projection to the SON has been reported (18), but the significance of such a limited projection is unknown. Since the direct retinofugal input to the SON is sparse, the loss of NAAG-IR observed in these nuclei cannot be explained solely by a loss of input fibers and their associated synaptic endings. Instead, it may be the case that this loss of cellular NAAG-IR results from some transsynaptic process which directly or indirectly changes the levels of NAAG in postsynaptic SON neurons.

In conclusion, the dramatic loss of NAAG-IR in the optic chiasm, the RHT fibers in the dorsomedial chiasm, and the ventral neuropil of the SCN after optic nerve transection demonstrates that this peptide is localized in the axon terminals of the RHT. NAAG is the only neuroactive compound to date which has been demonstrated to exist in high concentrations in the RHT, and as such, represents an outstanding candidate for the transmitter involved in circadian rhythm entrainment.

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