CHAPTER

2.1

N-Acetylaspartate and *N*-Acetylaspartylglutamate in Central Nervous System Health and Disease

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INTRODUCTION

Brain metabolites are most often defined by their functions, but one brain metabolite stands out as being defined by its utility to magnetic resonance spectroscopists; namely *N*-acetylaspartate (NAA). The utility afforded by NAA is that it represents the most reliable marker for neuronal health and integrity using water-suppressed proton magnetic resonance spectroscopy (MRS). Its utility as a marker for neuronal health is not based on a mature understanding of the functions served by NAA, but primarily through empirical evidence derived from MRS studies of brain injury, disease, mental disorders, and drug abuse, where NAA levels in the brain are typically reduced.

Also known as *N*-acetyl-L-aspartic acid, NAA is the acetylated form of the amino acid aspartate, and is present at very high concentrations in the brain. In rat brain the concentration is between 9.2 and 9.3 mM (Mlynarik et al., 2008). In humans it has been measured at approximately 12 mM (Rigotti et al., 2007, 2011b), making NAA one of the most concentrated metabolites in the human brain. The concentration of NAA in other tissues and serum is low, typically $<40-50$ μM (Miyake et al., 1981). One documented function of NAA in the nervous system is that it serves as a precursor for the biosynthesis of the neuronal peptide *N*-acetylaspartylglutamate or NAAG. NAAG has been described as the most concentrated neuroactive peptide in the human brain (Tsai & Coyle, 1995; Neale et al., 2000). NAAG can also be measured by MRS, but because the NAAG peak is smaller and appears as a shoulder on the NAA peak, its quantification is more difficult (Edden et al., 2007). Research into the functions of NAA and NAAG have begun to reveal roles in central nervous system (CNS) function, but controversies still surround many of the proposed functions.

Recently the genes for the biosynthetic enzymes for NAA (Wiame et al., 2010; Ariyannur et al., 2010b) and NAAG (Becker et al., 2010; Collard et al., 2010) have been identified providing much needed data on protein structure, mechanisms of synthesis, and tissue distribution, as well as providing new tools for identifying key functional roles. These recent discoveries raise hopes that definitive roles for both of these related metabolites will be determined in the relatively near future. Several earlier reviews of NAA and NAAG have been published (Neale et al., 2000; Demougeot et al., 2004; Moffett et al., 2006, 2007; Benarroch, 2008), so this review will focus somewhat more on recent developments and controversies.

NAA

NAA in MRS

NAA is a relatively small molecule, only 174 Da in its ionized form, and it has a methyl (CH_3) group associated with the acetate group. The three hydrogen atoms on the methyl group all resonate with a single frequency in water-suppressed proton MRS at a value

of 2.0 parts per million. Because the methyl hydrogen atoms all resonate with the same frequency, and because NAA is one of the most concentrated molecules in the brain, NAA provides the largest peak on proton MR spectrograms of healthy human brain tissue. For this reason most investigations have focused on determining the utility of NAA measurements in various brain diseases and disorders. NAA is reduced, often in specific brain areas, in a wide array of brain diseases, disorders, and neuropsychiatric conditions. Schizophrenia is one neuropsychiatric disorder in particular that has been relatively well studied with regard to reduced NAA levels in specific brain areas (Brugger et al., 2011). Reductions were consistently observed in frontal lobes, temporal lobes, and thalamus in both first episode and chronic patients. The observed reductions are generally modest, often around 5% relative to controls. In contrast, NAA is substantially reduced after severe brain injury or stroke, and levels may recover after several weeks, depending on the severity of the injury (Wardlaw et al., 1998; Signoretti et al., 2010, 2011; Vagnozzi et al., 2010). Recovery of NAA to normal levels is seen after milder brain injuries, but not in the case of severe injuries, or in the case of multiple milder injuries spaced closely in time as often occurs in contact sports. NAA decreases are also observed in a wide array of brain disorders including epilepsy (Savic et al., 2000, 2004; Riederer et al., 2006), multiple sclerosis (Gonzalez-Toledo et al., 2006; Rigotti et al., 2011a), Alzheimer's disease (Passani et al., 1997a; Kantarci & Jack, 2003; Watanabe et al., 2010), and human immunodeficiency virus (Suwanwelaa et al., 2000; Edden et al., 2007). NAA levels in the brain are increased in Canavan's disease (CD) due to genetic defects in the enzyme that degrades NAA (Wittsack et al., 1996), as will be discussed in detail below.

NAA Synthesis

NAA was first identified in brain extracts in 1956 (Tallan et al., 1956) and biosynthesis was tentatively observed in 1959 (Goldstein, 1959) and subsequently confirmed in 1969 (Goldstein, 1969). NAA is synthesized from L-aspartate and acetyl coenzyme A (acetyl CoA) by the enzyme aspartate *N*-acetyltransferase (Asp-NAT; Truckenmiller et al., 1985). In the adult brain, NAA synthesis occurs mostly in neurons (Urenjak et al., 1992). The corresponding gene for Asp-NAT is *Nat8l*, and is expressed predominantly in the brain (Wiame et al., 2010; Ariyannur et al., 2010b). It is not known if other cell types can synthesize NAA, but mRNA for Asp-NAT is expressed at low levels in the spleen and thymus (Wiame et al., 2010) suggesting that some immune cells may also synthesize NAA under some conditions. In fact, NAA levels have been reported to be relatively high in histamine-containing rat peritoneal mast cells, and it was found that compounds, which result in histamine depletion in these cells, also caused a reduction in NAA levels (Burlina et al., 1997).

