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Immunohistochemical localization of N-acetylaspartate in rat brain

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N-ACETYLASPARTATE (NAA) is one of the most prevalent compounds in the mammalian nervous system. As such, NAA largely contributes to the major peak on water-suppressed proton magnetic resonance spectra. Highly specific antibodies to NAA demonstrate that this compound is discretely localized in a substantial number of neurons throughout the extent of the rat CNS. N-acetylaspartylglutamate (NAAG) is a structurally related neuronal dipeptide which is less widely distributed than NAA. NAAG and NAA immunoreactivities were extensively colocalized in many brainstem areas, where NAAG containing neurons were more numerous than in forebrain structures.

Key words: Aspartate, Magnetic resonance spectroscopy, NAAG, Excitatory neurotransmission

Introduction

N-Acetylaspartate (NAA) is a ubiquitous compound in the mammalian nervous system, with an estimated concentration in human brain tissue of 13 to 18 mM.[1] Numerous functions have been proposed for NAA . Early reports suggested a role in the acetyl transport system for extramitochondrial fatty acid synthesis.[2] Recently, a congenital error in NAA metabolism has been associated with Canavan disease, a degenerative disorder characterized by leukodystrophy.[3] This condition results from a deficiency in the hydrolytic enzyme, aspartoacylase.[4] The enzyme that acetylates aspartate, aspartate N-acetyltransferase, has a regionally specific distribution in the nervous system.[5] NAA may act as a precursor for the synthesis of N-acetylaspartylglutamate (NAAG),[6] a neuron specific dipeptide which may be involved in neurotransmission.[7]

NAA has gained interest due to its prominent signature on water-suppressed proton magnetic resonance spectra of human brain. In patients with chronic localized encephalitis the locally decreased ratios of NAA to creatine (NAA/Cr) resonance intensities may reflect neuronal loss.[8] Also, magnetic resonance spectroscopy in patients with multiple sclerosis suggests that decreases in the NAA/Cr ratio occur only in plaques associated with permanent neuronal loss.[9] There are decreases in the ratio of NAA to choline in cases of herpes simplex encephalitis[10] and ischemic brain damage.[11] Determining which cell groups in the nervous system contain high levels of these acetylated compounds should aid interpretation of the changes in magnetic resonance spectra which are observed in a variety of CNS disorders. Here we report the specific immunohistochemical localization of NAA to neurons of the rat brain, and compare its observed neuronal distribution with that of NAAG in several brain regions.

Materials and Methods

Polyclonal antisera to NAA were produced in rabbits as previously described for NAAG.[6] The serum was affinity purified as previously described for NAAG[12] with some modifications. The antisera were diluted ten-fold in PBS (pH 7.2) and preincubated with 100 |ig mh[1] of NAAG, aspartate and glutamate, each coupled to bovine serum albumin (BSA). The serum was then applied to a NAA-coupled aminoalkyl-agarose gel and continuously circulated with a peristaltic pump for 18 h at 4°C. The column was washed with ten column volumes of 3M guanidine HC1 in PBS, eluted with the same volume of 6 M guanidine HCl, and the eluate was dialysed against PBS. Polyclonal sera against NAAG were affinity purified in a similar way on a NAAG-coupled aminoalkyl-agarose gel, after preblocking with BSA conjugates of NAA, glutamate and aspartate.

Three adult male rats were anaesthetized with Nembutal® (50 mg kg'[1] i.p.) prior to transcardial perfusion with 4% carbodiimide in PBS. Coronal sections (20 |im) were cut on a Bright cryostat. Immunohistochemistry was performed by the floating section technique.[12]

Specificity controls were two-fold. Working dilutions of the antibodies were incubated with nitrocellulose sheets previously adsorbed with serial dilutions of NAAG, NAA, aspartylglutamate, aspartate and glutamate conjugated to BSA. Also, working dilutions of the primary antibodies were incubated individually with the above protein-ligand conjugates in solution overnight at 4°C before application to sections.

