EXPRESSION OF *N*-ACETYLASPARTATE AND *N*-ACETYLASPARTYLGLUTAMATE IN THE NERVOUS SYSTEM

John R. Moffett and Aryan M. A. Namboodiri*

1. INTRODUCTION

N-Acetylaspartate (NAA) and *N*-acetylaspartylglutamate (NAAG) are highly concentrated acetylated compounds found predominantly in the nervous system of vertebrates and invertebrates.[1-3] Their high concentrations make them good candidates for localization by immunohistochemistry. The two compounds are assumed to be related to one another in terms of biosynthesis. NAA is thought to be a direct precursor for NAAG biosynthesis,[4] despite numerous failures in several laboratories to isolate or characterize a NAAG synthase enzyme capable of coupling NAA to glutamate. NAAG and NAA are found primarily in neurons,[5-11] although much lower levels may be present in some glial cells,[12,13] and in somatic tissues.[2] Because of their predominant neuronal localization, and the fact that the two molecules provide strong acetate signals in water-suppressed proton magnetic resonance spectra, reductions in their acetate signals have been used as a non-invasive diagnostic marker for neuronal loss or dysfunction.[14-17] The N-terminal acetyl groups of NAAG and NAA make their localization by immunohistochemistry problematic, because both molecules lack an amine group, and thus lack a reactive group that would permit standard fixation coupling with glutaraldehyde. This problem has been examined in detail previously.[10]

The only chemically reactive groups available on NAAG and NAA for coupling to proteins are carboxyl groups; NAAG has three carboxyl groups, and NAA has two. The most effective reagents for inducing peptide bond formation between carboxyl-containing compounds and proteins are known as carbodiimides. Historically, carbodiimides were first used for tissue fixation for immunohistochemistry in the early 1970's.[18,19] We have used the water-soluble carbodiimide, EDAC (1-ethyl-3 [3-dimethylaminopropyl] carbodiimide hydrochloride), for the immunohistochemical localization of NAAG and

* Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda MD, 20814, USA, email; jmoffett@usuhs.mil.

NAA. EDAC-based fixation for immunohistochemistry is complicated by the fact that this coupling reagent is water soluble, but does not penetrate lipid-rich tissues, such as white matter in brain. To overcome this problem, we found that 5% DMSO could be used in the fixative solution to increase penetration of EDAC into white matter and other lipid-rich tissues. DMSO was found to be critical for uniform labeling of NAAG and NAA in nervous system white matter, but was also found to destroy the internal ultrastructure of neurons, making its use unsuitable for electron microscopy. Other improvements to carbodiimide fixations for light microscopy were achieved by increasing the temperature of the fixative solution to 37^{o}C, and using the carbodiimide stabilizing agent; N-hydroxysuccinimide (1mM), to reduce non-productive hydrolysis of EDAC in solution.[10]

Another significant problem associated with small-molecule immunohistochemistry in general is cross-reactivity of the antibodies with non-specific epitopes in fixed tissue sections. The problems of cross-reactivity and high background staining in tissue sections were pronounced with affinity-purified NAAG and NAA antibodies. Solid phase immunoassays showed that affinity purified, polyclonal antibodies required an additional purification step to remove cross-reactive antibodies.[11] This was accomplished by the use of nitrocellulose-immobilized hapten-protein conjugates produced with EDAC. Structurally related compounds such as aspartate and aspartylglutamate were coupled to bovine serum albumin with EDAC, and then adsorbed to nitrocellulose strips. The nitrocellulose strips were then incubated first with the crude antisera to remove most of the cross-reactive antibodies. Then these partially purified anti-NAAG and NAA antibodies were affinity purified on a NAAG-coupled or NAA-coupled aminoalkyl-agarose gels respectively. Finally, the antibody solutions were incubated again with new nitrocellulose sheets containing related EDAC conjugates to eliminate remaining cross-reactivity. In the case of NAAG antibodies, NAA-BSA was the most cross-reactive conjugate, whereas in the case of NAA antibodies, NAAG-BSA was the most cross-reactive conjugate. This three-step purification process produced highly specific anti-NAAG and anti-NAA antibodies with less than 1% cross-reactivity to all related protein-hapten conjugates.

2. METHODS

Tissue fixation and immunohistochemistry protocols have been described in detail previously.[10,11,20] Briefly, carbodiimide fixations involved transcardial perfusion with 2 to 5 times the body volume of an aqueous solution of 6% EDAC and 5% DMSO containing 1mM N-hydroxysuccinimide. The fixative solutions were heated to 37^{o}C before initiating the perfusions, because the coupling reaction proceeds significantly faster at higher fixative temperatures. Post-fixations are done in 4% freshly depolymerized paraformaldehyde, or 10% neutral buffered formalin for 24 to 48 hours. For invertebrates or other animals that are difficult to perfuse transcardially, tissues were fixed by removal and rapid immersion in the standard EDAC fixative containing 5% DMSO and 1mM N-hydroxysuccinimide for 20 minutes, followed by further fixation in buffered formalin for at least 24 hours.

Immunohistochemistry was done by the avidin-biotin complex method (Vectastain Elite; Vector Labs, Burlingame, CA). Primary rabbit antibodies were diluted appropriately in 2% normal goat serum (NGS) and incubated with tissue sections for 48

to 72 hours at room temperature with constant rotary agitation (with 0.1% sodium azide as preservative). The secondary antibody and HRP-labeled avidin-biotin complex solutions were incubated with sections for 60-70 minutes each, and after washing were developed with a nickel and cobalt enhanced diaminobenzidine chromogen system (Pierce Biotechnology, Rockford, IL).

3. RESULTS

NAAG and NAA immunoreactivities (NAAG-IR and NAA-IR) have been found to be distinct in many areas of the CNS in the species studied to date. The rat has been the most thoroughly studied species in terms of the extent of the coverage of NAA and NAAG localization in various CNS regions. As compared with other species studied, the rat has relatively high levels of NAA (approximately 8mM) and relatively moderate levels of NAAG (approximately 0.75mM).[2] We consider the rat to be a good representative species for the description of the distributions of NAA and NAAG in the CNS, despite having lower levels than carnivores and primates. In the rat, the staining patterns for NAAG and NAA were very distinct in forebrain and cerebellar cortex, but were more similar in areas of the brainstem and spinal cord, as will be shown below. The greatest disparities in localization are observed in the cerebral and cerebellar cortices of the rat, where NAA is present in most neurons, but NAAG is only present in subpopulations of neurons. For a thorough examination of the disparities between NAAG and NAA expression, see Moffett and Namboodiri.[11]

3.1. Comparative NAA and NAAG Expression in Neocortex

The comparative distributions of NAA-IR and NAAG-IR in rat neocortex are shown in Figure 1. Both immunoreactivities appeared punctate, which may represent localization in intracellular organelles or vesicles. NAAG-IR was also observed in putative extracellular NAAG-positive synaptic contacts (see insert, Figure 6C). NAA-IR was not observed in synaptic-like extracellular puncta, and was more diffuse in the cytoplasm of neurons. However, NAA-IR was often observed in large, organelle-sized intracellular inclusions in certain types of neurons, including cortical pyramidal cells and principle neurons of the hippocampus (Figure 1F inserts). These could possibly be DMSO-based artifacts associated with the fusion of internal membrane structures or organelles that contained high concentrations of NAA (e.g., neuronal mitochondria).

NAAG-IR was most prevalent in apparent interneurons in all cortical layers, and in the rat, was not observed significantly in pyramidal cells (Figure 1A, C, E). This is in contrast with carnivores and primates, where both interneurons and pyramidal cells were strongly immunoreactive for NAAG (see Figures 6 and 7 below). NAAG staining was present in the proximal dendrites of immunoreactive cells in the rat, but was not seen in distal dendrites. This also contrasts with carnivores and primates, where cortical pyramidal cells were immunoreactive for NAAG throughout their dendritic arborizations. NAA-IR was present in most or all neurons in all layers of neocortex, and was also observed in the apical and basal dendrites of pyramidal neurons (Figures 1B, D, F).

