
Localization and Synaptic Release of *N*-Acetylaspartylglutamate in the Chick Retina and Optic Tectum

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Abstract

The neuropeptide, *N*-acetylaspartylglutamate (NAAG), was identified in the chick retina (1.4 nmol/retina) by HPLC, radioimmunoassay and immunohistochemistry. This acidic dipeptide was found within retinal ganglion cell bodies and their neurites in the optic fibre layer of the retina. Substantial, but less intense, immunoreactivity was detected in many amacrine-like cells in the inner nuclear layer and in multiple bands within the inner plexiform layer. In addition, NAAG immunoreactivity was observed in the optic fibre layer and in the neuropil of the superficial layers of the optic tectum, as well as in many cell bodies in the tectum. Using a newly developed, specific and highly sensitive (3 fmol/50 μ X) radioimmunoassay for NAAG, peptide release was detected in isolated retinas upon depolarization with 55 mM extracellular potassium. This assay also permitted detection of peptide release from the optic tectum following stimulation of action potentials in retinal ganglion cell axons of the optic tract. Both of these release processes required the presence of extracellular calcium. Electrically stimulated release from the tectum was reversibly blocked by extracellular cadmium. These findings suggest that NAAG serves an extracellular function following depolarization-induced release from retinal amacrine neurons and from ganglion cell axon endings in the chick optic tectum. These data support the hypothesis that NAAG functions in synaptic communication between neurons in the visual system.

Introduction

N-acetylaspartylglutamate (NAAG) has been identified in retinal neurons of the frog (Kowalski *et al.*, 1987), rat (Anderson *et al.*, 1987; Moffett *et al.*, 1990, 1991), monkey (Tieman *et al.*, 1987a), cat (Tieman *et al.*, 1987b) and human (Tieman *et al.*, 1988). The dipeptide's distinct neuronal localization, together with its acidic amino acid character led to speculation that NAAG may have a role in synaptic communication within the visual system (Anderson *et al.*, 1987; Tieman *et al.*, 1987a). This has been supported by the identification of peptide immunoreactivity within synaptic vesicles (Williamson and Neale, 1988a) of amacrine, bipolar and photoreceptor cells in the amphibian retina. Consistent with this, we have observed the calcium-dependent release of newly synthesized peptide from this retinal tissue following depolarization (Williamson and Neale, 1988b). Additionally, the depolarization-induced release of endogenous peptide from excised rat brain tissue (Zollinger *et al.*, 1988), synaptosomes (Pittaluga *et al.*, 1988) and superior colliculus (Tsai *et al.*, 1990) have been reported. Despite these data, the physiological role of NAAG in the visual system remains unclear. Peptidase activity against NAAG suggested

a brain mechanism for catabolism of this dipeptide and this activity compromised equilibrium binding assays (Riveros and Orrego, 1984). It has been proposed that NAAG is metabolized by an enzyme with specificity for *N*-acetylated, α -linked acidic dipeptides (Robinson *et al.*, 1987). However, a more recent study demonstrated that the enzyme prefers the deacylated acidic dipeptide and that some additional, membrane-bound, brain peptidases also hydrolyse NAAG (Serval *et al.*, 1990). Extracellular peptidase activity against NAAG in excised chick retinas (Neale and Williamson, 1989) provides a mechanism for conversion of NAAG to glutamate and *N*-acetylaspartate in the synaptic space of this tissue. Thus, the peptide may serve as a sequestered form of glutamate involved in neurotransmission. This may be relevant in those cell systems where the amino acid appears to possess more potent excitatory activity than the peptide (Westbrook *et al.*, 1986). Alternatively, the rapid transport of glutamate, following hydrolysis of NAAG, may constitute a two-step system of extracellular peptide inactivation. An initial study suggesting a direct excitatory synaptic action for

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NAAG in the lateral olfactory tract (French-Mullen *et al.*, 1985) seems likely to have been confounded by the presence of elevated levels of potassium in the peptide preparation (Whittemore and Koerner, 1989). Beyond this, there is little agreement as to the excitatory potency of the peptide in several systems that respond to acidic amino acid agonists and that contain substantial amounts of endogenous NAAG (Luini *et al.*, 1984; Bernstein *et al.*, 1985; Westbrook *et al.*, 1986; Mori-Okamoto *et al.*, 1987; Sekiguchi *et al.*, 1987; Henderson and Salt, 1988; Schneider and Perl, 1988). Equilibrium binding studies, which purported to demonstrate affinity between the peptide and an acidic amino acid receptor subtype (Koller and Coyle, 1985; Schoepp and Johnson, 1989), were not definitive inasmuch as they failed to account for the influence of the binding of glutamate released via peptidase activity (Riveros and Orrego, 1984; Blakely *et al.*, 1988).

Central to understanding NAAG's function in the visual system are data on synaptic release of endogenous peptide. In the present study, we established the presence of this peptide within ganglion and amacrine cells of the chick retina. This system was used to test the hypothesis that NAAG is released synaptically from amacrine cells upon retinal depolarization and following initiation of action potentials in ganglion cell axons of the optic nerve. Some of these findings were published in abstract form (Eagles *et al.*, 1989).

Materials and methods

Immunohistochemistry

Four 6-day-old chicks (White Leghorn, Truslow Farms) were fixed by transcardial perfusion with phosphate buffered saline (PBS), pH 7.4, containing 4% carbodiimide following anaesthesia with Nembutal. Eyes and brain were removed and postfixed overnight in 4% carbodiimide in PBS and then fixed for a further 48 h in 4% paraformaldehyde in PBS at 4°C. The tissue was saturated through a series of 10, 20 and 30% sucrose solutions in PBS before freezing to -20°C. Frozen tissue sections (20 µm thick) were cut from glycerol-embedded tissue at -20°C using a cryostat (Hacker-Bright) and processed as floating sections. Tissue sections were incubated in PBS with 2% normal goat serum for 1 h and incubated overnight with affinity purified antibodies in PBS with 2% goat serum plus 0.01% sodium azide. The immunoreactivity was developed using an avidin—biotin bridged peroxidase protocol (Elite Vectastain, Vector Labs) with diaminobenzidine as substrate.

In preparation of the affinity purified antibodies for immunohistochemistry, 4-fold diluted rabbit anti-NAAG serum (Cangro *et al.*, 1987) was preincubated overnight at 4°C with 10 µg/ml of all of the following: *N*-acetylaspartate, aspartate and glutamate, each coupled to bovine serum albumin by a carbodiimide-mediated reaction. Using a peristaltic pump, this mixture was equilibrated for 24 h at 4°C with an affinity column containing NAAG coupled to Affigel 102 (BioRad) via carbodiimide. The column was washed with 15 column volumes of PBS and 15 volumes of 3 M guanidine hydrochloride in PBS. Specifically bound antibodies were eluted with 15 volumes of 6 M guanidine hydrochloride into a tube containing 1 ml of normal goat serum to yield a final concentration of — 2% goat serum. The purified antibodies were dialysed overnight against three 100-volume changes of PBS. Incubation of the affinity purified antibodies with 50 µg/ml of glutamate, aspartate or *N*-acetylaspartate, coupled to bovine serum albumin failed to reduce the immunoreactivity but similar preincubation of the serum with 10 µg/ml NAAG coupled to bovine serum albumin completely eliminated it. Production, characterization and application

of the primary anti-NAAG serum, from which the antibodies were purified, have been previously reported (Anderson *et al.*, 1987; Cangro *et al.*, 1987; Kowalski *et al.*, 1987; Williamson and Neale, 1988a).