Asp-NAT has not been extensively studied because of the difficulties in purifying and characterizing the enzyme activity from brain. An early examination of the tissue distribution of enzyme activity found it present only in the brain, spinal cord, and retina, but in this early study the spleen and thymus were not among the peripheral organs tested (Truckenmiller et al., 1985). It was found that caudal CNS structures such as the spinal cord and brainstem had the highest Asp-NAT activity levels, whereas retina had the lowest level. Now that the gene for Asp-NAT has been identified as *Nat8l*, it will be possible to study the enzyme in much greater detail. For example, recent studies have shown that the Asp-NAT protein sequence can be subdivided into five functional domains (regions 1–5) including a putative membrane associated domain (region 4), which is closely associated with the catalytic domains. This close association between membrane and catalytic domains may explain the lability of Asp-NAT activity in detergent-solubilized brain homogenates (Tahay et al., 2012). Enzyme purification studies using mild detergent homogenization, native gel electrophoresis, and size-exclusion chromatography indicated that the functional enzyme existed as a large, multiprotein complex with an apparent molecular weight exceeding 600 kDa (Madhavarao et al., 2003; Ariyannur et al., 2008). These findings suggest that in the mammalian brain Asp-NAT is part of a membrane-associated protein complex, and that dissociation from the complex or the membrane disrupts the catalytic site and abolishes enzyme activity.

The fact that NAA levels are decreased in many neurological disorders and disease states raises the question of whether the loss of NAA results from reduced NAA synthesis or increased NAA catabolism. The synthesis of NAA can be monitored noninvasively using ^{13}C MRS using 1-^{13}C-labeled glucose administration. NAA synthesis rates measured by this method indicate a reduction by approximately 60% in CD patients, whereas synthesis rates appear to be modestly increased in Alzheimer's disease and schizophrenia patients (Moreno et al., 2001; Harris et al., 2006). This provides *in vivo* evidence for the regulation of NAA synthesis under different pathological conditions. Based on the observations that brain NAA levels are reduced in Alzheimer's disease (Passani et al., 1997a; Watanabe et al., 2010) and schizophrenia (Brugger et al., 2011), the finding that synthesis rates

are modestly increased under these conditions could suggest that increased catabolism is responsible for the observed decreases. However, in conditions where significant neuronal loss is involved, such as late stage Alzheimer's disease, it is also possible that the observed reductions in NAA levels are associated directly with the loss of biosynthetic capacity, with a concomitant increase in the rate of synthesis in the remaining neurons.

Using ^{13}C MRS and 1-^{13}C-labeled glucose administration in rats, Choi and Gruetter (2004) studied NAA synthesis *in vivo* and found that incorporation was detected in the acetyl group of NAA about 1.5 h earlier than in the aspartate group, indicating a delay in labeling of aspartate as compared to acetyl CoA. This finding would be expected based on the rapid conversion of glucose to pyruvate and subsequently acetyl CoA, which then could be used for the synthesis of NAA. The incorporation of label from glucose into aspartate would follow later after acetyl CoA entered the citric acid cycle, eventually forming oxaloacetate and then aspartate. Based on their findings, Choi and Gruetter (2004) concluded that NAA synthesis occurs in a single metabolic compartment (neurons), that it exhibits a relatively low turnover rate (up to 72 h for complete turnover), and that it is not likely to be involved as a major source of energy when the brain is in a resting state. These findings are in agreement with earlier studies that showed NAA is a substantial source of acetyl groups for lipid synthesis (Burri et al., 1991), which is a metabolic process that is relatively slow compared with energy derivation. In the same study, Burri and coworkers (1991) found that between 7 and 9% of the acetate moiety of NAA ended up in the protein fraction after 4 h incubation. Although this finding was not discussed by these investigators, their finding of rapid protein labeling from NAA-derived acetate suggests that some portion of this acetate may be employed in protein acetylation reactions because this is the most rapid route by which acetate could be incorporated into the protein fraction. This will be discussed in more detail in the following sections.

NAA and Asp-NAT Cellular Localization

Early studies of the levels of NAA in different dissected brain regions suggested that NAA might be localized predominantly in neurons (Tallan, 1957). An examination of human nervous system tumors and peripheral neural tissues from cows strongly suggested that NAA was predominantly localized in neurons (Nadler & Cooper, 1972b). Gas chromatography of various tissue extracts indicated that NAA was present in the brain at concentrations over 100 times greater than found in non-neural tissues suggesting that neurons were a major source of NAA (Miyake et al., 1981). A later *in vitro* study on purified brain cell types using MRS and high-performance liquid chromatography (HPLC) indicated that neurons such as cerebellar granule cells contained high levels of NAA, whereas astrocytes and mature oligodendrocytes contained undetectable levels (Urenjak et al., 1992). They also found high levels of NAA in oligodendrocyte-type-2 astrocyte progenitor cells and immature oligodendrocytes.

Antibodies to protein-coupled NAA used in conjunction with specialized tissue fixation methods for coupling NAA to tissue proteins provided a method of looking at NAA localization directly in brain tissue via immunohistochemistry (Moffett et al., 1991; Simmons et al., 1991). NAA was found to be present predominantly in neurons throughout the CNS. The methods were refined by the use of highly purified antibodies to protein-coupled NAA and enhanced NAA-fixation techniques allowing for the visualization of NAA in neurons and their processes (Moffett et al., 1993; Moffett & Namboodiri, 1995, 2006). NAA in the rat brain was present in most neurons, some dendritic processes, and in the axons of most fiber pathways throughout the CNS. In the rat, NAA was found to be present at different levels in different neuronal populations, with pyramidal neurons having higher levels than smaller interneurons (Fig. 2.1.1). This suggests that NAA may be more important in larger projection neurons with longer axons and more extensive myelination.