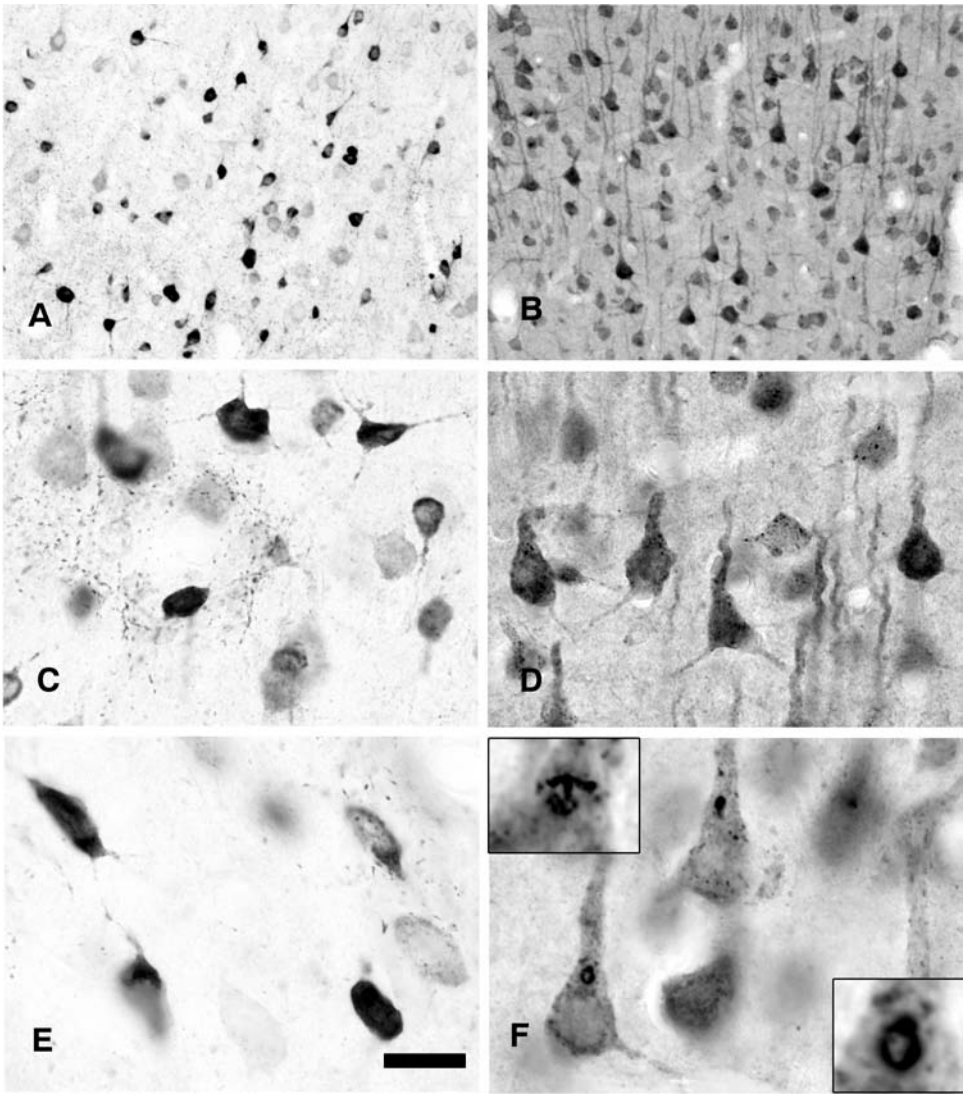

Figure 1. NAA and NAAG immunoreactivity in rat neocortex. NAAG-IR is relatively limited in rat neocortex, being present mostly in small interneurons (A), but NAA-IR is observed in most or all neurons, with high levels in cortical pyramidal cells (B). NAAG-IR is present in cell bodies, proximal dendrites, and probable synaptic contacts in the neuropil (C, E), whereas NAA-IR is present in cell bodies and throughout the dendritic arborizations of neurons (D). In cortical areas, many pyramidal neurons contained large NAA-IR inclusions, often located at the base of the apical dendrite (F). These ranged in shape from round to complex (inserts in F), and could represent DMSO-fused organelles, such as mitochondria, that contained high concentrations of NAA. Bar = 100μm A, B; 30μm C, D and 20μm E, F.

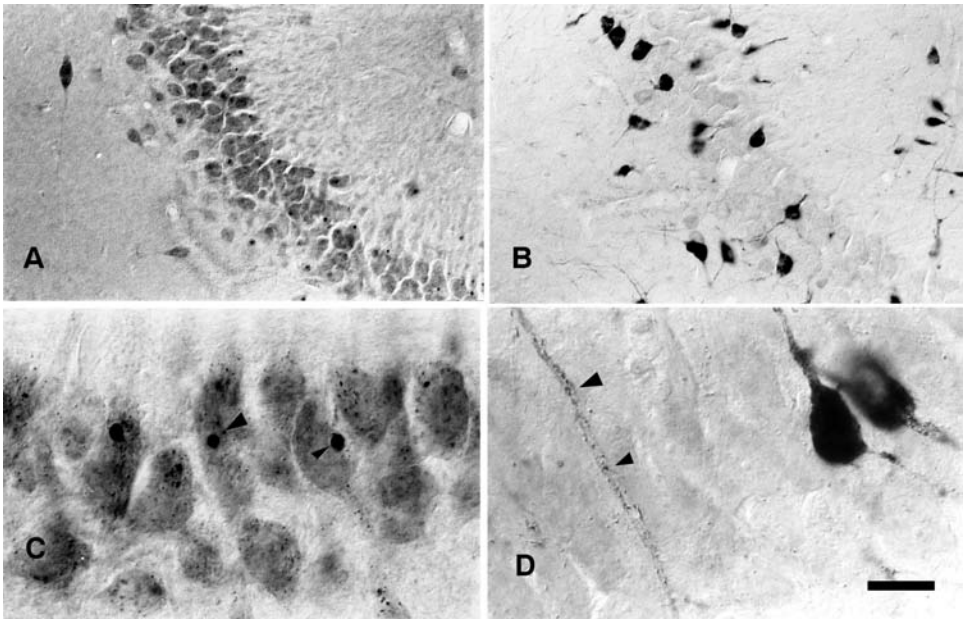

Figure 2. NAA-IR and NAAG-IR in rat hippocampus. NAA is present in most neurons in the rat hippocampus (A). Large intracellular NAA-stained elements were observed in many hippocampal pyramidal cells (arrowheads C). NAAG distribution is relatively restricted in the rat hippocampus, being expressed in scattered neurons in the pyramidal layer in all subdivisions (B), and in cells of the polymorph layer. Many unstained pyramidal cell dendritic shafts were covered with NAAG-stained puncta that appeared to be NAAG-containing synaptic contacts (arrowheads in D). Bar = 100μm A, B and 20μm C, D.

3.2. Comparative NAA and NAAG Expression in Hippocampus

NAA was much more widely distributed than NAAG in hippocampus, as was the case in neocortex (Figures 2A, B). NAAG staining was often opaque in the cytoplasm (Figure 2B), and punctate in processes such as dendrites and axons (Figure 2D, arrowheads). NAA appeared both diffusely in cytoplasm, and was also expressed in heavily-stained puncta within neurons (Figures 2A, C). Large, NAA-IR inclusions were also observed in many hippocampal pyramidal neurons (Figure 2C, arrowheads), as was the case in neocortical pyramidal neurons. NAAG-IR was restricted to scattered large neurons in the pyramidal cell layer, whereas NAA-IR was present in all pyramidal cells, and was moderate to strong in granule cells. No NAAG-IR was observed in the granule cell layers, but many cells in the polymorph layer expressed moderate to high levels of NAAG.

3.3. NAA, NAAG and GAD_{67} Expression in Forebrain and Midbrain

Figure 3 shows the expression of NAAG, NAA and glutatmic acid decarboxylase (GAD_{67}) in rat neocortex and hippocampus as compared with midbrain structures. NAAG was distributed differentially, with midbrain and hindbrain having substantially higher levels than telencephalic cortical structures (Figure 3A). NAA distribution was relatively ubiquitous, and similar levels were observed in forebrain and hindbrain (Figure 3B).