Assay of retinal NAAG by HPLC

Retinas were homogenized in 90% methanol to extract the dipeptide for direct assay of retinal NAAG. The methanol extracts were dried, resuspended in water and subjected to cation exchange chromatography (Bio Rad AG 50W-X8). The effluent was dried, resuspended in water and applied to C-18 reversed phase (Sepharosorb ODS, 5 µm, 250 x 10 mm) high performance liquid chromatography (HPLC) and eluted with 0.1% trifluoroacetic acid. Fractions containing molecules that comigrated with external standard NAAG were collected, dried, resuspended in water and applied to Whatman Partisil SAX (10 µm, 250 x 5 mm) anion exchange HPLC with isocratic elution in 100 mM phosphate, pH 4.9 (Cangro *et al.*, 1987). Peptide elution was monitored by absorbance at 214 nm. NAAG concentration in the retinal extracts was estimated by comparison with absorbance values for NAAG obtained under similar conditions.

Radioimmunoassay

For use in radioimmunoassay, the rabbit anti-NAAG serum was affinity purified by direct application to the Affigel—NAAG matrix, washed with 1 M sodium thiocyanate, eluted with 6 M guanidine hydrochloride and dialysed against PBS.

Affinity purified antibodies (100 µl of 1:50 dilution relative to original antiserum) were incubated for 24 h at 4°C with the sediment from 50 /d of protein A (PANSORBIN) cells. The mixtures were sedimented at 7000 g for 5 min and the antibody solution was removed for reuse up to two more times. The pellets were resuspended with 50 /d of tissue extract or release media and with 100 µl of [³H]NAAG (50 000 cpm/100 µl, 50.7 Ci/mmol, NEN) resulting in a 0.01 µM NAAG concentration. The suspensions were agitated for 24 h at 4°C, sedimented at 7000 g for 5 min, the supernatants aspirated, the pellets rinsed twice with 0.5 ml of PBS and the bound radioactivity determined by liquid scintillation spectrometry. Assays were performed in duplicate and included NAAG standard curves ranging from 0.003 to 6.5 pmols. Displacement data were analysed by a RIA DATA Reduction System program, which employs a four-parameter logistic function and a 2 + 2 linear regression approach to curve fitting (Jaffe and Associates, Silver Spring, MD). Specificity was determined from competitive displacement curves for NAAG (0.05 nM — 100 nM), related amino acids and related peptides (100 nM — 1 mM). Validity of the RIA was determined by assay of chick retinal homogenates extracted with 90% methanol and purified by cation and anion exchange HPLC, as described above. Parallelism of the RIA was confirmed by assay of dilutions of chick retinal extracts.

Retinal peptide release

Six- to ten-day-old chicks were killed by cervical dislocation and retinas were excised. Pairs of retinas were sequentially incubated in a series of modified Tyrode's buffers. Low potassium was the standard medium, containing (mM): NaCl, 126; KCl, 2.68; MgCl₂·6H₂O, 0.49; NaH₂P₀₄·H₂O, 0.36; NaHCO₃, 22 and D-glucose, 5.6. Low potassium plus ethylene glycol-bis (β-aminoethyl ether) *N,N,N,N'*-tetra-acetic acid (EGTA) medium additionally contained 1 mM EGTA. High potassium medium contained the standard medium salts but with

55 mM KCl and 70 mM NaCl. High potassium plus calcium medium contained high potassium salts plus 2.5 mM CaCl₂. Each incubation medium contained 20 μ M aspartylglutamate to inhibit peptidase activity against NAAG (Robinson *et al.*, 1987). The incubation media (pH 7.2–7.4) were warmed to 37°C and equilibrated with 95% O₂ and 5% CO₂. This gas mixture was perfused through the media during incubation. Pairs of submerged, freely suspended retinas were incubated for 5 min intervals in 1-ml volumes of media in multiwell culture plates with wells 17 mm in diameter x 15 mm deep. The media were collected after incubation with tissue, passed through BioRad AG50W-X8 cation exchange resin, lyophilized and the NAAG content of the release media determined by RIA. Retinas were homogenized in 90% methanol, sedimented at 13 000 *g* for 30 min and the supernatants passed through cation exchange resin prior to RIA, as described above.

Optic tract release

Optic lobes with associated optic nerves were dissected from 6- to 10-day-old chicks and placed in one compartment of a release chamber (two compartments, each 1.5 cm x 0.5 cm x 0.5 cm). The optic lobe preparation, consisting of a single optic lobe with optic tract, optic chiasm and both optic nerves, was submerged in 0.5 ml of Tyrode's medium at 37°C (pH 7.4); this had been equilibrated with 95% O₂ and 5% CO₂. The optic nerves were passed through an opening to the adjacent compartment. A Vaseline® bridge was used to separate the stimulating electrode compartment from the recording compartment. Aspartylglutamate (15 μ M) was added to this medium to inhibit peptidase activity (Robinson *et al.*, 1987) in some preparations. The tissue was equilibrated with buffer for 1.5–2 h prior to assay. A suction electrode (1–2 mm diameter) was fitted to the cut optic nerve end and a glass recording electrode, consisting of a microelectrode with a broken tip, was inserted into the contralateral optic tectum at the level of the optic fibre layer to determine the optimal stimulation paradigm for eliciting action potentials and to permit continuous assay of the viability of the preparation. The magnitude of the compound action potentials recorded in the optic tectum was dependent on the stimulus duration and voltage. Placement of the recording electrode deeper in the tectum, outside of the boundaries of the optic projection, resulted in loss of the response. Stimulation of the optic nerve on one side failed to result in compound action potentials in the optic fibre layer of the ipsilateral tectum. At the end of each experiment, the optic nerve was crushed at a point between the stimulating electrode and the optic chiasm. In every case, this resulted in loss of the stimulus-induced action potentials in the tectum. Suitable stimulation for eliciting compound action potentials was obtained at 0.7–1.8 V for a duration of 1 ms. Optic nerves were stimulated for 30 s at 40 Hz, followed by a 9.5 min interval without stimulation to allow for diffusion of the released substances into the incubation medium. Media were replaced every 10 min and assayed for NAAG by RIA following cation exchange chromatography.