Results

Immunoblots indicated both purified antibodies had a high degree of specificity for the corresponding

protein coupled compounds. NAAG antibodies crossreacted approximately 1-2% with aspartylglutamate and NAA conjugates as indicated by similar staining intensity in spots with concentration differences of 50 to 100 times. Similarly, the purified NAA antibodies showed 1-2% crossreactivity with the BSA-NAAG conjugate. In blocking experiments, 10 jig ml"[1] of the NAA conjugate completely eliminated the immunoreactivity (IR) for NAA in tissue sections, while 50 jug ml"[1] for the other conjugates, failed to inhibit binding. The antibodies to NAAG were similarly inhibited by 10 jag ml"[1] of NAAG coupled to BSA, but were not blocked by 50 jig ml"[1] of the other conjugates.

NAA-IR was found in neurons in every major area of the rat brain. This staining was punctate, restricted to cell bodies and basal dendrites, and was excluded from cell nuclei. Cresyl violet counterstaining showed that many neurons throughout the brain were not immunoreactive for NAA. NAAG-IR was less widespread than that of NAA, particularly in forebrain, but was also punctate. Regions in which NAAG staining was low included neocortex, central thalamus, and striatum. NAA-IR was moderate to intense in each of these regions. *Neocortex.* In neocortex, neuronal NAA-IR was extensive (Fig. 1A), but there were also non-immunoreactive neurons in all layers of cortex. Pyramidal neurons in layer V ranged from highly immunoreactive to non-immunoreactive (Fig. 1C). In contrast, NAAG-IR was much more restricted in neocortex (Fig. IB). Scattered neurons were highly immunoreactive for NAAG, but they were only a small fraction of the total number of cortical neurons. While many pyramidal neurons were immunoreactive for NAA, the scattered NAAG-IR neurons in cortex appeared to be interneurons (based on size and morphology, Fig. ID).

Hippocampus. The staining patterns for NAA and NAAG in the hippocampus were distinct (Fig. 2A and 2B). NAA-IR was more ubiquitously distributed, with staining in most pyramidal cells, granule cells, and polymorphic cells. Pyramidal cell staining was most intense in the CA3 region (Fig. 2E). Granule cells displayed moderate NAA staining, and many of the polymorphic cells were also immunoreactive (Fig. 2C). The pattern of NAAG-IR was quite restricted (Fig. 2B), encompassing the majority of polymorphic cells (Fig. 2D), and small numbers of scattered neurons in the pyramidal cell layer of all regions (Fig. 2F). *Brainstem.* NAAG-IR and NAA-IR had similar