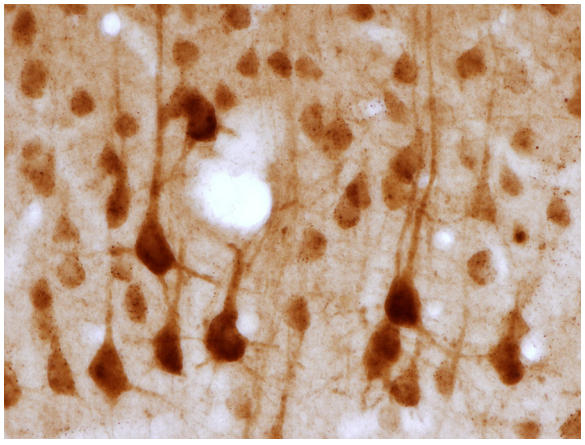

FIGURE 2.1.1 NAA immunoreactivity in motor cortex of the rat. Highly purified antibodies to protein-coupled NAA were used to visualize NAA in carbodiimide-fixed brain tissue. NAA was observed predominantly in neurons, with staining in cell bodies, dendrites, and axons. Much lighter staining was observed in oligodendrocytes, ependymal cells, and some blood vessels (40× objective with enhanced depth of field).

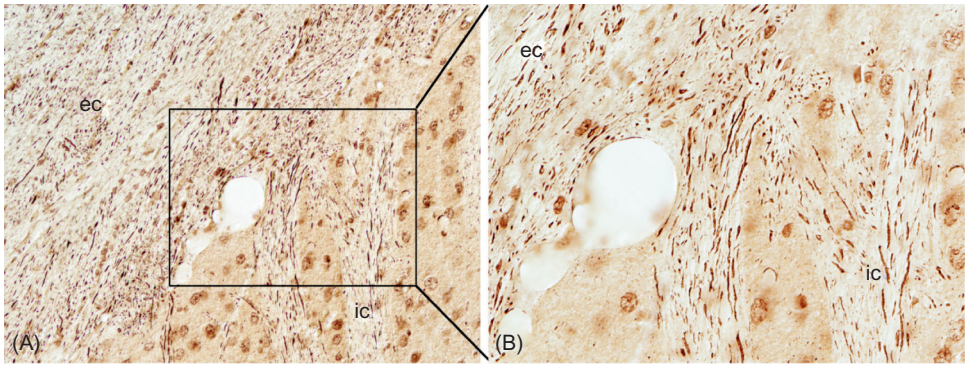

FIGURE 2.1.2 Immunostaining for the NAA biosynthetic enzyme Asp-NAT in the corpus callosum and internal capsule of the rat. Affinity-purified polyclonal antibodies to a 19 amino acid sequence unique to Asp-NAT were used to demonstrate cellular expression levels. Asp-NAT immunoreactivity was present at low levels in neuronal cell bodies, and at substantially higher levels in axons throughout the brain. Staining was present in discontinuous patches along the length of axons. Image in (A) is shown at higher magnification in (B) (20× objective, A; 40×, B). ec, external capsule; ic, internal capsule.

Detailed studies of Asp-NAT cellular localization in brain have not been done to date. Using uncharacterized and unpurified antibodies, Niwa and coworkers (2007) showed that neurons of the nucleus accumbens expressed *Nat8l*, but at the time it had not been determined that the corresponding gene encoded Asp-NAT. Our laboratory has affinity purified the same antibody obtained from the Niwa group (2007) and applied it to rat brain slices, and have found the expression to be generally light in neuronal somata, and stronger in neuronal axons. Interestingly the expression in axons was not homogeneous, but appeared to be present in discontinuous patches (Fig.2.1.2).

Asp-NAT Subcellular Localization

Pioneering studies on NAA production in the brain provided strong evidence for mitochondria being one major site of NAA synthesis. Using purified mitochondrial preparations Patel and Clark (1979) found that brain mitochondria oxidizing pyruvate or 3-hydroxybutyrate to acetyl CoA produced and exported NAA in an inverse relationship to aspartate. As more pyruvate or 3-hydroxybutyrate was added to the incubation medium, they observed increasing NAA export and decreasing aspartate export. They concluded that NAA synthesis depended on and was regulated by aspartate synthesis in mitochondria. They noted that intramitochondrial aspartate synthesis depended on the supply of cytoplasmic glutamate, which is transported into the mitochondrial matrix in brain by the aspartate-glutamate exchanger now known as aralar1.

This conclusion brings up an interesting connection between mitochondrial function and NAA synthesis that was discovered during studies of aralar1 knockout mice. Aralar1 is the main mitochondrial aspartate-glutamate carrier expressed in the brain and skeletal muscle, and it is part of a larger complex that comprises the so-called mitochondrial malate-aspartate shuttle. This key metabolite exchanger system acts in bulk to move reducing equivalents into the mitochondrial matrix in the form of malate. Aralar1, as a component of this complex, acts to move cytoplasmic glutamate into mitochondria, while also moving mitochondrially synthesized aspartate out. Without aralar1, mitochondrial glutamate import and aspartate export are crippled. Studies with aralar1 knockout mice showed a dramatic drop in aspartate levels, and in turn dramatic reductions in NAA synthesis in the brain (Satrustegui et al., 2007). The finding that lack of aralar1 dramatically reduces NAA synthesis has at least two possible explanations. First, it could be due to the lack of aspartate output from mitochondria, which would limit the ability of microsomal Asp-NAT to synthesize NAA due to a lack of substrate. Second, it could be that the lack of glutamate uptake into mitochondria prevents intramitochondrial aspartate synthesis due to the lack of substrate for the mitochondrial aspartate aminotransferase reaction, which interconverts glutamate and oxaloacetate with aspartate and α-ketoglutarate. In this case the lack of intramitochondrial aspartate synthesis limits NAA production. It is possible that both of these mechanisms are involved in the drop in brain NAA levels in aralar1-deficient mice. The earlier work by Patel and Clark (1979) would suggest that the second alternative was more likely; however, more recent work with *Nat8l* transfection and bioinformatics studies call this conclusion into question, as discussed later.