Results

Ganglion cells

Using affinity purified antibodies prepared against NAAG, the peptide was localized in carbodiimide-fixed retinal and brain tissue by immunohistochemistry. Intense NAAG immunoreactivity was found in many, but not all, cell bodies in the retinal ganglion cell layer (Fig. 1A). All size classes of ganglion cells were immunoreactive. The proportion of NAAG immunoreactive ganglion cells appeared higher

in the central retina than in the peripheral retina. Occasional neurons with substantially larger perikarya and more intense NAAG immunoreactivity were observed among the smaller immunoreactive cells in the inner nuclear layer. These probably represented displaced ganglion cells (Fig. 1A, arrow). Immunoreactivity also was observed in the inner plexiform layer of the retina and in the optic fibre layer carrying axons to the optic nerve. The photoreceptors and the cells in the external half of the inner nuclear layer were not immunoreactive for NAAG. The NAAG immunoreactivity in retina was completely blocked by incubation of the antisera with NAAG coupled to bovine serum albumin at a concentration of 10 μ g/ml of protein (Fig. 1B) but 5-fold greater concentrations of glutamate, aspartate and *N*-acetylaspartate conjugates failed to reduce the NAAG immunoreactivity. The staining pattern in the peripheral retina was slightly different from that observed towards the central portion. In the periphery, the external plexiform layer was mildly to moderately immunoreactive for NAAG (Fig. 1C).

Fibres in the inner plexiform layer

A substantial proportion of cells in the inner half of the inner nuclear layer exhibited NAAG immunoreactivity. When examined at multiple planes of focus, these presumptive amacrine cells could be seen to extend very fine diameter neurites into the inner plexiform layer of the retina (Fig. 2A and B). In cross-section, the inner plexiform layer exhibited a laminated pattern of NAAG immunoreactivity within the neuropil (Figs 1A and 2A). The laminated appearance of this layer was more easily resolved in some retinal sections than in others—an apparent consequence of the plane of section. Some large ganglion cells also could be seen to extend branched processes into the inner plexiform layer (Fig. 2C).

Optic tectum

Extensive neuropil staining for NAAG was observed in the receptive layers of the tectum of 6-day-old chicks. NAAG immunoreactivity displayed a laminated appearance in the superficial layers receiving optic fibre input (Fig. 3A). The avian optic tectum can be divided into six layers (Ariens Kappers *et al.*, 1936), beginning at the pial surface with the optic fibre layer (stratum opticum). This is followed by a zone with alternating cell and fibre layers that receives the optic fibre input (stratum griseum et fibrosum superficiale), a central efferent cell zone (the stratum griseum centrale), followed by an efferent fibre layer (stratum album centrale) and finally the periventricular cell and fibre layers. The retinal ganglion cell fibres in the outermost layer—the stratum opticum—were consistently stained for NAAG. The neuropil of the main receptive zone of the tectum (stratum griseum et fibrosum superficiale) displayed both diffuse and punctate NAAG immunoreactivity (Figs 3B and C). Immunoreactive puncta suggestive of synaptic contacts were visible on many of the tectal neurons in the layers receiving optic fibre input (Fig. 3D). When viewed at multiple focal planes, these puncta appeared to conform to the external surface of the cell bodies with which they were associated.

The majority of neurons in the receptive tectal layers were NAAG positive. Some neurons in the deeper efferent layers of the tectum were also immunoreactive for NAAG. However, many of the larger neurons of the central cell zone (stratum griseum centrale) were not immunoreactive, as shown by cresyl violet counterstaining. Cells of the periventricular zone were generally more heavily labelled than those

of the central cell layer. Many axons in the central fibre layer (stratum album centrale) were also immunoreactive for NAAG.

Anion exchange and reversed phase HPLC To confirm the presence of NAAG in the chick retina, tissue extracts were purified using cation exchange chromatography followed by either HPLC anion exchange chromatography or reversed phase HPLC. Using absorbance values obtained from known quantities of the synthetic

peptide, which were applied to these HPLC systems, the NAAG content of chick retina was estimated to be 1.41 nmol/retina (± 0.26 SD, $n = 5$).

Radioimmunoassay

To more readily trace the release of NAAG from this tissue, a radioimmunoassay was developed using affinity purified antibodies against NAAG and [3 H]NAAG (50 Ci/mmol). The sensitivity of the assay for detection of synthetic NAAG had a lower limit of 3 fmol/50 μ l of sample (Fig. 4). The IC_{50} values for unlabelled NAAG, *N*-acetylaspartate and aspartylglutamate in this assay were 2.5 nM, 100 μ M and 1 mM, respectively. Glutamate, aspartate, *N*-acetylglutamine, *N*-acetyllysine, *N*-acetylalanylalanine, *N*-acetylglycylleucine and *N*-hydroxysuccinimide exhibited IC_{50} values of greater than 1 mM. To test the validity of the radioimmunoassay in the visual system, chick retinal tissue was homogenized and the methanol-soluble molecules were resolved by sequential cation and anion exchange HPLC. Endogenous retinal NAAG immunoreactivity, as identified by the RIA, migrated on HPLC in a position identical to that of synthetic NAAG (Fig. 5). To confirm the efficacy of the radioimmunoassay in defining the concentration of endogenous tissue NAAG, the displacement curves for dilutions of chick retinal homogenate and synthetic NAAG were compared and found to be parallel (Fig. 6).

NAAG content of the chick retina (30 mg wet weight, 4.6 mg protein) determined by this radioimmunoassay was 1.43 ± 0.10 nmol/retina ($n = 3$), a value consistent with that obtained from absorbance estimates of NAAG purified from retina.

Retinal release

In order to determine whether this retinal NAAG was available for release, excised retinas were incubated in a series of balanced salt solutions. The efflux of NAAG was measured by RIA. Following sequential incubation in media without calcium (low potassium with EGTA; low potassium without EGTA), elevated extracellular potassium (high potassium, 55 mM) did not alter the basal appearance of the peptide immunoreactivity in the incubation medium (Fig. 7). However, with the addition of 2.5 mM calcium (high potassium plus calcium), there was a 10-fold increase in release of NAAG into the medium. Similar calcium-dependent release was observed during a second cycle