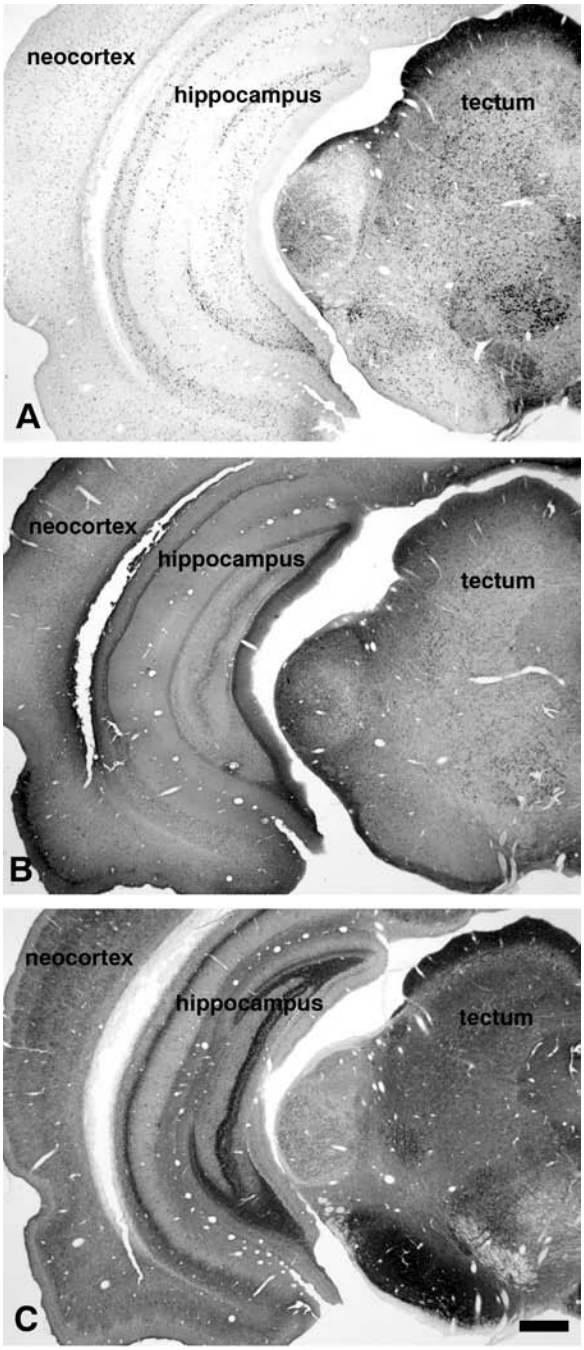

Figure 3. NAAG, NAA and GAD_{67} expression in rat forebrain and midbrain. NAAG expression is relatively low in neocortex and hippocampus as compared with the midbrain tectum, tegmentum and hindbrain (A). NAA immunoreactivity, in contrast, is expressed differentially in different neuronal groups, but in general, high levels are found throughout the CNS (B). GAD_{67} expression is extremely varied, as was the case with NAAG, with high levels in hippocampal granule cell and pyramidal cell layers, and very high expression in the reticular part of the substantia nigra and tectum (C). Bar = 400μm.

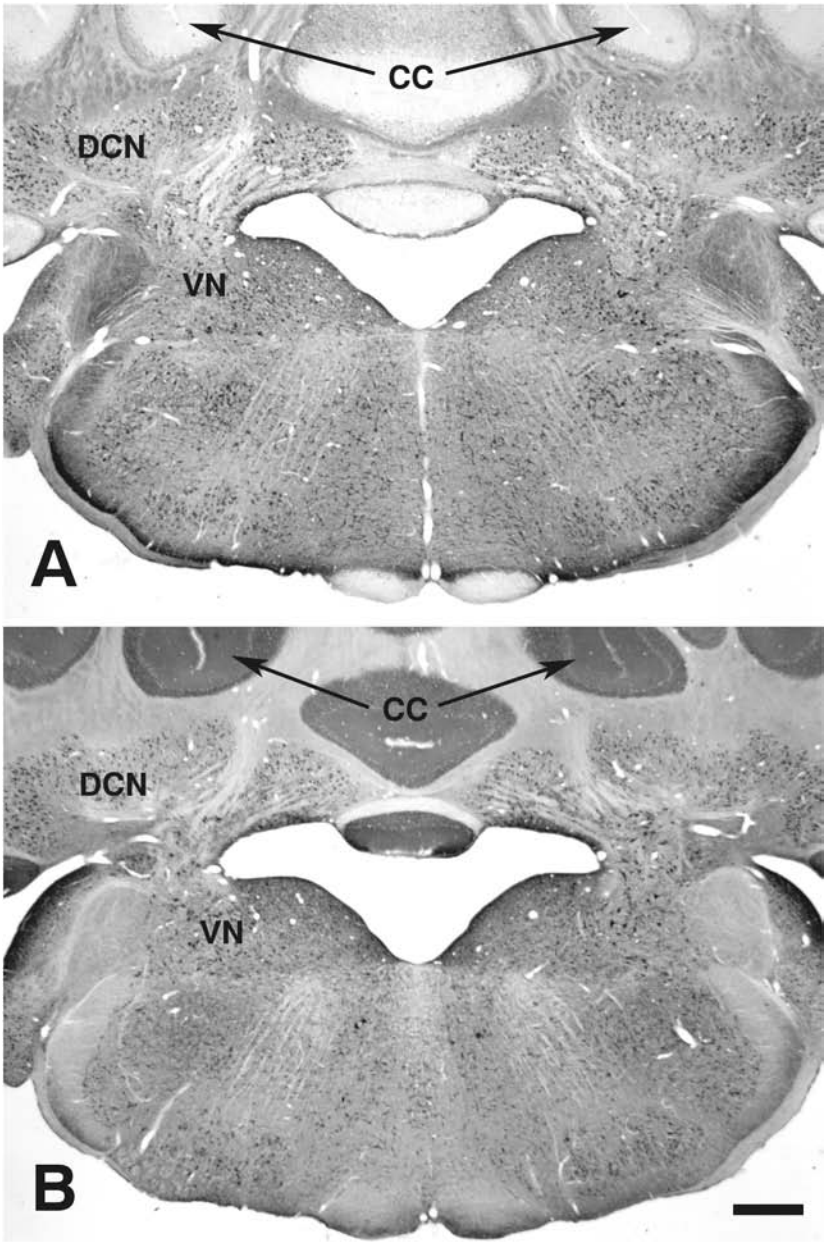

Figure 4. NAAG (A) and NAA (B) are both extensively distributed throughout the brainstem. NAAG expression is very high in the brainstem and deep cerebellar nuclei (DCN), but was much more limited in the cerebellar cortex (CC). In contrast, NAA expression was very high in the CC. Both NAAG and NAA were expressed in many cell groups throughout the brainstem, such as the vestibular nuclei (VN). Many NAAG and NAA-containing axons are present throughout the cerebellar white matter. Bar = 400μm.

GAD_{67} expression was somewhat higher in many midbrain structures than in most cortical layers, but the increase along the rostro-caudal axis of the CNS was not as apparent as was the case with NAAG expression (Figure 3C).

3.4. NAA and NAAG Expression in the Cerebellum and Brainstem

NAAG and NAA were both extensively distributed throughout the cerebellum, brainstem and spinal cord (Figure 4). NAAG levels were low in cerebellar cortex, but were very high in the deep cerebellar nuclei and medulla (Figure 4A). NAA levels were high throughout cerebellar cortex, deep nuclei and medulla (Figure 4B).

3.5. NAA, NAAG and Aspartoacylase Expression in Corpus Callosum

Aspartoacylase is an enzyme that specifically de-acetylates NAA, and which was observed almost exclusively in oligodendrocytes (Figure 5A). In contrast, NAA and NAAG were expressed primarily in neurons (Figures 5B, C). Aspartoacylase-stained cells were arranged in characteristic rows between axon bundles in the corpus callosum. Low levels of NAA can also be seen in rows of oligodendrocytes. The level of NAA-IR in adult rat oligodendrocytes is substantially lower than the level in neurons. NAAG is present in scattered axons in the corpus callosum, whereas NAA is present in most or all axons in the corpus callosum, although at only a moderate level. It remains to be determined if the NAA present in oligodendrocytes is synthesized within the glial cells themselves, or if it is made in neurons, and passed from axon to glia.

3.6. NAAG Expression in Rhesus Monkey Motor Cortex

NAAG expression in Rhesus monkey motor cortex was more extensive than observed in the rat (Figure 6A). In particular, most cortical pyramidal cells in the Rhesus monkey were moderately to strongly stained, whereas in the rat, these cells were either unstained, or faintly stained.[11] NAAG-containing small neurons were relatively numerous in layer III of motor cortex, and the dendritic shafts of motor neurons could be seen coursing through this layer (Figure 6B). Medium and large motor neurons in layer V were moderately to strongly stained for NAAG (Figure 6 C). Many dendritic and axonal elements were immunoreactive for NAAG in layer V, including probable NAAG-containing synaptic contacts on apical motor neuron dendrites. Numerous NAAG-IR neurons and fibers were observed in layer 6b of motor cortex, including axons entering the corpus callosum (Figure 6D).