One of the more interesting outcomes of aralar1 deficiency in addition to the large decrease in brain NAA levels is hypomyelination (Jalil et al., 2005; Wibom et al., 2009). The hypomyelination is hypothesized to

result from the lack of availability of NAA, and this conclusion is supported by the fact that galactocerebrosides were reduced in aralar1 knockout mice, one of the myelin lipid classes that are reduced in CD (Madhavarao et al., 2005; Arun et al., 2010). CD will be discussed in more detail in the next section.

The majority of studies on NAA synthesis have suggested that NAA is synthesized in both mitochondria and in the microsomal fraction, which probably represents endoplasmic reticulum synthesis (Patel & Clark, 1979; Clark, 1998; Wang et al., 2007; Ariyannur et al., 2008; Arun et al., 2009). However, recent bioinformatics and *Nat8l* transfection studies have suggested that based on the protein sequence Asp-NAT is always retained in the endoplasmic reticulum (ER), and is not targeted to mitochondria (Wiame et al., 2010). These investigations included *Myc*-tagged *Nat8l* construct transfection experiments *in vitro*, which showed Asp-NAT to have predominantly perinuclear and ER localization, without any detectable mitochondrial localization (Tahay et al., 2012). The investigators concluded that Asp-NAT is localized almost exclusively in the ER when expressed under *in vitro* conditions. Verifying that Asp-NAT is also present in neuronal mitochondria *in vivo* awaits future studies. Nonetheless, dual targeting of proteins to both the ER and mitochondria is a common expression pattern for many proteins. Unlike proteins with N-terminal mitochondrial targeting sequences, a number of proteins with dual localization expression patterns have subcellular targeting sequences that are internal to the protein, and these are known as cryptic targeting sequences (Anandatheerthavarada et al., 2008; Sangar et al., 2010; Avadhani et al., 2011). Also there are instances in which proteins lacking any targeting sequence are associated with other proteins that have mitochondrial targeting sequences. Because Asp-NAT appears to exist as a part of a multiprotein complex, this may be how Asp-NAT could also be targeted to mitochondria (Madhavarao et al., 2003).

NAA and CD

Prior to identifying the connection between NAA and CD, NAA was considered metabolically inert, and therefore attracted little attention from neuroscientists or clinicians (Nadler & Cooper, 1972a). CD was first described in the 1930s (Canavan, 1931) and was recognized as a unique infantile disorder in the 1940s, but the cause remained unknown (van Bogaert & Bertrand, 1949). It was not until the 1980s that the connection to NAA catabolism was made (Hagenfeldt et al., 1987; Matalon et al., 1988). The enzyme that catabolizes NAA is designated aspartoacylase (ASPA), and is also known as amidohydrolase II. Amidohydrolases including ASPA act to remove acetate from acetylated amino acids in order to allow for metabolism and recycling of the deacetylated amino acids, and reclamation of the released acetate (Lindner et al., 2000; Perrier et al., 2005). In CD mutations in the ASPA gene result in greatly reduced enzyme activity, and therefore NAA catabolism is substantially blocked because there are no alternative enzyme pathways. As such, NAA levels build up in the brain and greatly increased levels of NAA appear in the urine. Despite the understanding that CD is associated with ASPA deficiency, resulting in increased NAA levels in the brain, the pathophysiology of CD remains uncertain because the functions of NAA in the nervous system are far from completely understood.

CD is classified as an autosomal recessive spongiform leukodystrophy, and a number of previous reviews have detailed the clinical symptoms, and possible biochemical mechanisms, involved in this fatal genetic disorder (Kaul et al., 1994b; Matalon & Michals-Matalon, 1998; Traeger & Rapin, 1998; Kumar et al., 2006a; Moffett et al., 2007). CD infants appear normal at birth, and symptoms typically occur by 4–6 months of age. The clinical symptoms of CD include poor head control, macrocephaly, marked developmental delay, optic atrophy, seizures, hypotonia, and for most of those afflicted, death in childhood. Postmortem analyses of the brains of CD patients indicate severe vacuolation in both white and gray matter and enlargement of the ventricles. Cloning of the human ASPA gene has enabled molecular genetic studies of CD (Kaul et al., 1993; Namboodiri et al., 2000). Two mutations were found to be prevalent among Ashkenazi Jewish patients with CD (Kaul et al., 1994b). A missense mutation in codon 285 causing substitution of glutamic acid to alanine accounts for 83.6 % mutations identified in 104 alleles from 52 unrelated Ashkenazi Jewish patients. A nonsense mutation on codon 231, which converts tyrosine to a stop codon, was found in 13.4% of the alleles from the Jewish patients. Among non-Jewish patients, the mutations are different and more diverse (Kaul et al., 1996; Sistermans et al., 2000). The most common is in codon 305, a missense mutation substituting alanine for glutamic acid. This mutation was observed in 35.7% of the 70 alleles from 35 unrelated non-Jewish patients (Kaul et al., 1994a). Fifteen other mutations were detected in 24 other CD patients. Additional mutations, some with the children dying immediately after birth, have also been reported (Zeng et al., 2002). The diverse mutations limit the use of prenatal diagnosis to couples who are both carriers with known mutations. Very recently a study has looked at the correlation between enzyme activity levels associated with various ASPA

mutations and the severity of CD phenotype, and observed that a good correlation existed between reduction in enzyme activity and disease severity (Zano et al., 2012). However, there were exceptions to this rule where two mutations in which enzyme activity was very low but the disease progression was relatively mild. It was found that enzyme stability may be a more significant factor in disease progression than catalytic activity wherein milder forms of CD are associated with ASPA enzyme that is conformationally stable despite the mutations.