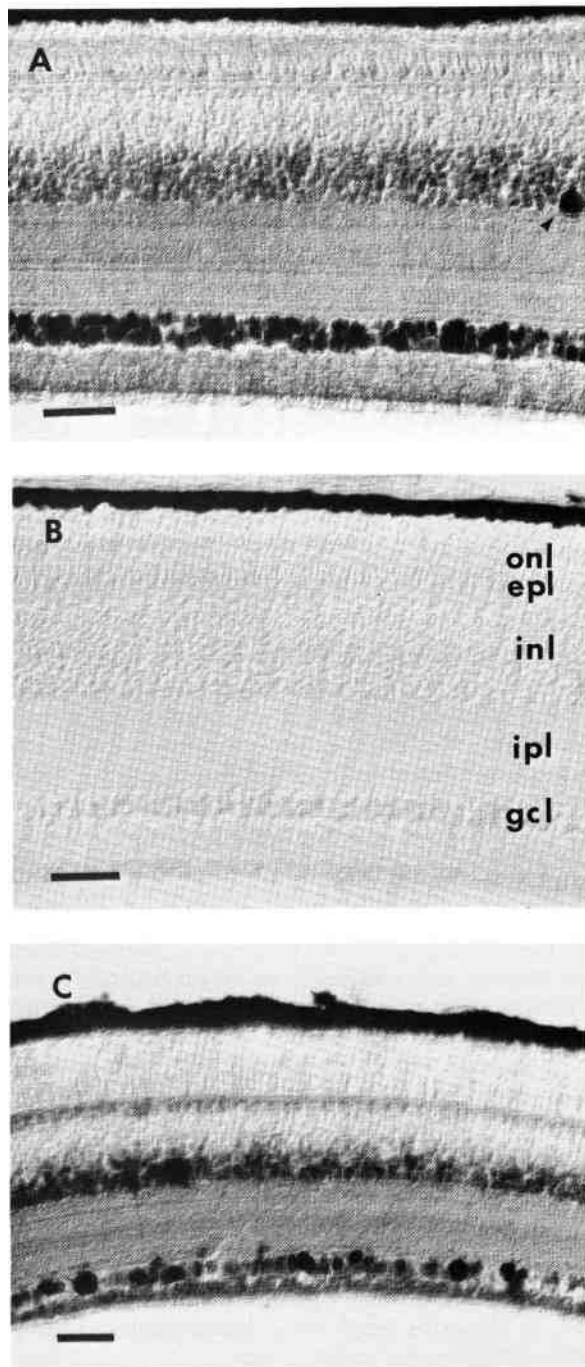


FIG. 1. Immunohistochemical localization of NAAG in the chick retina. (A) NAAG immunoreactivity is indicated by the presence of dark peroxidase reaction product in most retinal ganglion cell (gcl) bodies and, to a lesser extent, in their neurites. Moderate NAAG immunoreactivity was present within synaptic bands in the inner plexiform layer (ipl), which could be seen most clearly when the sectioning plane was perpendicular to the plane of the retina. Immunoreactivity also was present in numerous, presumptive amacrine cell bodies in the inner half of the inner nuclear layer (inl). No cells were seen to exhibit any NAAG immunoreactivity in the outer nuclear layer (onl) containing the photoreceptor cell bodies, or in their outer segments. The size and structure of the cell marked with a large arrow in (A) suggests that it is a displaced ganglion cell. (B) A retinal section is shown following incubation with affinity purified antibodies, which were blocked with 1 μ g/ml of NAAG conjugated to bovine serum albumin and processed for IR as in (A) (Bar = 50 μ m for A and B). The sections were photographed using differential interference contrast optics to enhance the resolution of unreactive retinal cells and synaptic layers. (C) The pattern of NAAG immunoreactivity was slightly different in the periphery of the retina, wherein the external plexiform layer (epl) displayed light to moderate staining that was not observed in the central retina. (Bar = 50 μ m).

through the same series of incubation media. About 2% of the retinal NAAG immunoreactivity was released during each exposure to elevated potassium with calcium.

Optic tectum release

In an attempt to examine the question of NAAG release from retinal ganglion cell axon endings under physiological conditions, the intact

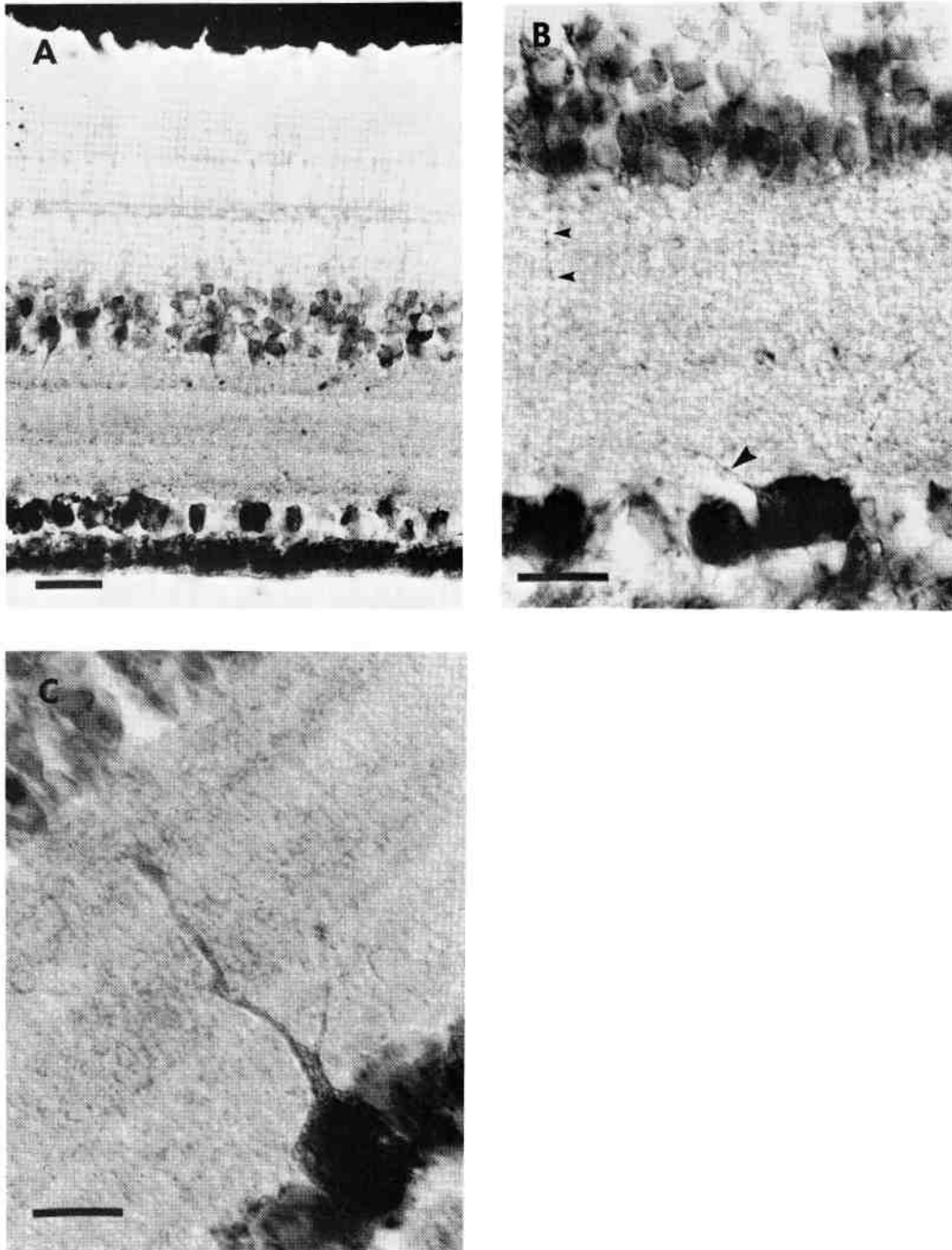


FIG. 2. NAAG immunoreactive fibres in the inner plexiform layer of the chick retina. Fine, mildly immunoreactive fibres could be seen entering the inner plexiform layer from both amacrine cells and ganglion cells (A and B). Probable amacrine cells along the inner margin of the inl, which were immunoreactive for NAAG could occasionally be seen to extend immunoreactive fibres into the inner plexiform layer (B; small arrowheads), as could immunoreactive fibres originating from ganglion cells (B; large arrowhead). The overall staining pattern in the inner plexiform layer was laminated, with multiple bands of increased NAAG immunoreactivity. The most obvious fibres entering the inner plexiform layer were those of large ganglion cells in either the ganglion cell layer or the inner nuclear layer. These could be seen to extend large branched, immunoreactive processes into the inner plexiform layer, with different branches often entering different layers in the neuropil (C). (Bar in A = 30 μ m; B and C = 15 μ m).