3.7. NAAG and GAD_{67} Expression in Rhesus Monkey Motor Cortex

The distribution of small neurons stained for NAAG in neocortex was very similar to the distribution of GAD-positive interneurons (Figure 7). However, double labeling experiments showed that fewer than 50% of the NAAG-positive neurons in monkey

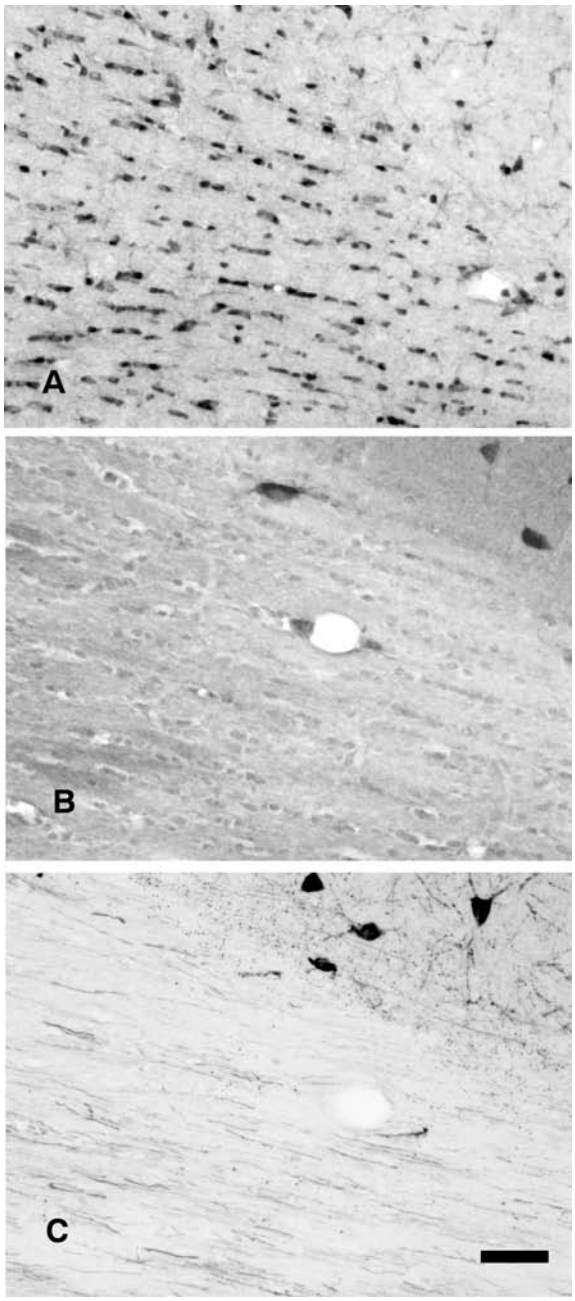

Figure 5. ASPA (A), NAA (B) and NAAG (C) in rat corpus callosum. Aspartoacylase (ASPA) is the only enzyme known in the brain that acts to deacetylate NAA. ASPA expression was predominantly observed in oligodendrocytes in white matter such as corpus callosum (A), as well as throughout the brain and spinal cord. NAA expression in oligodendrocytes was very low, possibly because it is broken down rapidly by ASPA (B). NAAG expression was relatively low in the corpus callosum of the rat, being present in a relatively small number of axons (C). In the cortical white matter from monkey, far more NAAG containing axons were observed, reflecting the greater expression of NAAG in pyramidal cells. Bar = 100μm.

neocortex were double-labeled for GAD_{67} (data not shown), suggesting that many small, NAAG-expressing neurons in cortex may be peptidergic rather than GABAergic.

3.8. NAAG and NAA in GABAergic Neuronal Groups

Unlike the partial colocalization of NAAG and GAD_{67} in neocortex, NAAG was expressed in virtually all GABAergic neurons in GABA projection areas such as the globus pallidus, thalamic reticular nucleus and lateral hypothalamus (Figure 8A, C, E). NAA-IR was also expressed at moderate to high levels in these cell groups (Figure 8B, D, F). One feature in common among many GABAergic projection systems containing NAAG was that NAAG was typically observed only in the cell body and basal dendrites, but not in the axons. This is in contrast to many glutamatergic projection systems containing NAAG, such as the visual projections, where NAAG is present throughout the axons and terminals.

3.9. NAAG in Groups with Known Neurotransmitters

NAAG is strongly expressed in several neuronal groups known to utilize specific neurotransmitters. Examples include the vertical limb of the diagonal band of Broca, which is known to use acetylcholine as the primary neurotransmitter (Figure 9A). The locus coeruleus is a noradrenergic nucleus in the brainstem which is strongly immunoreactive for NAAG (Figure 9B), and the substantia niagra pars compacta is a major dopaminergic nucleus in the midbrain which expresses high levels of NAAG-IR (Figure 9C). NAAG is also present in serotoninergic neurons of the raphe (Figure 9D).

3.10. Phylogeny

NAAG and NAA appear to be phylogenetically ancient molecules, being found in the nervous system of invertebrates,[3,21] and teleost vertebrates.[2] No data are available on elasmobranch vertebrates, but both compounds have been found in the brains of amphibians and reptiles. NAA and NAAG, in general, exhibit a concentration ratio in birds and mammals of approximately 10:1 respectively.[2] This ratio is reversed in amphibians such as the frog, where the ratio is approximately 1:10. In teleosts, such as the goldfish, the ratio is 100:1, with only trace amounts of NAAG [2] and only scattered NAAG-IR neurons present in the brain (Figure 10B, D and F). In contrast, NAA-IR was observed in most neurons throughout the goldfish brain (Figure 10A, C, and E).

4. DISCUSSION

NAAG and NAA have different distributions in the nervous system, and this disparity is most apparent in cortical areas of the CNS. NAA has a ubiquitous presence in most neurons in the nervous system, and yet NAA levels in different cell groups can vary