Mechanisms of CD Pathogenesis

There are two primary hypotheses on the pathogenic mechanisms of CD that are not mutually exclusive. One hypothesis posits that the accumulation of excess NAA in the brain impairs osmotic regulation in neurons (Baslow, 1999, 2003), or causes neuronal overexcitation (Kitada et al., 2000). This hypothesis suggested that ASPA gene therapy with a neurotrophic viral vector could overcome the problem and serve as a cure for the disease by reducing brain NAA levels. Similarly, efforts toward decreasing the synthesis of NAA by selective inhibitors of the NAA synthesizing enzyme have been attempted. In the other hypothesis it is proposed that NAA-derived acetate is a significant source of acetyl CoA during brain development, which is synthesized in neurons and transferred to oligodendrocytes as a trophic support mechanism. The transferred NAA is then used in the synthesis of fatty acids and myelin lipids in the brain, as well as in protein acetylation reactions including histone acetylation. Under this hypothesis, a lack of ASPA activity in the brain impairs oligodendrocyte development and maturation through an acetyl CoA deficit leading to oligodendrocyte death and defective myelin synthesis during the period of postnatal myelination.

Our laboratory has directly tested the acetate deficiency hypothesis in ASPA $-/-$ mice providing data showing significant decreases in both brain acetate levels, and the synthesis of myelin lipids, during the peak period of postnatal myelination (Madhavarao et al., 2005). More recently we have shown that dietary acetate supplementation using a potent, hydrophobic acetate source, glyceryl triacetate (GTA), significantly improves the phenotype in the tremor rat model of CD (Arun et al., 2010). Tremor rats are a naturally occurring mutant strain that lacks the entire genetic sequence for ASPA (Kitada et al., 2000). Motor performance of the GTA-treated tremor rats was significantly improved, and brain vacuolation was modestly reduced. Further, galactocerebroside levels in myelin were also improved with GTA treatment. Nonetheless, the pathogenic mechanisms of CD remain a controversial issue. This is primarily due to our lack of understanding the role of NAA in the nervous system. Our laboratory has proposed that NAA acts as a storage form of acetyl CoA that can be transported from the site of synthesis in neurons, to the site of utilization in oligodendrocytes, representing a trophic interaction between the two cell types (Ariyannur et al., 2010b). Therefore, CD results in a lack of acetyl CoA in oligodendrocytes, especially during the period of postnatal myelination, impairing oligodendrocyte maturation, possibly by disrupting histone acetylation and epigenetic gene regulation (Kumar et al., 2006b, 2009; Mattan et al., 2010). It is also likely that the excess levels of NAA in the brain are responsible for some of the pathological consequences of ASPA deficiency, for example epileptic seizures (Klugmann et al., 2005).

Increased NAA and CD Pathogenesis

An unresolved issue concerning NAA surrounds its potential toxicity to neurons or oligodendrocytes when the concentration is elevated in the brain, as is the case with CD patients (Matalon & Michals-Matalon, 1998). Based on the assumption that the primary etiology of CD involves toxic NAA buildup in the brain, adenoviral transfer of the ASPA gene to the brains of humans has been performed in an attempt to reverse brain edema and vacuolation (Janson et al., 2002). The only available viral vectors for gene introduction into the CNS are neurotrophic in nature, and will primarily target genes to neurons rather than oligodendrocytes. Adenoviral gene transfer studies using the tremor rat ASPA-null mutant strain have yielded generally negative results on the efficacy of this approach using current technology. In one study, NAA levels were reduced, and seizure activity was diminished, but brain vacuolation, motor performance, and dysmyelination were unaffected, suggesting that some of the pathological features of the disease are not mediated only by excessive NAA concentrations (Klugmann et al., 2005). A very recent follow-up study of the children who underwent ASPA gene therapy has indicated that NAA levels in the brain were modestly reduced, and that this was accompanied by reduced seizure activity and a stabilization of the children's condition (Leone et al., 2012). These results strongly suggest that excess NAA is in part responsible for the pathogenesis of CD, especially with regard to seizure activity.

NAA and Osmoregulation

NAA synthesis and breakdown have been proposed as an osmoregulatory mechanism for removing metabolic water from neurons (Baslow, 1997, 1999, 2002; Baslow et al., 2007). The existing empirical data on an osmoregulatory role for NAA are sparse and suggest that NAA is not one of the major osmolytes that move

in response to changes in extracellular tonicity (reviewed in Moffett et al., 2007). It is well documented that many inorganic ions and organic metabolites, including a number of amino acids, move in response to osmotic stress (Gullans & Verbalis, 1993), so NAA represents a viable candidate for this role. However, studies of the organic compounds that respond to hyponatremia often do not include NAA in the list of responsive metabolites (Soupart et al., 2007). Studies that looked directly at NAA responsiveness to osmotic stress indicate that it is a minor contributor to osmoregulatory responses in the brain when compared with other osmolytes such as taurine (Taylor et al., 1994, 1995; Verbalis, 2006).

The NAA osmolyte hypothesis is distinct from standard models of water homeostasis in the brain in that it does not view NAA as responsive to osmotic stress, but rather that it acts as a water cotransporter that removes metabolically produced water from neurons (Baslow et al., 2012). This is hypothesized to be a continuous process linked only to neuronal depolarization, which is said to move NAA-bound water to the extracellular space by way of an uncharacterized transporter. So technically NAA would not be considered a standard osmolyte under this hypothesis, but rather that in conjunction with an uncharacterized transporter protein would be classified specifically as a "molecular water pump" found only in nerve cells. Catabolism by ASPA is thought to dewater the NAA, and allow the water to exit the extracellular fluid more rapidly than bound water. Therefore, under this hypothesis CD is an osmotic disorder of the nervous system associated with the inability to move metabolic water generated in neurons to the vasculature in a timely manner. Currently there is no data on depolarization-induced NAA release from neurons or any potential NAA transporter protein that moves both NAA and its bound water out of neurons during neuronal depolarization. Until such a transport protein is identified and characterized the molecular water pump hypothesis remains both intriguing and hypothetical.