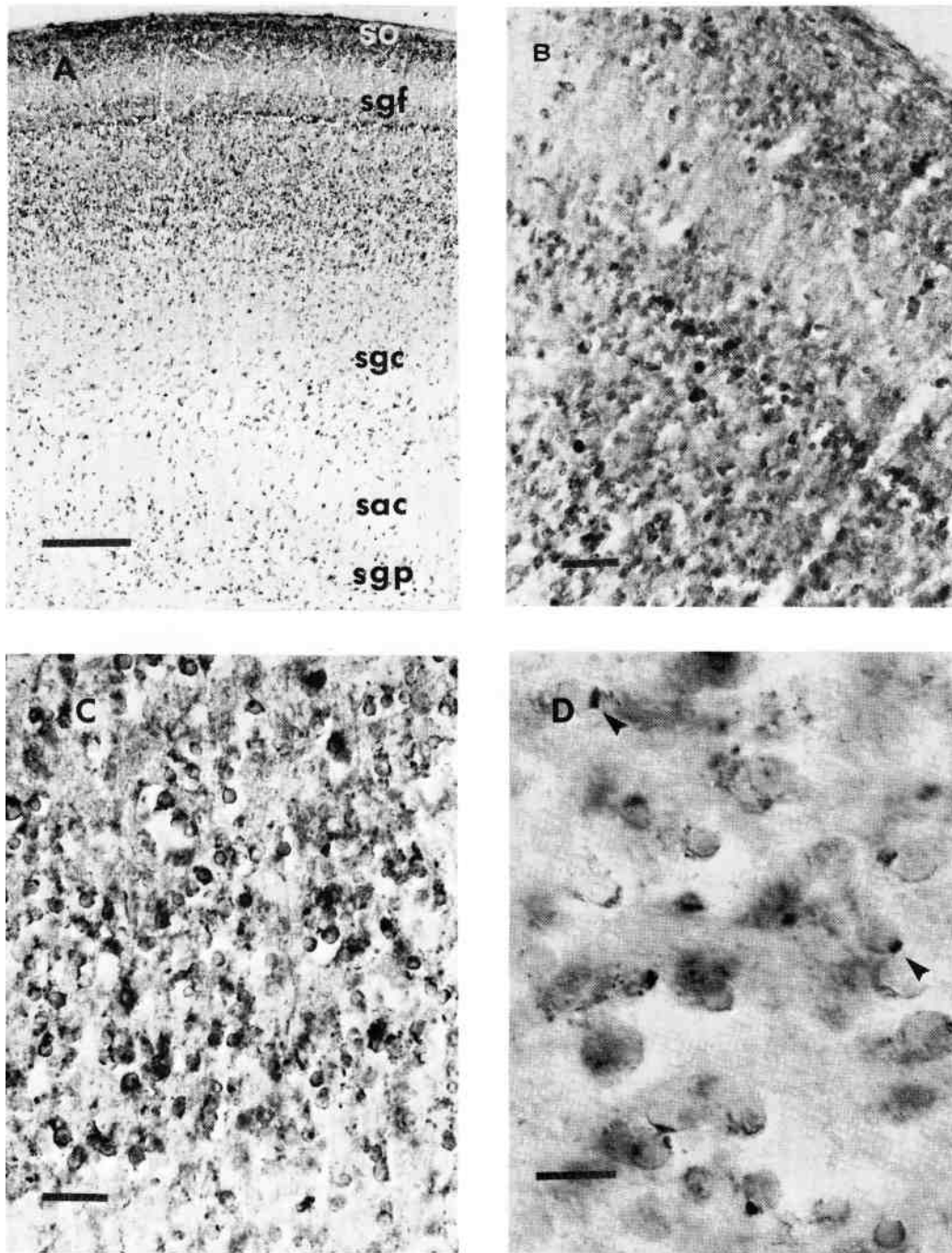


FIG. 3. NAAG immunoreactivity in the tectum of a 6-day-old chick. Ganglion cell axons in the optic fibre layer (stratum opticum, so) were consistently stained for NAAG (A and B). The optic fibres displayed NAAG immunoreactivity throughout the optic projections. The NAAG immunoreactivity in the optic tectum was most intense in the superficial layer beneath the optic fibre layer (A). The immunoreactivity in the neuropil of the superficial layers (stratum griseum et fibrosum superficiale, sgf) exhibited a striated appearance that corresponded to the external cellular layers (A). (Bar = 250 *µm*). Many of the neurons in the superficial layers of the tectum were immunoreactive for NAAG (B). (Bar = 50 *µm*). A smaller proportion of cells in the deeper layers of the tectum, including the stratum griseum centrale (sgc), stratum alba centrale (sac) and stratum griseum periventriculare (sgp) were moderately immunoreactive for NAAG. In all the superficial layers of the optic tectum (C and D), large immunoreactive puncta, which were suggestive of synaptic contacts, were associated with the tectal neurons. (Bar = 30 *µm* in C, 10 *µm* in D).

optic nerves, optic tracts and associated optic tecta were isolated in stimulus and recording chambers. The midbrain, including the optic lobe with attached optic chiasma and nerves was isolated and bisected

and one optic lobe was placed in the recording chamber. The distal cut ends of the optic nerves were isolated from the tectum with a Vaseline® barrier. The cut optic nerve end was stimulated with a