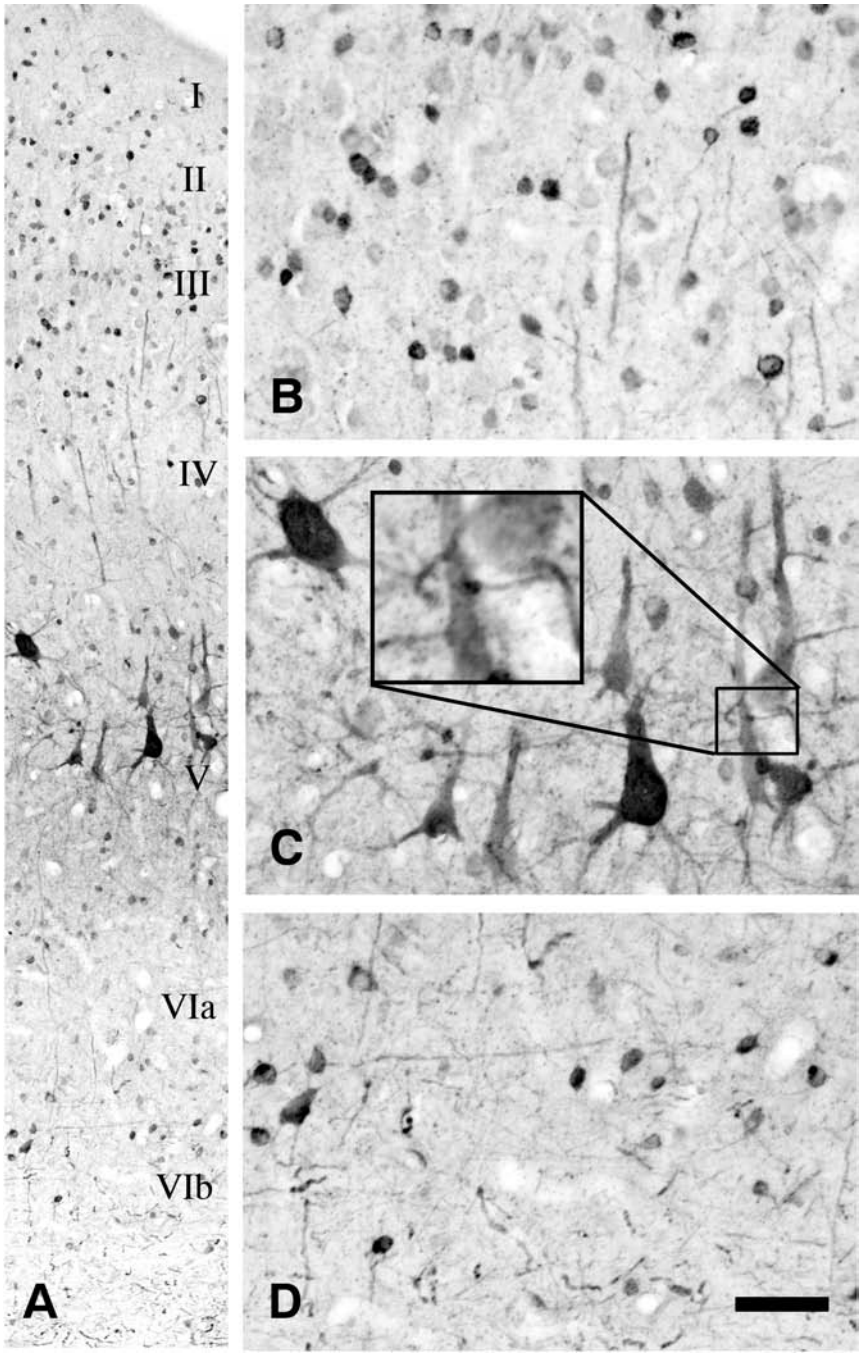

Figure 6. NAAG expression in rhesus monkey motor cortex. NAAG is expressed in all layers of neocortex in the monkey (A). In superficial cortical layers, NAAG was expressed in many small neurons, and in the apical dendrites of pyramidal cells (B). Unlike the rat, NAAG expression was highest in layer V, where it is present in pyramidal cells, and a dense plexus of fibers and synapses in the neuropil (C). In deeper layers, many small neurons expressed high levels of NAAG, as did axons entering the white matter and corpus callosum (D). Bar = 200μm A; 60μm C, D and E.

substantially (Figures 1B, 4B, 9B, D and F). NAAG has a more restricted distribution, and exhibits an increasing concentration gradient from the rostral to the caudal CNS (see Fig 3). NAAG-IR *in vivo* is only expressed in neurons, but it has been reported that oligodendrocytes and microglia can express low levels of NAAG *in vitro*.[12] In addition to being localized in most neurons, NAA was observed at low levels in oligodendrocytes (Figure 5B). Some brain capillary endothelial cells were also stained moderately to strongly for NAA, but only a small percentage of the total endothelial population of the CNS was NAA-positive (data not shown).

NAA has been shown to have a more ubiquitous distribution in the CNS as compared with NAAG, but both compounds are present in most or all regions of the brain and spinal cord of the rat.[8,11,20] Several broad generalizations can be made concerning their comparative localization, to which there are exceptions. First, both compounds are expressed at high levels primarily in neurons, although NAA is found in other cell types in the brain. Astrocytes and microglia did not stain for either compound in any species examined. NAA-IR is present in most neurons to a greater or lesser degree, and is particularly prominent in cortical pyramidal cells, and in granule cell layers in many brain regions, including hippocampus, retrosplenial cortex and cerebellar cortex. The distribution in NAAG in forebrain is also widespread, but only a relatively small percentage of neurons are NAAG-positive in many areas of the telencephalon. While cortical areas have relatively low numbers of NAAG immunoreactive neurons, NAAG is expressed in the majority of neurons in many brainstem and spinal cord regions. For example, virtually all the neurons of the deep cerebellar nuclei are strongly stained for NAAG, whereas a much lower density of stained neurons is seen throughout neocortex.

NAAG is colocalized extensively with GABA in GABAergic projection systems such as the globus pallidus, lateral hypothalamus, thalamic reticular nucleus (Figure 8) and the reticular region of the substantia nigra, but it is only partially colocalized with GABAergic interneurons in cortical brain areas. NAAG is known to be colocalized with other neurotransmitters, including acetylcholine, dopamine, norepinephrine and serotonin (see Figure 9). NAAG is also localized extensively in the retinal projections and retinorecipient terminals areas, which are known to be glutamatergic, perhaps to provide a means of preventing excitotoxicity associated with prolonged glutamate release.[22]

4.1. Comparative Phylogenetic Distribution

Miyake and colleagues were the first to show the concentrations of NAA and NAAG in various classes of vertebrates ranging from fish to mammals.[2] They demonstrated that teleost fish have very low levels of NAAG, but that amphibians, reptiles and mammals have relatively high NAAG levels in the CNS. Birds were found to have intermediate concentrations of NAAG in the brain. NAAG has also been reported in the nerve cord of a crustacean,[23] suggesting that it is present in the nervous system of most or all animals. Our immunohistochemical studies have confirmed that there are very few NAAG-positive neurons in the brains of teleosts (Figure 10), whereas birds have moderate numbers of NAAG positive neurons.[24] Among mammals, rats have lower levels of NAAG, and fewer NAAG-positive neurons (Figure 1) than primates (Figures 6 and 7). In

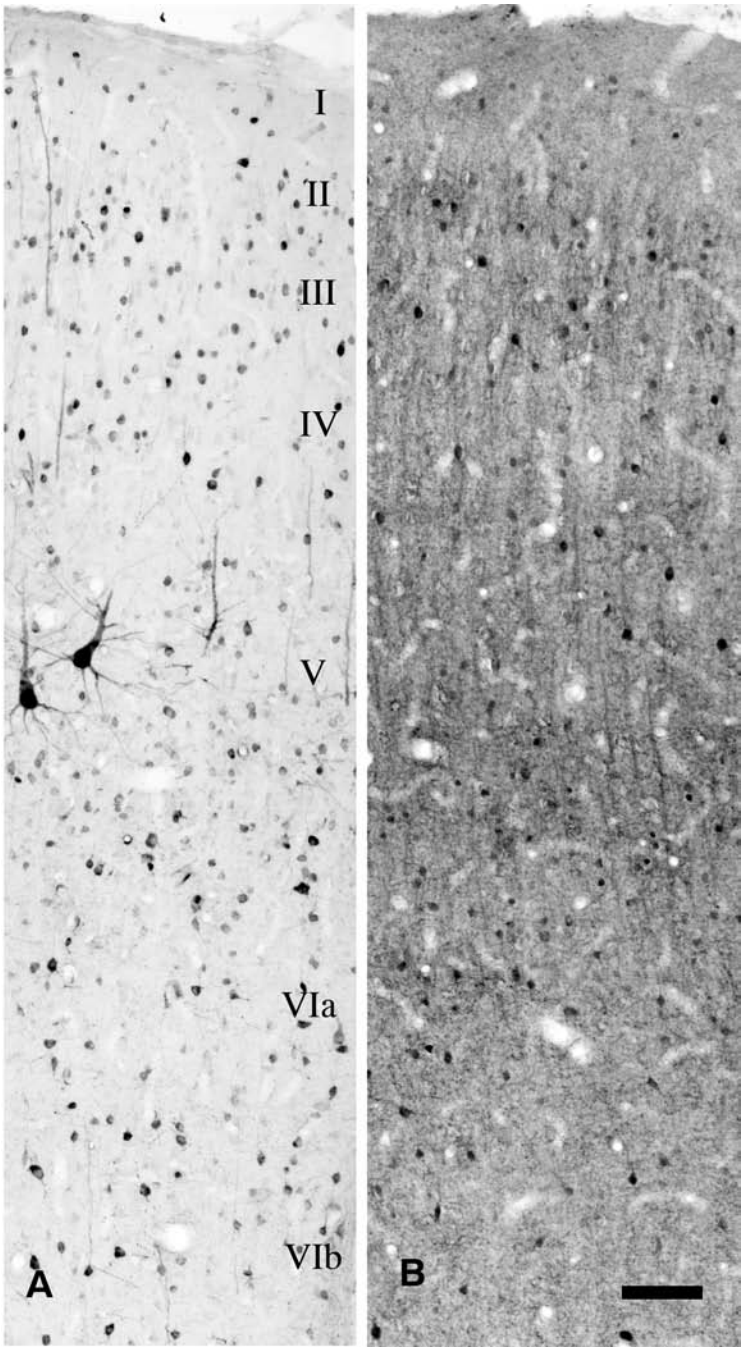

Figure 7. NAAG (A) and GAD_{67} (B) in rhesus monkey cortex. NAAG and the 67 kilodalton form of glutatmic acid decarboxylase (GAD_{67}; the enzyme that synthesizes GABA) were present in small neurons throughout all layers of monkey neocortex (A, B). Bar = 200μm.