NAA, Acetyl CoA, and Lipid Synthesis

Neurons are known to provide metabolites, which include choline, palmitate, acetate, phosphate and ethanolamine (Ledeen, 1984), as well as NAA (Chakraborty et al., 2001) their ensheathing oligodendrocytes for the purpose of myelination. It has also been known for some time that NAA supplies acetate groups for the synthesis of acetyl CoA (D'Adamo & Yatsu, 1966; Burri et al., 1991; Mehta & Namboodiri, 1995), but the quantitative contribution to total acetyl CoA synthesis under different physiological conditions is uncertain. The synthesis of NAA requires the utilization of existing acetyl CoA and therefore NAA is not a primary source of acetyl CoA as is the case with pyruvate. Because NAA concentrations in the brain are exceptionally high, NAA synthesis probably consumes a substantial proportion of the acetyl CoA pool in brain. It seems likely that NAA may be acting in part as a storage and transport form of acetate in the CNS that can be used for subsequent *de novo* synthesis of acetyl CoA (Ariyannur et al., 2010b). This arrangement makes sense when viewed in light of the localization of the biosynthetic and degradatory enzymes for NAA wherein Asp-NAT is present primarily in neurons (Truckenmiller et al., 1985; Madhavarao et al., 2003; Wiame et al., 2010), and ASPA is present primarily in oligodendrocytes (Klugmann et al., 2003; Madhavarao et al., 2004; Moffett et al., 2011). Elegant studies by Ledeen and colleagues (1984), using NAA radiolabeled on the acetate moiety, showed that when NAA was injected into the eye it was transported down the optic nerves and the radioactivity was incorporated into the ensheathing myelin lipids (Chakraborty et al., 2001). These findings indicate that NAA in neurons supplies acetyl groups for the synthesis of myelin lipids in oligodendrocytes. However, the current evidence indicates that NAA supplies only a portion of the requisite acetyl CoA for myelin lipid synthesis, with the majority coming from citrate produced in oligodendrocyte mitochondria. As such NAA appears to be a parallel pathway that may be more critical during the period of intensive myelination that begins shortly after birth. This is the time of suckling in mammals when glucose availability in the diet is low, and the brain relies more on ketone bodies for energy derivation.

It has been noted that it is unusual that oligodendrocytes would use NAA rather than exclusively using glucose for lipid synthesis during development (Ramos et al., 2011). In adult animals glucose is the primary energy deriving metabolite in the brain. However, it is well documented that during brain development ketone bodies are preferred over glucose as an energy source for neurons and oligodendrocytes (Edmond et al., 1987). It is also known that acetate is released from the liver along with ketone bodies to provide this substrate to other tissues of the body (Ballard, 1972; Yamashita et al., 2001). Using radiolabeled precursors Edmond and colleagues (1987) showed that ketone bodies were nine times more effective than glucose for supporting oligodendrocyte respiration during brain development. These findings indicate that ketone bodies and acetate are more critical energy metabolites for oligodendrocytes during brain development than in adults. This may help explain why NAA-derived acetate appears to be more critical for oligodendrocytes during postnatal myelination.

Acetate Deficiency and CD Pathogenesis

There is accumulating evidence that NAA is involved in lipid synthesis and myelination in the CNS (reviewed in Moffett et al., 2007). But the correlation with myelination is only partial because substantial brain and spinal cord vacuolation is observed in many gray matter areas with sparing of many white matter tracts in ASPA-deficient animal models of CD (Surendran et al., 2005). This gray matter vacuolation is first observed between postnatal days 14 and 21 in the *Nur7* ASPA-deficient mouse model coinciding with the peak of postnatal CNS myelination (Traka et al., 2008). Studies on the maturation of oligodendrocytes during postnatal myelination demonstrate the important role of histone acetylation and deacetylation in the epigenetic control of differentiation from oligodendrocyte precursor cells to mature oligodendrocytes (Ye et al., 2009; Copray et al., 2009; MacDonald & Roskams, 2009). We have proposed that NAA-derived acetate is an important source of acetyl CoA in oligodendrocytes for histone acetylation reactions that regulate chromatin structure and gene transcription (Arun et al., 2010; Ariyannur et al., 2010b). The dramatic reduction in acetate availability in oligodendrocytes during brain development that results from ASPA deficiency may impact histone acetyltransferase reactions required for epigenetic gene regulation. The resultant disruption of oligodendrocyte differentiation and maturation would explain the observed loss of mature oligodendrocytes in the cerebellum and brainstem of *Nur7* ASPA-deficient mice (Traka et al., 2008), and the death of immature oligodendrocytes in ASPA $-/-$ mice (Kumar et al., 2009). In addition, histone hyperacetylation has been reported during postnatal myelination in animal models of CD (Kumar et al., 2009; Mattan et al., 2010) suggesting that control over cellular differentiation is disrupted via ASPA deficiency. In preliminary studies we have also found that histone acetylation is substantially increased in the tremor rat model of CD, and that acetate supplementation with GTA significantly reverses these abnormalities (Fig. 2.1.3).

Free acetate cannot be metabolized until it is converted to acetyl CoA. Acetate derived from NAA hydrolysis is enzymatically converted to acetyl CoA by the enzyme acetyl coenzyme A synthase-1 (AceCS1). We observed that AceCS1 is present in the nuclei and cytoplasm of many oligodendrocytes during postnatal