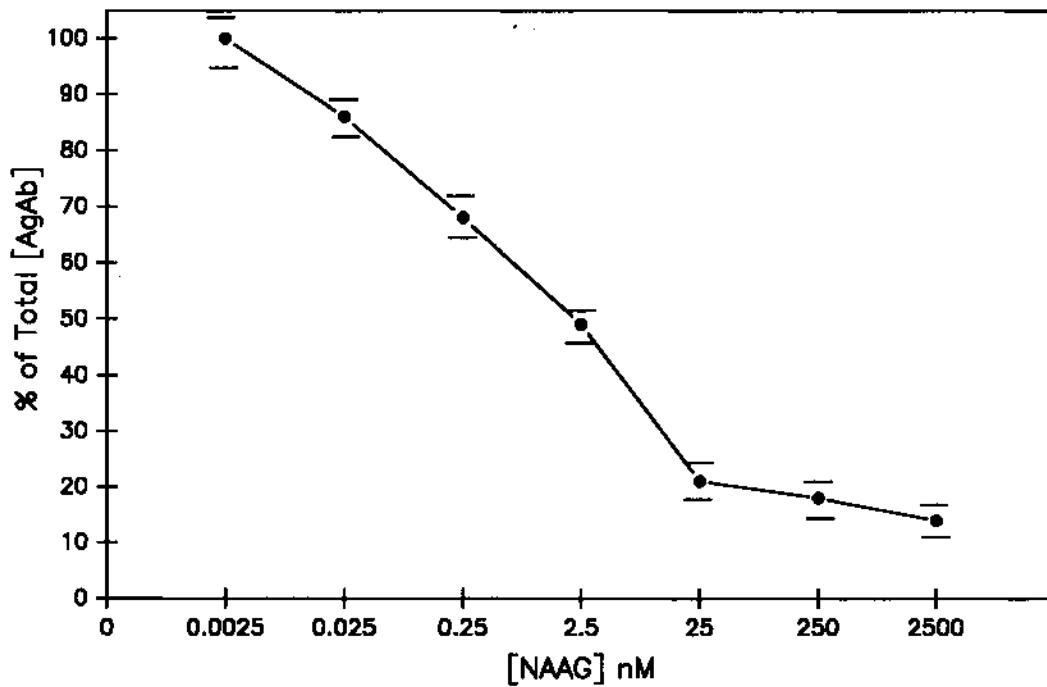


FIG. 4. NAAG standard curve in radioimmunoassay. The displacement of radiolabeled NAAG binding by increasing concentrations of synthetic NAAG is presented as the percentage of total radiolabel bound in the absence of unlabelled peptide. The values (\pm 95% confidence limits) are means of eight separate determinations. With assay volumes of 150 μ l, of which 50 μ l was sample, the lower limit of sensitivity was taken as that concentration of NAAG that would consistently produce a displacement of at least 15% of the radiolabeled NAAG. That value was \sim 0.025 nM NAAG in a 150 μ l assay volume, or \sim 3 fmol/assay.

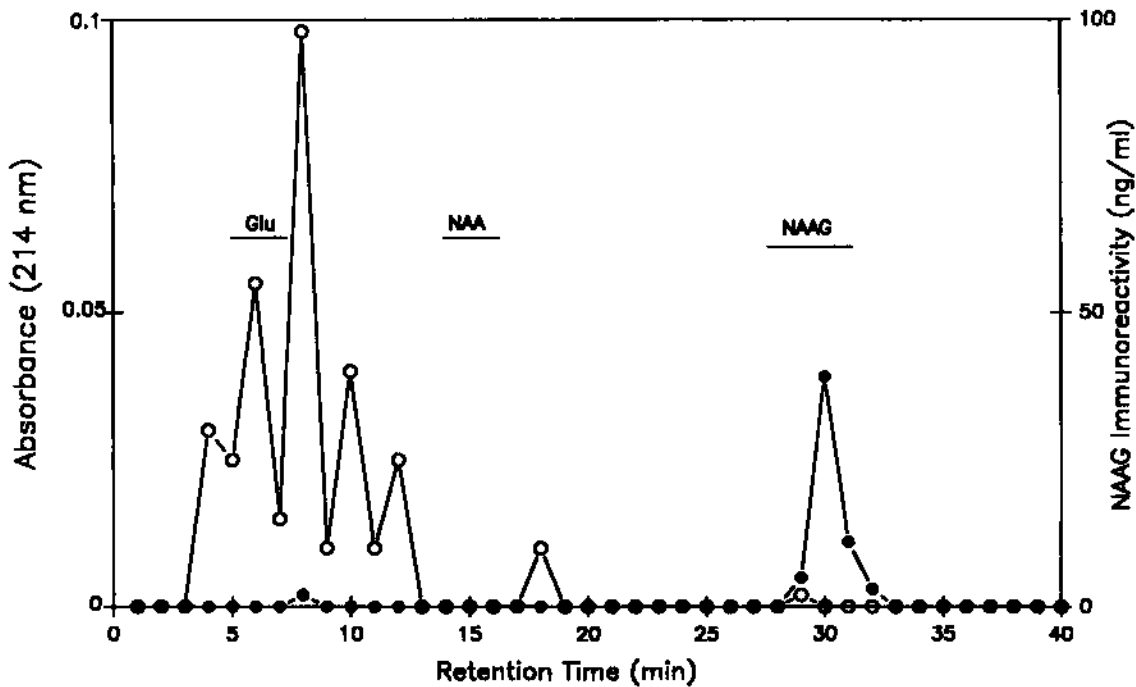


FIG. 5. RIA of chick retinal extract following HPLC. Chick retinal tissue was extracted in methanol, the molecules with primary amines were removed via cation exchange chromatography and the remaining molecules resolved using weak anion exchange HPLC. HPLC fractions were assayed for NAAG by RIA (filled circles). The absorbance of the retinal tissue extract (open circles) is presented with bars that indicate the retention time of glutamate (glu), N-acetylaspartate (NAA) and NAAG, as determined by the retention times of synthetic standards.

was crushed or severed between the stimulating and recording electrodes. When media were collected for 10 min intervals without stimulation, a consistent basal release of NAAG immunoreactivity was observed. Generally, stimuli with amplitudes of 1 — 1.6 V, durations of 1 ms and frequencies of 40 Hz, applied during the initial 30 s at the onset of each 10 min incubation interval, resulted in a substantial increase in the appearance of NAAG immunoreactivity in the incubation medium relative to the unstimulated intervals. In 40 unstimulated release intervals obtained from five separate optic tectum preparations, the mean (\pm SD) basal release of NAAG was 4.6 ± 1.2 pmol/tectum, while the mean release upon stimulation was 11.2 ± 3.8 pmol/tectum ($n = 20$). Removal of calcium from the incubation medium had a delayed effect in eliminating the stimulus-induced release of NAAG (Table 1, Expt. A). This effect was reversible with synaptic release resuming upon reincubation with calcium. In three preparations where the removal of calcium was studied, the mean unstimulated NAAG release with calcium was 4.9 ± 0.9 pmol/tectum ($n = 12$), the mean stimulated release with calcium was 17.7 ± 5.6 pmol/tectum ($n = 12$), the mean unstimulated release in the absence of calcium was 4.1 ± 0.7 pmol/tectum ($n = 10$) and the mean stimulated release in the absence of calcium was 6.3 ± 4.5 pmol/tectum ($n = 5$). Addition of $50 \mu\text{M}$ cadmium in the normal Tyrode's incubation medium reversibly eliminated the stimulus-induced release of peptide (Table 1, Expt. B). Very similar data demonstrating the reversible blockade by cadmium of stimulated NAAG release were obtained in two additional optic tectum preparations. EGTA was used in other release studies to increase the rate of removal of extracellular calcium ions in the tectal tissue but this approach did not provide useful data because it interfered with the conduction properties of the optic tract.

Antidromic retinal stimulation

Both retinal ganglion and amacrine cells exhibit NAAG immunoreactivity and the question therefore arises as to which cell type was responsible for peptide release following potassium-induced depolarization of the isolated retinas. To determine whether the ganglion cell bodies or their neurites in the retina were capable of depolarization-induced release, ganglion cell axons were stimulated antidromically. Suction electrodes were attached to the end of the cut optic nerve, which remained associated with the retina, in three preparations. Suction electrode stimulus levels (1 V, 10 ms, 40 Hz), which routinely stimulated action potentials in the distal optic tract, were applied to the optic nerve axons, which remained in continuity with their retinal ganglion cell bodies. The medium bathing these stimulated retinas was assayed for NAAG release by RIA. No increases in peptide concentration were detected in the induction medium bathing the antidromically stimulated retinas beyond the level that was released under the non-stimulated conditions.