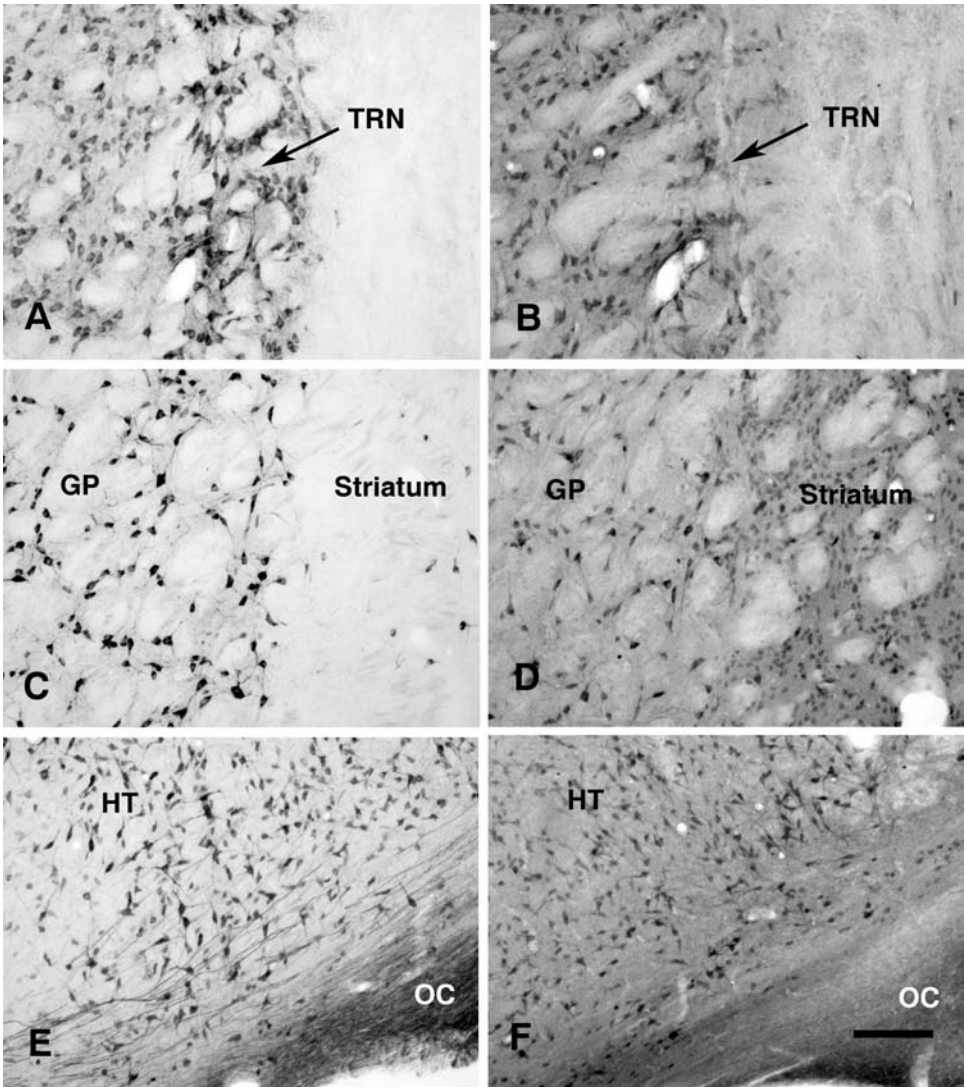

Figure 8. NAAG (A, C and E) and NAA (B, D and F) in GABAergic neuronal groups. NAAG and NAA are both expressed at relatively high levels in GABAergic projection groups, including the thalamic reticular nucleus (TRN), globus pallidus (GP) and the lateral hypothalamus (HT). Bar = 150μm.

contrast, NAA seems to be present in high concentrations in the CNS of most species of animals (with the notable exception of frogs), including invertebrates.

The expression level of NAAG in teleost cortex is extremely limited, with only scattered neurons stained for NAAG (Figure 10B). Substantially higher expression levels are seen in reptiles [25] and birds [24]. Among the mammalian species studied to date, the rat has the lowest levels of NAAG in cortical areas, whereas carnivores (cat) and primates (rhesus monkey) have higher levels. In the rat, very few cortical pyramidal cells

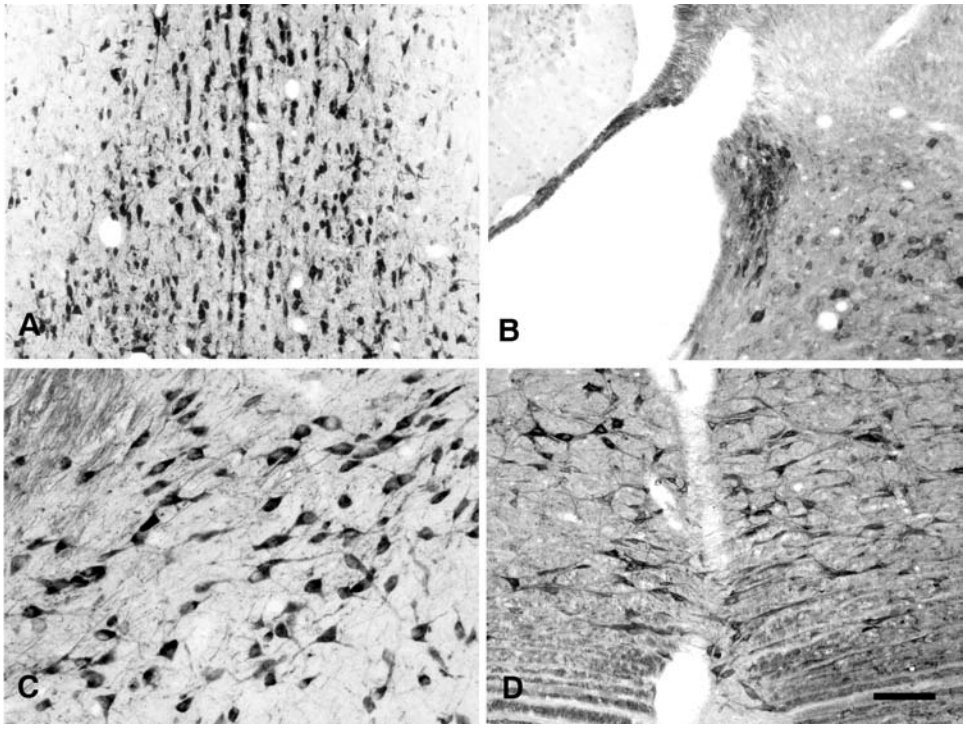

Figure 9. NAAG in groups with known neurotransmitters. NAAG is colocalized with virtually all major neurotransmitters including GABA (Figure 8), acetylcholine in the vertical limb of the diagonal band of Broca (A), norepinephrine in the locus coeruleus (B), dopamine in the compact part of the substantia nigra (C) and serotonin in the raphe (D). Bar = 100 μm.

contain NAAG, but in cat[26,27] and monkey, most layer V pyramidal cells express high levels of NAAG (see Figures 6 and 7).

4.2. Conclusions

Based on their cellular localization and known actions, several conclusions can be drawn concerning the roles played by these two compounds in brain function. In general, the distribution of NAA is consistent with it having a metabolic or housekeeping role in the nervous system, whereas the distribution of NAAG is more consistent with a role in neurotransmitter release modulation.

Because NAA is present at high concentrations in most neurons, and because it is not released from neurons in a calcium dependent manner after depolarization, it can not be acting as a classical neurotransmitter. The high concentration, lack of electrophysiological actions, and ubiquitous distribution all argue in favor of a metabolic role. These facts have led some researchers to propose that NAA acts to counter the "anion deficit" in neurons, [28] or acts as a "molecular water pump" to extrude metabolic water