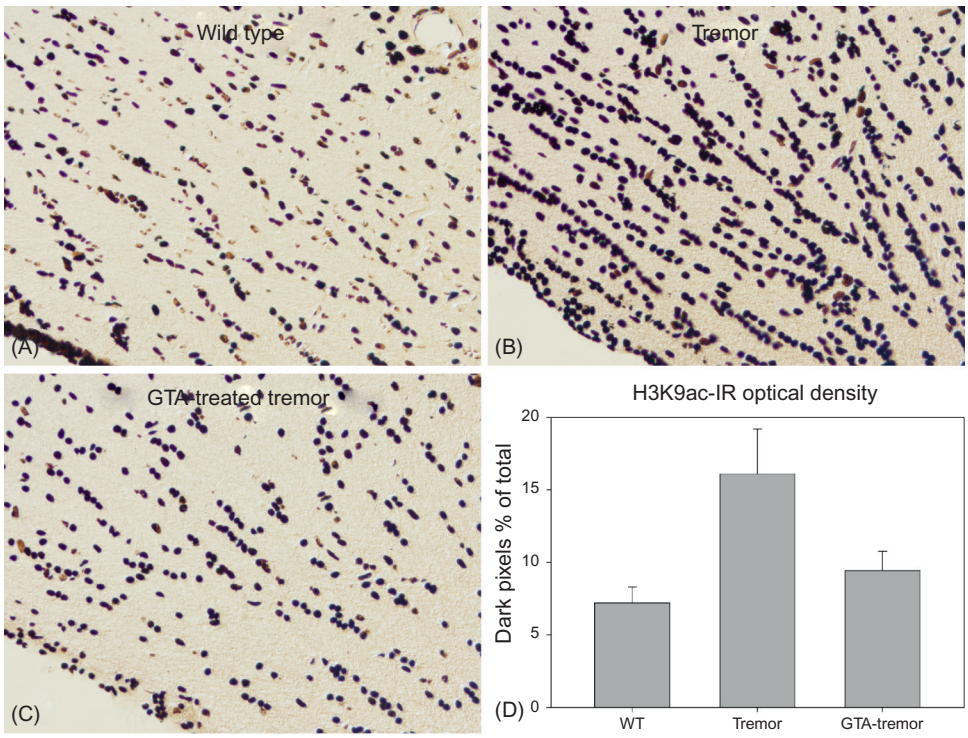

FIGURE 2.1.3 Acetylated histone H3 immunoreactivity (H3ac-IR) in the rat external capsule. Antibodies to histone H3 acetylated on the lysine-9 residue were used to examine histone acetylation in tremor and wild-type (WT) rats. (A) Shows H3ac-IR in the external capsule of a wild-type rat, with many oligodendrocyte cell nuclei expressing acetylated H3. (B) shows the same region of the external capsule from a tremor rat (~70 days old). Many more oligodendrocyte nuclei express acetylated H3. (C) Shows the same region from a GTA-treated tremor rat (~70 days old). The number of oligodendrocyte nuclei expressing acetylated H3 is reduced to near normal levels. (Methods: 20× objective; ChIP grade anti-H3K9ac antibodies, (Abcam#10812; 0.25 μg/ml).

myelination in the rat (Ariyannur et al., 2010a). AceCS1 expression was upregulated in neurons and oligodendrocytes in adult tremor rats as compared with wild-type controls, and was returned to near-normal levels after acetate supplementation with GTA. AceCS1 has recently been shown to be one of the enzymes involved in histone acetylation reactions necessary for cell differentiation (Wellen et al., 2009) where citrate was the predominant source of acetyl CoA, but AceCS1 also provided some of the required substrate. The reduced substrate availability for AceCS1 in CD could negatively impact histone acetylation critical for proper oligodendrocyte maturation (Copray et al., 2009; Kumar et al., 2009). We propose that ASPA deficiency leads to improper regulation of histone acetylation in developing oligodendrocytes, preventing normal differentiation and leading to oligodendrocyte cell death, dysmyelination, neuronal injury, and inflammation and possibly even contributing to vacuole formation. The death of immature oligodendrocytes has been documented in ASPA-deficient mice (Kumar et al., 2009). These findings suggest that AceCS1 levels may be regulated in part by substrate availability whereby low acetate concentrations associated with ASPA deficiency result in upregulation of expression, and supplementation with exogenous acetate in ASPA deficiency leads to a normalization of expression. One important conclusion that can be drawn from these observations is that NAA-derived acetate is not only involved in brain myelination, but that it is also involved in providing some of the acetyl CoA required for histone acetylation and gene regulation associated with oligodendrocyte maturation during postnatal brain development.

Other Acetyl CoA Uses That May be Tied to NAA

Because NAA can act as a source of acetate groups for acetyl CoA synthesis (Burri et al., 1991; Mehta & Namboodiri, 1995), NAA can participate indirectly in many acetylation reactions, including not only nuclear histone acetylation, but also cytoplasmic protein acetylation reactions. Cytoplasmic and ER protein acetylation is becoming recognized as an important regulatory mechanism for controlling protein stability and function (Kouzarides, 2000; Costantini et al., 2007). We have proposed that another potential mechanism linking reduced acetyl CoA availability and the neuropathologies in CD could involve cytoplasmic acetylation reactions, including cotranslational and post-translational acetylation of proteins, particularly in oligodendrocytes (Arun et al., 2010). Oligodendrocytes have very active protein secretory pathways through the ER and are sensitive to disorders of protein misfolding. Recent work indicates that acetylation and deacetylation of certain nascent polypeptide chains in the ER secretory pathway of cells is required for stabilization and proper folding (Costantini et al., 2007; Spange et al., 2009; Guan & Xiong, 2010; Jonas et al., 2010). Acetyl CoA is required for the acetyltransferase reactions involved in acetylation at lysine sites on proteins, and the substantial drop in brain acetate levels that occurs in CD could have a negative impact on protein folding and stabilization, thus targeting proteins for ER-associated degradation. A dramatic loss of myelin basic protein and PLP/DM20 proteolipid proteins has been observed in the ASPA knockout mouse model combined with perinuclear retention of myelin protein staining. These findings indicate impairment in protein trafficking in oligodendrocytes (Kumar et al., 2009). Oligodendrocytes are highly susceptible to ER stress associated with disruptions in protein synthesis and trafficking (Lin & Popko, 2009). Recent studies in the newer mouse model of CD are consistent with the acetate/acetyl CoA deficiency hypothesis of CD (Francis et al., 2012).