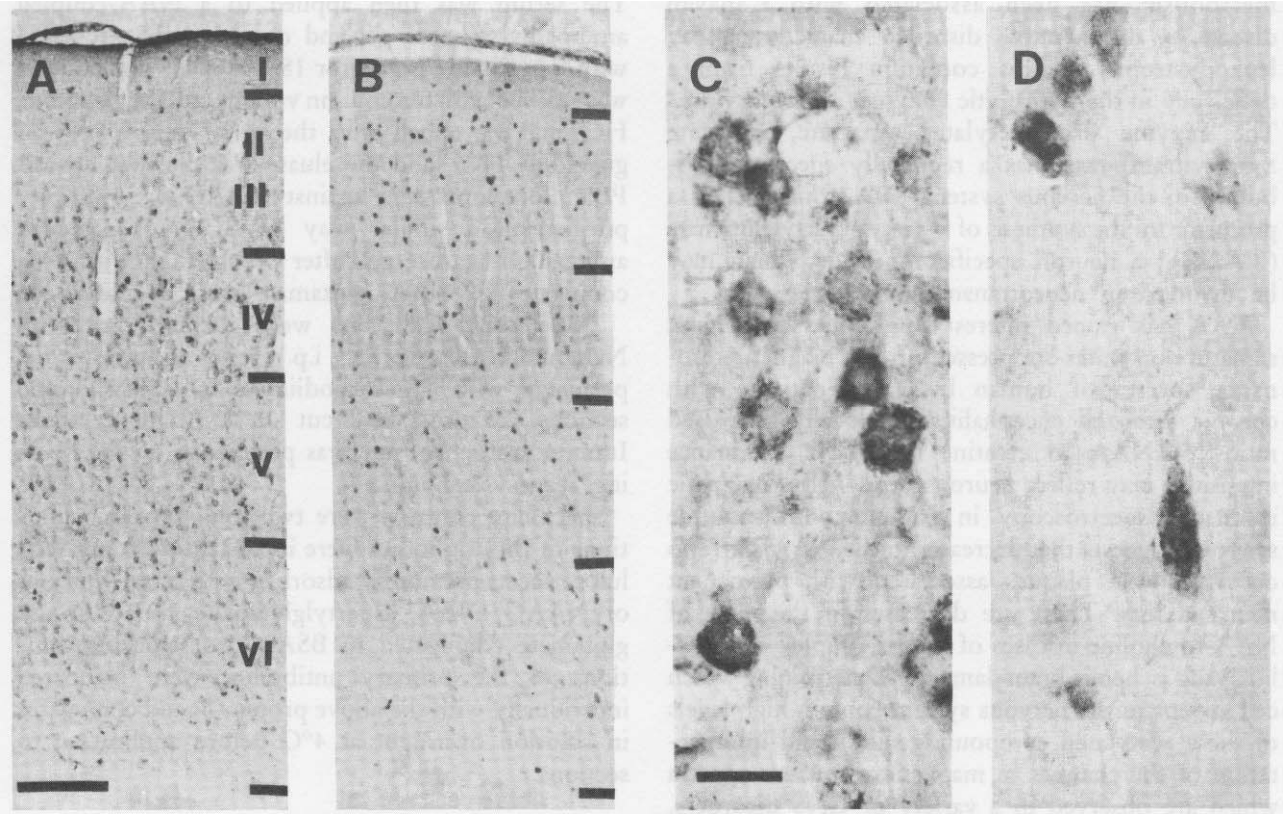

FIG. 1. The distributions of NAA (A) and NAAG (B) in temporal cortex. The distribution for NAAG was limited, being present in a relatively small number of neurons in layers 11—IN/ in neocortex. The distribution for NAA was much more extensive, being present in the majority of cells in layers II-VI. Many neurons did not stain, however, indicating that NAA is not a ubiquitous compound in neocortical neurons. While many pyramidal neurons, such as those of layer V, were labelled for NAA (C), most neurons in this layer did not stain for NAAG, and those that did appeared to be interneurons (D). [Bars = 250 \im A and B; 30|im C and D].