Discussion

The excitatory neurotransmitter that mediates communication from the retina in vertebrates is not established, although elevated levels of substance P (Brecha *et al.*, 1987), LANT-6 (Eldred *et al.*, 1988), adenosine (Braas *et al.*, 1987), corticotropin releasing factor (Williamson and Eldred, 1989) and GABA (Yu *et al.*, 1988) have been localized in retinal ganglion cells of various vertebrate species. In the cat, acidic amino acid receptors appear to mediate postsynaptic responses to optic nerve stimulation in the lateral geniculate nucleus

Exp. A	NAAG	Medium	Exp. B	NAAG	Medium
-	5.4	Tyrode's	-	7.2	Tyrode's
+	18.9	Tyrode's	+	13.6	Tyrode's
-	4.2	Tyrode's	-	5.4	Tyrode's
-	5.6	Tyrode's	-	4.7	Tyrode's
+	16.5	Tyrode's	+	14.1	Tyrode's
-	5.1	Tyrode's	-	4.9	+ 50 μM cadmium
-	4.5	-Ca ²⁺	-	4.3	+ 50 μM cadmium
+	10.4	-Ca ²⁺	+	4.2	+ 50 μM cadmium
-	4.3	-Ca ²⁺	-	3.9	+ 50 μM cadmium
-	4.2	-Ca ²⁺	-	4.2	+ 50 μM cadmium
+	4.9	-Ca ²⁺	+	4.4	+ 50 μM cadmium
-	3.5	-Ca ²⁺	-	4.4	Tyrode's
-	3.6	-Ca ²⁺	-	4.3	Tyrode's
+	3.7	-Ca ²⁺	+	15.9	Tyrode's
-	3.1	-Ca ²⁺	-	3.8	Tyrode's
-	7.2	+ Ca ²⁺			
+	23.3	+ Ca ²⁺			
-	5.2	+ Ca ²⁺			
-	3.4	+ Ca ²⁺			
+	12.2	+ Ca ²⁺			
-	3.3	+ Ca ²⁺			

Tyrode's media bathing the optic tecta were collected at 10 min incubation intervals, which included 30 s with (+) or without (-) optic nerve stimulation. Stimuli consisted of 1 ms pulses at 1 V and 40 Hz, except the stimuli during and after cadmium exposure, which were at 5 V to test the strength of the cadmium block. Incubation of optic tecta in Tyrode's medium without calcium is shown as — Ca

(Kemp and Sillito, 1982; Sillito *et al.*, 1990a,b). In avian species, data from ablation (Fonnum and Henke, 1981), uptake (Bondy and Purdy, 1977), transport (Beaudet *et al.*, 1981) and release (Canzek *et al.*, 1981) support a role for aspartate and glutamate in neurotransmission from ganglion cells, although similar release data were not obtained in the rabbit superior colliculus (Sandberg and Corazzi, 1983). The presence of NAAG immunoreactivity in the great majority of retinal ganglion cells in several vertebrate species (Anderson *et al.*, 1987; Tieman *et al.*, 1987a,b, 1988; Moffett *et al.*, 1990, 1991) suggests that this neuropeptide might contribute to synaptic communication from all subtypes of ganglion cells. We report here that NAAG is present in the great majority of chick retinal ganglion cells and, further, that this endogenous peptide is released from the optic tectum—a terminal field of retinal ganglion cell axons. Data supporting the conclusion that this release involves vesicular synaptic release include: (i) its increase following initiation of action potentials in the optic tract; (ii) its requirement for extracellular calcium and (iii) its reversible block by cadmium. It could be argued that postsynaptic, NAAG immunoreactive neurons in the tectum contribute to the observed NAAG release in response to optic nerve stimulation. However, the presence of NAAG in retinal ganglion cells, retinal ganglion cell axons and in the neuropil of the superficial layers of the tectum, points to the ganglion cell terminals as the likely source of the released NAAG. Further support for the conclusion that NAAG is released synaptically is derived from the localization of NAAG immunoreactivity within synaptic vesicles (Williamson and Neale, 1988a). These data lend additional support to the idea that NAAG is important in visual system communication and call into question the idea that free glutamate is the principal substance released by ganglion cell terminals in avian species in response to retinal stimulation. We have been unable to detect increased levels of glutamate

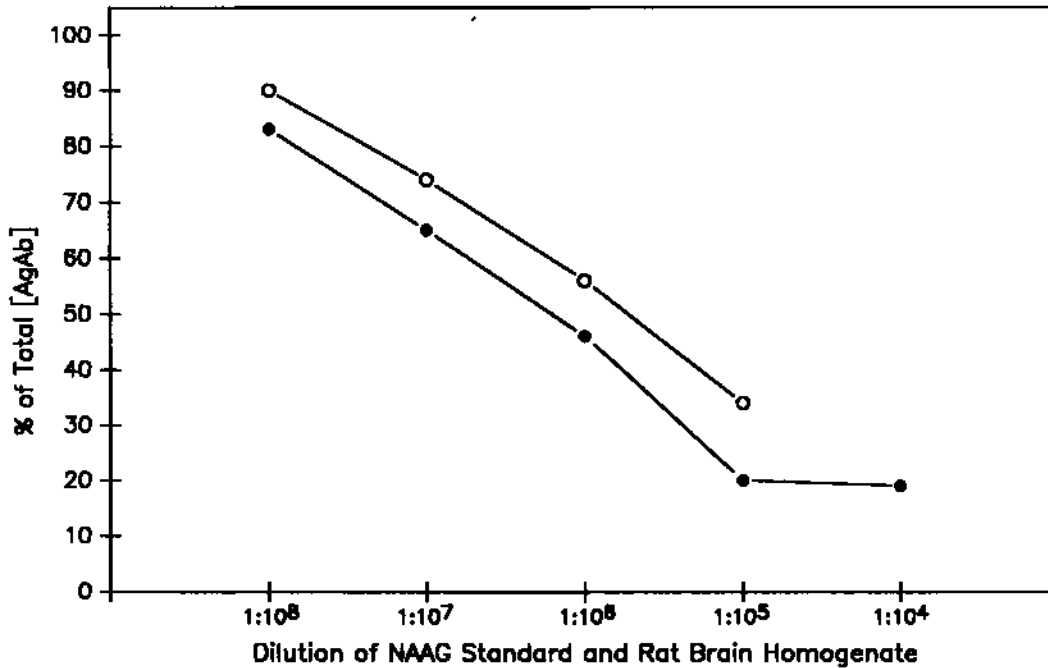


FIG. 6. Parallelism in RIA between synthetic NAAG and endogenous NAAG in retinal tissue. The displacement of radiolabeled NAAG binding in the RIA by dilutions of a 1 mM solution of synthetic NAAG (closed circles) and by dilutions of the cation exchange effluent (concentrated to 100 μ l before dilution) obtained from the methanol extract of a single chick retina (open circles). The values are means of two separate assays performed in duplicate at each concentration.