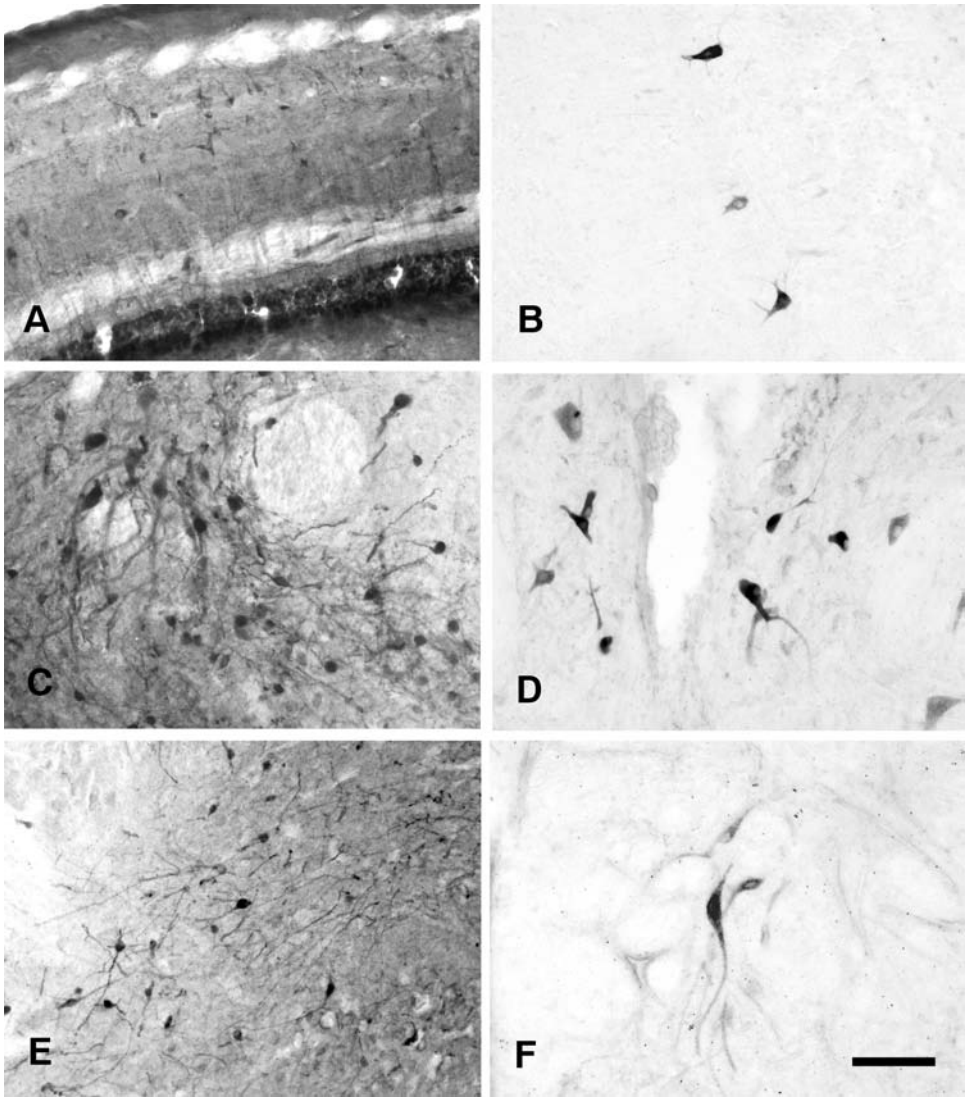

Figure 10. NAA (A, C and E) and NAAG (B, D and F) in goldfish brain. Both NAA and NAAG are phylogenetically conserved brain molecules found in the brains of teleost fish. NAA is expressed strongly throughout the goldfish brain, including cortex (A), hypothalamus (B) and hindbrain (C). NAAG, in contrast, has a very limited expression, with only scattered neurons being strongly stained in cortex (B, rotated 90 degrees compared with A), hypothalamus (D) and hindbrain (F). Bar = 100 μm

from neurons.[29,30] Recent findings clearly demonstrate that NAA provides a significant source of acetate for myelin lipid synthesis during CNS development,[31] and that NAA possibly plays a significant role in neuronal energy metabolism.[32]

The evidence for NAAG acting as a neurotransmitter or neuromodulator is convincing. NAAG meets all the criteria of a classical neurotransmitter except for one. It

is released in a calcium dependent manner after neuronal depolarization, it is broken down extracellularly by a specific enzyme (carboxypeptidase II) and it is known to act at postsynaptic NMDA receptors at high concentrations. However, NAAG application fails the test of eliciting the same postsynaptic actions as are elicited by stimulation of the afferent fibers to various brain regions receiving NAAG input (e.g., the lateral geniculate). Indeed, NAAG application often results in mixed actions on postsynaptic neurons. Electrophysiological studies have shown that different neurons in various brain regions could be depolarized, hyperpolarized, or not respond at all to exogenous NAAG application.[33,34]

The most compelling reason to think that NAAG is a peptide involved in neurotransmitter release modulation, rather than being a classical neurotransmitter, is that it is extensively colocalized with every major neurotransmitter known. Further, the specific and potent action at certain metabotropic glutamate receptors ($mGluR_3$) which are linked to neurotransmitter release modulation[35,36] argues strongly in favor of a modulatory role for NAAG. Finally, the extensive colocalization with both glutamatergic and GABAergic systems implicates NAAG in the regulation of the balance between excitatory and inhibitory neurotransmission in the nervous system.

5. ACKNOWLEDGEMENTS:

This work supported by the Samueli Institute for Information Biology, and by grants from the NIH (ROI, NS39387) and the National Eye Institute (EY 09085).

6. QUESTION AND ANSWER SESSION

PARTICIPANT: I was curious about your data with the monkey cortex.

DR. MOFFETT: Yes.

PARTICIPANT: Are you not finding NAAG in pyramidal neurons?

DR. MOFFETT: Yes, we do in the monkey. Those are all pyramidal cells in layer 5, these large ones. It was only in rat cortex where we did not see NAAG in pyramidal cells.

PARTICIPANT: You showed a monkey cortex, where you showed the comparison to GAD_{67}.

DR. MOFFETT: Yes. Here are some layer V pyramidal cells. And these pyramidal cells were chopped off, and are not visible in this particular 20-micron-thick section, but you can still clearly see that their apical dendrites are heavily labeled. So that's just a particular place where we only picked up two in that spot.

PARTICIPANT: You see it in pyramidal neurons in other layers, also?

DR. MOFFETT: Yes.

SESSION CO-CHAIR COYLE: That raises an interesting question in terms of animal models. We may potentially get misled about the salience of NAAG in cognitive processing because of the under representation of NAAG in mouse/rodent pyramidal neurons.

DR. MOFFETT: Yes. But in terms of non-cortical NAAG expression, the rodent brain is very similar to the situation in carnivores and primates. Even in rat cortex, NAAG immunoreactivity in non-pyramidal neurons is virtually identical to that in primates.

The one other question - we can't really answer too many functional questions with structural studies like this - but the one question that keeps coming back to me is that we have extremely high levels of NAA in virtually all neurons, especially in pyramidal neurons, but there is no deacetylase known that can metabolize NAA in neurons. So that's a question we still have to figure out. Why is there so much NAA in a cell type that cannot further metabolize it?

SESSION CO-CHAIR COYLE: I think you find that in terms of GABAergic neurons in particular, when you raise the question of what is colocalization all about; GABA is certainly an inhibitory neurotransmitter, plus NAAG activating mGluR3; Barbara Wroblewska has shown that, which further down-regulates excitatory neurotransmission, so that colocalization is coherent.

DR. MOFFETT: Yes.

Dr. WEINBERGER: It's also even beyond that level of coherence because mGluR3 is a heteroreceptor that is also found on all of these other neurotransmitter terminals. So there may actually be a local circuit of NAAG regulation of mGluR3 acting at various heteroreceptor terminals.

DR. MOFFETT: I did not focus on it, but you can quite clearly see examples of synaptic-like contacts on dendrites, where there are no obvious axons coming in. And to me, this suggests that these might be presynaptic on a dendrite. In other words, NAAG is being secreted from presynaptic sites on dendrites, which would also be unusual.

SESSION CO-CHAIR BURLINA: Other questions?

PARTICIPANT: Is there anything known about the localization of NALADase?

DR. MOFFETT: Primarily astrocyte, isn't it?

SESSION CO-CHAIR COYLE: Yes, it's in astrocytes, but it looks like it's on the end feet. When you make synaptosomal preparations you get NALADase is enriched in the synaptosomes, which could mislead you to think it is in nerve terminals, but those preparations also contain astrocyte end feet.

DR. MOFFETT: End feet, yes.

SESSION CO-CHAIR COYLE: Thank you very much.

DR. MOFFETT: Thank you.

7. REFERENCES

1. E. Miyamoto, Y. Kakimoto and I. Sano, Identification of N-acetylaspartylglutamic acid in the bovine brain. *J. Neurochem.*, **13**, 999-1003 (1966).
2. M. Miyake, Y. Kakimoto and M. Sorimachi, A gas chromatographic method for the determination of N-acetyl-L-aspartic acid, N-acetyl-aspartylglutamic acid and beta-citryl-L-glutamic acid and their distributions in the brain and other organs of various species of animals. *J. Neurochem.*, **36**, 804-810 (1981).
3. A. K. Urazaev, R. M. Grossfeld, P. L. Fletcher, H. Speno, B. S. Gafurov, J. G. Buttram and E. M. Lieberman, Synthesis and release of N-acetylaspartylglutamate (NAAG) by crayfish nerve fibers: implications for axon-glia signaling. *Neurosci.*, **106** (1), 237-247 (2001).
4. L. M. Gehl, O. H. Saab, T. Bzdega, B. Wroblewska and J. H. Neale, Biosynthesis of NAAG by an enzyme-mediated process in rat central nervous system neurons and glia. *J. Neurochem.*, **90** (4), 989-997 (2004).
5. C. B. Cangro, M. A. Namboodiri, L. A. Sklar, A. Corigliano-Murphy and J. H. Neale, Immunohistochemistry and biosynthesis of N-acetylaspartylglutamate in spinal sensory ganglia. *J. Neurochem.*, **49**, 1579-1588 (1987).