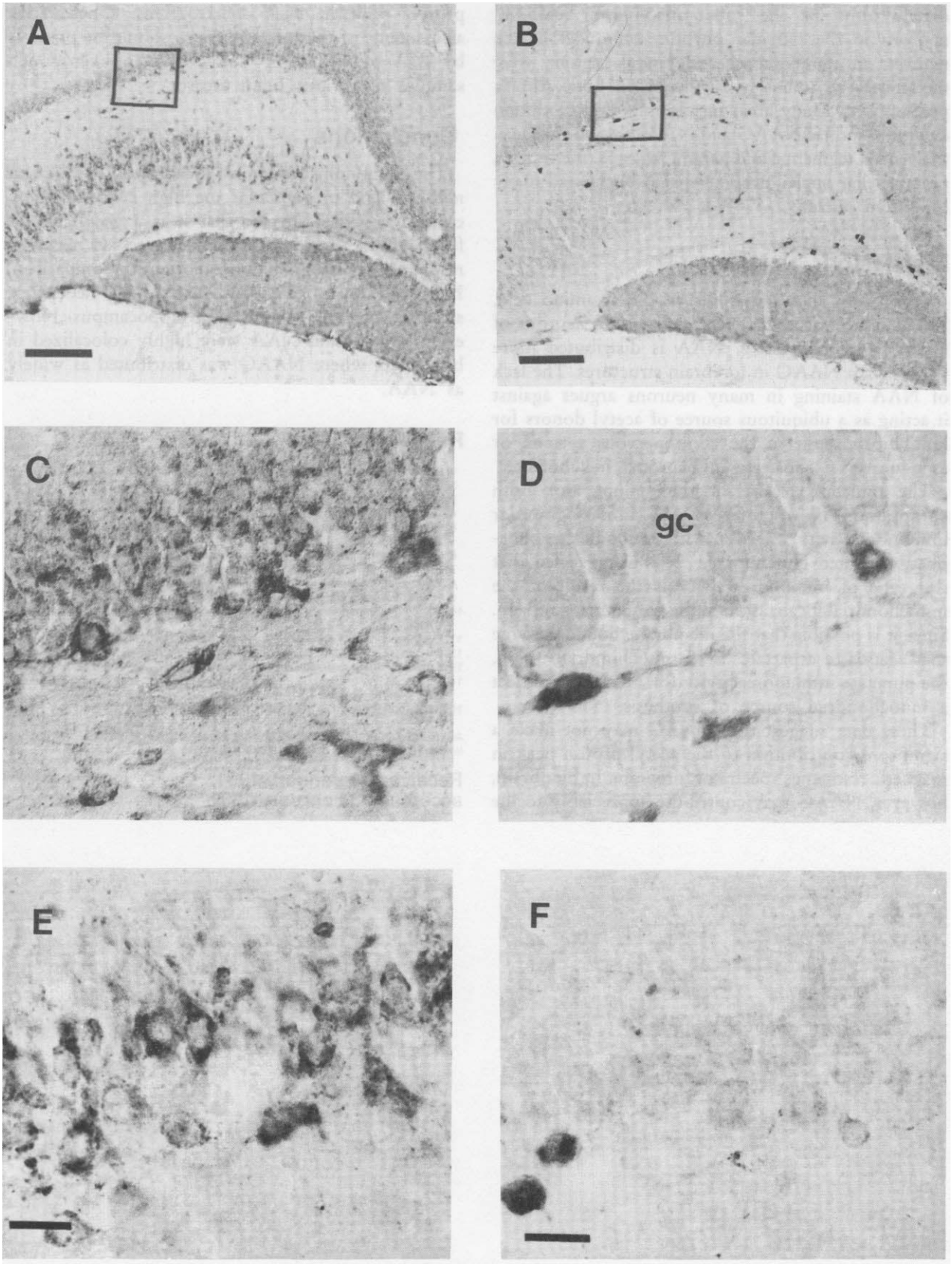

FIG. 2. The staining patterns in hippocampus for NAA (A) and NAAG (B) were quite distinct. All cell types were immunoreactive for NAA, including pyramidal cells, granule cells, and polymorphic cells (A). The granule cells displayed moderate punctate staining (C, differential interference contrast, area shown by box in A). The polymorphic cells were moderately to heavily stained for NAA (C). The highest level of NAA-IR in the pyramidal cell layer occurred in CA3 (E), and the lowest in CA1. The majority of neurons did not stain for NAAG in the hippocampus (B). However, many large neurons of the polymorphic layer were highly immunoreactive for NAAG (D, differential interference contrast, area shown by box in B). The granule cells (gc) did not stain for NAAG (D). Scattered neurons in the pyramidal cell layer were immunoreactive for NAAG, but these appeared to be interneurons (F). [Bars = 250 *im,* A and B; 30 jim, C, D, E and F].

distributions in the substantia nigra. Scattered neurons in the reticular portion, and most of the neurons in the compact and lateral regions were moderately to intensely labelled with both affinity preparations. Many areas in midbrain and hindbrain exhibited similar NAAG and NAA distributions, e.g. the ventral tegmental and paranigral areas, the tectum, precerebellar nuclei, deep cerebellar nuclei and many brainstem nuclei.

Discussion

This study shows that the modified amino acid, NAA, is specifically concentrated within neurons of the rat nervous system. NAA is distributed more widely than NAAG in forebrain structures. The lack of NAA staining in many neurons argues against it acting as a ubiquitous source of acetyl donors for lipid biosynthesis in the adult nervous system, or as a universal substrate in neuronal metabolism.

The punctate nature of the staining for both compounds is of interest. A similar granular immunoreactivity was seen with antibodies to phosphate activated glutaminase.[13] It was suggested that this type of immunoreactivity is the result of the mitochondrial localization of the glutaminase antigen. Since it is possible that the membrane bound enzyme that acetylates aspartate[5] is of mitochondrial origin, the punctate immunoreactivity for NAA may reflect a mitochondrial source of synthesis.

These data suggest that NAAG may not make a significant contribution to the acetyl proton peak in magnetic resonance spectra of forebrain. In hindbrain, however, NAAG may contribute appreciably to the proton peak in such spectrograms. Quantitative assessments of these distributions should be possible by RIA of NAAG[14, 15] and NAA in micropunch samples of various brain areas.

Conclusions

The immunohistochemical localization of NAA in neurons, and in particular the high levels in many cortical neurons, suggest that it is a good marker for neuronal viability in water-suppressed magnetic resonance spectrograms of forebrain. In comparison, NAAG-IR was relatively reduced in neocortex, striatum, central thalamus and hippocampus. However, NAAG and NAA were highly colocalized in brainstem, where NAAG was distributed as widely as NAA.

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