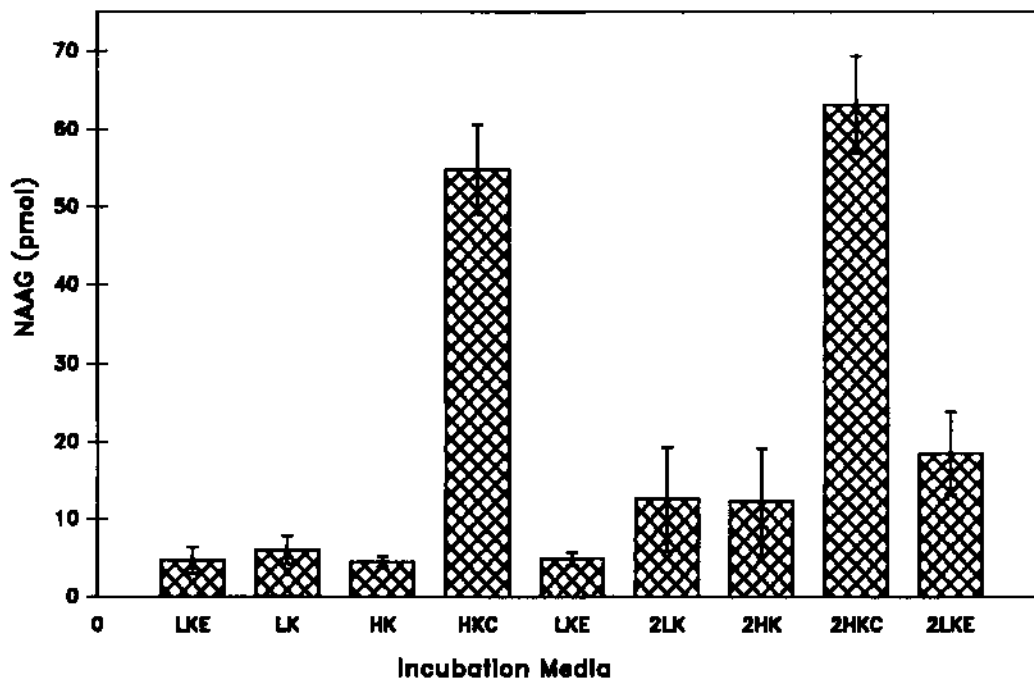


FIG. 7. Chick retinal release of endogenous NAAG. Pairs of chick retinas were incubated for 5 min intervals in two cycles of incubation media: LKE, 2.6 mM potassium without calcium and with EGTA; LK, 2.6 mM potassium without calcium; HK, 55 mM potassium without calcium; HKC, 55 mM potassium with 2.5 mM calcium. Data obtained from the second cycle of incubations are indicated as 2LK, 2HK etc. The bar heights represent mean values for NAAG released by four pairs of retinas. The standard errors ($n = 4$) are indicated. NAAG was determined by RIA.

suction electrode and compound action potentials were recorded with an extracellular electrode placed in the optic fibre layer of the tectum. The stimulus conditions were adjusted to produce a large compound

action potential which: (i) remained constant in appearance during the experiment; (ii) was dependent on both the stimulus intensity and the duration of the stimulus pulse and (iii) disappeared when the optic nerve

immunoreactivity in retinal ganglion cells or their termination zones in either chick or rat (Moffett *et al.*, unpublished data).

The immunohistochemical demonstration of NAAG in ganglion cells and presumptive amacrine cells in the chick retina, combined with the observation that antidromic stimulation did not increase basal NAAG release in the retinal preparations, suggests that retinal NAAG release occurred from the amacrine-like cells upon depolarization with increased potassium. Subpopulations of avian amacrine cells express a diversity of neurotransmitters, such as GABA (Watt *et al.*, 1984), acetylcholine (Miller *et al.*, 1985), dopamine and serotonin (Floren, 1979), as well as neuropeptides such as enkephalin (Brecha *et al.*, 1979), neurotensin and somatostatin (Brecha *et al.*, 1981). Given the rather uniform distribution of NAAG in the inner half of the inner nuclear layer, it seems probable that it is colocalized with several different transmitters and peptides in amacrine cells. Our observations on NAAG release following retinal depolarization and the absence of detectable NAAG release following antidromic stimulation of retinal ganglion cells imply that some portion of this amacrine cell NAAG is within a synaptic pool involved in intraretinal neurotransmission. Thus, the avian retina provides a potentially useful experimental model in which to examine the influence of NAAG on synaptic release of several different neurotransmitters and peptides, as well as on the second messenger responses of this tissue.

We detected a significant steady basal release of NAAG into the incubation medium in the isolated retinas and in the optic tecta with associated optic tracts. While the stimulated release was generally 3-fold above this basal level, these data suggest that the peptide may be chronically released from neurons and that this process neither requires normal levels of extracellular calcium nor is blocked by cadmium. We cannot rule out the possibility that this release is occurring from damaged cells in the acutely isolated retinal or tectal tissue. However, the steady-state secretion of NAAG into the extracellular space merits further consideration in light of data on the tonic activation of NMDA receptors by an endogenous acidic amino acid receptor agonist in the hippocampus (Sah *et al.*, 1989).

Understanding the functional consequences of NAAG release as a result of action potential propagation within the optic nerve will require analysis of the kinetics of peptide release, uptake and catabolism, together with glutamate transport following its extracellular release via NAAG hydrolysis. Similarly, the extracellular fate of N-acetylaspartate, a product of enzyme-mediated NAAG hydrolysis, may be an issue of considerable importance.

Equilibrium binding data, which can be useful in identifying potentially relevant interactions between neuroactive molecules and membrane-bound receptors, have yet to provide rigorous support for the hypothesis that NAAG is possessed of high affinity for neuronal receptors (Valivullah *et al.*, 1990). Beyond this, there is a relative paucity of consistent and unambiguous data on the direct interaction of NAAG with amino acid receptors under physiological conditions. This contrasts with the very extensive cellular distribution of the dipeptide and of neurons that respond to acidic amino acid receptor agonists. Taken together with the growing body of data on the synaptic release of NAAG, we are led to several non-exclusive hypotheses as to the role of NAAG in nervous system function. First, NAAG may serve as a sequestered form of glutamate, which is physiologically activated by peptidase action in the extracellular space. Secondly, this dipeptide may act through a relatively low affinity receptor, which has yet to be detected in binding assays and that mediates a pre- or postsynaptic response that is not directly coupled to changes in

membrane conductance. Finally, we speculate that, in some neuronal circuits containing NAAG, it may reach sufficient concentrations as to directly affect acidic amino acid receptors. Such multiplicity of action is consistent with our growing appreciation of both the diversity of transmitter function and the considerable heterogeneity of receptors.

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Abbreviations

EGTA, ethylene glycol-bis (β -aminoethyl ether) *N, N, N'*, *N'*, tetraacetic acid; HPLC, high performance liquid chromatography; NAAG, *N*-acetylaspartylglutamate; PBS, phosphate buffered saline

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