6. K. J. Anderson, M. A. Borja, C. W. Cotman, J. R. Moffett, M. A. Namboodiri and J. H. Neale, N-acetylaspartylglutamate identified in the rat retinal ganglion cells and their projections in the brain. *Brain Res.*, **411**, 172-177 (1987).
7. S. B. Tieman, K. Butler and J. H. Neale, N-acetylaspartylglutamate. A neuropeptide in the human visual system. *JAMA*, **259**, 2020 (1988).
8. J. R. Moffett, M. A. Namboodiri, C. B. Cangro and J. H. Neale, Immunohistochemical localization of N-acetylaspartate in rat brain. *Neuroreport*, **2**, 131-134 (1991).
9. S. B. Tieman, J. H. Neale and D. G. Tieman, N-acetylaspartylglutamate immunoreactivity in neurons of the monkey's visual pathway. *J. Comp. Neurol.*, **313**, 45-64 (1991).
10. J. R. Moffett, M. A. Namboodiri and J. H. Neale, Enhanced carbodiimide fixation for immunohistochemistry: Application to the comparative distributions of N-acetylaspartylglutamate and N-acetylaspartate immunoreactivities in rat brain. *J. Histochem. Cytochem.*, **41**, 559-570 (1993).
11. J. R. Moffett, M. A. Namboodiri, Differential distribution of N-acetylaspartylglutamate and N-acetylaspartate immunoreactivities in rat forebrain. *J. Neurocytol.*, **24**, 409-433 (1995).
12. L. Passani, S. Elkabes and J. T. Coyle, Evidence for the presence of N-acetylaspartylglutamate in cultured oligodendrocytes and LPS activated microglia. *Brain Res.*, **794** (1), 143-145 (1998).
13. A. K. Urazaev, J. G. Buttram, Jr., J. P. Deen, B. S. Gafurov, B. S. Slusher, R. M. Grossfeld and E. M. Lieberman, Mechanisms for clearance of released N-acetylaspartylglutamate in crayfish nerve fibers: implications for axon-glia signaling. *Neurosci.*, **107** (4), 697-703 (2001).
14. R. Burri, P. Bigler, P. Straehl, S. Posse, J. P. Colombo and N. Herschkowitz, Brain development: 1H magnetic resonance spectroscopy of rat brain extracts compared with chromatographic methods. *Neurochem. Res.*, **15**, 1009-1016 (1990).
15. S. H. Graham, D. J. Meyerhoff, L. Bayne, F. R. Sharp and M. W. Weiner, Magnetic resonance spectroscopy of N-acetylaspartate in hypoxic- ischemic encephalopathy. *Ann. Neurol.*, **35**, 490-494 (1994).
16. P. J. Pouwels, J. Frahm, Regional metabolite concentrations in human brain as determined by quantitative localized proton MRS. *Magn Reson. Med.*, **39** (1), 53-60 (1998).
17. S. M. Leary, C. A. Davie, G. J. Parker, V. L. Stevenson, L. Wang, G. J. Barker, D. H. Miller and A. J. Thompson, 1H magnetic resonance spectroscopy of normal appearing white matter in primary progressive multiple sclerosis. *J. Neurol.*, **246** (11), 1023-1026 (1999).
18. P. A. Kendall, J. M. Polak and A. G. Pearse, Carbodiimide fixation for immunohistochemistry: observations on the fixation of polypeptide hormones. *Experientia*, **27**, 1104-1106 (1971).
19. J. M. Polak, P. A. Kendall, C. M. Heath and A. G. Pearse, Carbodiimide fixation for electron microscopy and immunoelectron cytochemistry. *Experientia*, **28**, 368-370 (1972).
20. J. R. Moffett, M. Palkovits, M. A. Namboodiri and J. H. Neale, Comparative distribution of N-acetylaspartylglutamate and GAD_{67} immunoreactivities in the cerebellum and precerebellar nuclei of the rat utilizing enhanced carbodiimide fixation and immunohistochemistry. *J. Comp. Neurol.*, **347** (4), 598-618 (1994).
21. Tsukada Y, Uemura K, Hirano S and Nagata Y. Distribution of amino acids in different species. In: Richter D, ed. *Comparative Neurochemistry*. (Pergamon Press, Oxford, 1964), 79-183.
22. J. R. Moffett, Reductions in N-acetylaspartylglutamate and the 67 kDa form of glutamic acid decarboxylase immunoreactivities in the visual system of albino and pigmented rats after optic nerve transections. *J. Comp Neurol.*, **458** (3), 221-239 (2003).
23. B. Gafurov, A. K. Urazaev, R. M. Grossfeld and E. M. Lieberman, N-Acetylaspartylglutamate (NAAG) is the probable mediator of axon-to- glia signaling in the crayfish medial giant nerve fiber. *Neurosci.*, **106** (1), 227-235 (2001).
24. L. C. Williamson, D. A. Eagles, M. J. Brady, J. R. Moffett, M. A. Namboodiri and J. H. Neale, Localization and synaptic release of N-acetylaspartylglutamate in the chick retina and optic tectum. *Eur. J. Neurosci.*, **3**, 441-451 (1991).
25. M. M. Kowalski, M. Cassidy, M. A. Namboodiri and J. H. Neale, Cellular localization of N-acetylaspartylglutamate in amphibian retina and spinal sensory ganglia. *Brain Res.*, **406**, 397-401 (1987).
26. S. B. Tieman, C. B. Cangro and J. H. Neale, N-acetylaspartylglutamate immunoreactivity in neurons of the cat's visual system. *Brain Res.*, **420**, 188-193 (1987).
27. S. B. Tieman, J. R. Moffett and S. M. Irtenkauf, Effect of eye removal on N-acetylaspartylglutamate immunoreactivity in retinal targets of the cat. *Brain Res.*, **562**, 318-322 (1991).
28. Cooper JR, Bloom FE and Roth RH. Metabolism in the central nervous system. *The Biochemical Basis of Neuropharmacology*. (Oxford University Press, New York, 1991) **,** 75-87, .
29. M. H. Baslow, Evidence supporting a role for N-acetyl-L-aspartate as a molecular water pump in myelinated neurons in the central nervous system. An analytical review. *Neurochem. Int.*, **40** (4), 295-300 (2002).

30. M. H. Baslow, Brain N-acetylaspartate as a molecular water pump and its role in the etiology of canavan disease: a mechanistic explanation. *J. Mol. Neurosci.*, **21** (3), 185-190 (2003).
31. C. N. Madhavarao, P. Arun, J. R. Moffett, S. Suczs, S. Surendran, R. Matalon, J. Garbern, D. Hristova, A. Johnson, W. Jiang and M. A. Namboodiri, Defective N-acetylaspartate catabolism reduces brain acetate levels and myelin lipid synthesis in Canavan's disease. *Proc. Natl. Acad. Sci. U. S. A*, **102** (14), 5221-5226 (2005).
32. B. Tavazzi, S. Signoretti, G. Lazzarino, A. M. Amorini, R. Delfini, M. Cimatti, A. Marmarou and R. Vagnozzi, Cerebral oxidative stress and depression of energy metabolism correlate with severity of diffuse brain injury in rats. *Neurosurgery*, **56** (3), 582-589 (2005).
33. H. E. Jones, A. M. Sillito, The action of the putative neurotransmitters N- acetylaspartylglutamate and L-homocysteate in cat dorsal lateral geniculate nucleus. *J. Neurophysiol.*, **68**, 663-672 (1992).
34. E. R. Whittemore, J. F. Koerner, An explanation for the purported excitation of piriform cortical neurons by N-acetyl-L-aspartyl-L-glutamic acid (NAAG). *Proc. Natl. Acad. Sci. USA*, **86**, 9602-9605 (1989).
35. B. Wroblewska, J. T. Wroblewski, O. H. Saab and J. H. Neale, N-acetylaspartylglutamate inhibits forskolin-stimulated cyclic AMP levels via a metabotropic glutamate receptor in cultured cerebellar granule cells. *J. Neurochem.*, **61**, 943-948 (1993).
36. B. Wroblewska, J. T. Wroblewski, S. Pshenichkin, A. Surin, S. E. Sullivan and J. H. Neale, N-Acetylaspartylglutamate selectively activates mGluR3 receptors in transfected cells. *J. Neurochem.*, **69**, 174-181 (1997).