Summary

Together, the currently available data suggest that NAA acts in part as a trophic support system for ensheathing oligodendrocytes, whereby neuronal axons supply NAA to oligodendrocytes, probably through a coordinated release-uptake system between neurons and oligodendrocytes. This trophic support appears to be most critical during the period of postnatal myelination when metabolic demands on oligodendrocytes are maximal, and when glucose supply is low. Despite the mounting evidence in favor of the trophic hypothesis, even this potential role for NAA requires further validation and experimental support before it becomes generally accepted. Additional roles in the nervous system such as osmoregulation await future studies.

NAAG

NAAG Synthesis

It has been postulated for some time that NAAG was synthesized non-ribosomally by the action of a peptide synthase enzyme, similar, for example, to how the tripeptide glutathione is synthesized. This conclusion was based on the observation that NAAG was synthesized in explanted neural tissue even in the presence of protein synthesis inhibitors (Cangro et al., 1987). NAAG synthesis in brain tissue was tentatively

reported about 40 years ago (Reichelt and Kvamme 1973), but this area of research has progressed slowly in the ensuing years because most laboratories were unable to detect biosynthesis in broken cell preparations. Synthesis of NAAG from radiolabeled precursors has also been demonstrated in neuronal and glial cell culture systems (Arun et al., 2004, 2006, 2008; Gehl et al., 2004). However, until recently the identification and characterization of the synthetic enzyme remained elusive.

Recently, two members of the ATP grasp protein family were identified to be the NAAG synthetase enzymes. They are known as rimK-like family member B (*Rimklb*) and A (*Rimkla*) (Becker et al., 2010; Collard et al., 2010). The enzymes of this family (RIMK) are ATP-dependent glutamate ligases that ligate the α-amino group of glutamate to the carboxylic group of an acceptor. These ligases have been found to be specifically dependent upon NAA and ATP for their function and are inactive in the absence of either one. Both the enzymes have very high K_m values (1.48 mM NAA for RIMKLA) indicating that very high concentrations of NAA are required for enzyme activity (Collard et al., 2010). RIMKLB also has a low level of glutamate ligase activity toward citrate, forming β-citrylglutamate. RIMKLA was found to have about three times lower K_m value for NAA compared to RIMKLB, while the K_m values for citrate are comparable for both the enzymes. Both NAAG synthase enzymes share about 65% sequence identity. Also, RIMKLA was recently found to have an additional glutamate ligase activity to synthesize *N*-acetylaspartyl-glutamyl-glutamate ($NAAG_2$), a tripeptide. From the publically available database on protein localization, the newly identified RIMKLB and RIMKLA proteins have a primary neuronal localization with the highest expression levels in hindbrain and spinal cord. This expression pattern matches the expression levels observed using antibodies to protein-coupled NAAG (Moffett & Namboodiri, 1995, 2006). Future gene knockout studies should help to advance our understanding of the functional roles of NAAG in the CNS.

NAAG Localization

NAAG was first identified by Miyamoto and colleagues (1966) in bovine brain extracts in the mid-1960s. Using gas chromatography Miyake and Kakimoto (1981) found that NAAG increased in concentration in the brain and spinal cord from birth through adulthood in both rats and guinea pigs. Further studies showed that NAAG was most likely present in neurons, for example, the concentration of NAAG in gray matter was more than twice that found in white matter, and the concentration in the CNS was over 100 times greater than the concentrations seen in peripheral organs (Miyake et al., 1981). In these studies NAAG was identified in the CNS of all mammalian, avian, reptilian, and amphibian species studied, with the lowest concentration noted in the CNS of fish. Further evidence for neuronal localization came from HPLC analyses of piriform cortex in the rat 3 days after unilateral olfactory bulbectomy showing a 22% drop in NAAG concentrations indicating that some of the NAAG in piriform cortex was associated with olfactory fibers or nerve endings (ffrench-Mullen et al., 1985). A number of evoked release studies showed that NAAG was released after depolarization in a calcium-dependent manner from terminals in the retina and retinal target areas in the brain (Tsai et al., 1988; Williamson et al., 1991; Williamson & Neale, 1992), as well as from tissue slices of striatum, cerebellum, and spinal cord (Zollinger et al., 1994). These and similar investigations demonstrated that NAAG is released synaptically from neurons in the same manner as classical neurotransmitters.

Development of antibodies and specialized fixation techniques permitted the visualization of NAAG in brain sections (Anderson et al., 1987; Cangro et al., 1987; Tieman et al., 1987). These studies showed that NAAG was present in neurons, axons, and synaptic terminals, but not in glia. Subsequent improvements to the methods provided very detailed neuroanatomical information on NAAG localization in the CNS of the rat (Moffett et al., 1993; Moffett et al., 1994; Moffett & Namboodiri, 1995, 2006; Moffett et al., 1993, 1994), cat (Tieman et al., 1987, 1991a), monkey (Tieman et al., 1991b; Moffett & Namboodiri, 2006), and human (Tieman & Tieman, 1996; Passani et al., 1997b). NAAG expression generally showed an increasing rostrocaudal concentration gradient, with lower levels in many forebrain areas, and higher levels in the medulla and spinal cord. However the visual system was an exception in the forebrain in that there were very high levels of NAAG expression in the optic nerve fibers and in synapses in visual target areas including the lateral geniculate, superior colliculus, suprachiasmatic nucleus, and accessory optic nuclei (Moffett et al., 1990; Moffett, 2003; Tieman, 2006). Ultrastructural studies have shown NAAG to be present in synaptic vesicles in the visual system and cerebellum (Williamson & Neale, 1988; Renno et al., 1997).

NAAG is not expressed in all neurons, as is apparently the case with NAA. NAAG expression is much more restricted than that of NAA. In some brain regions NAAG expression is relatively sparse, for example, only a relatively small percentage of

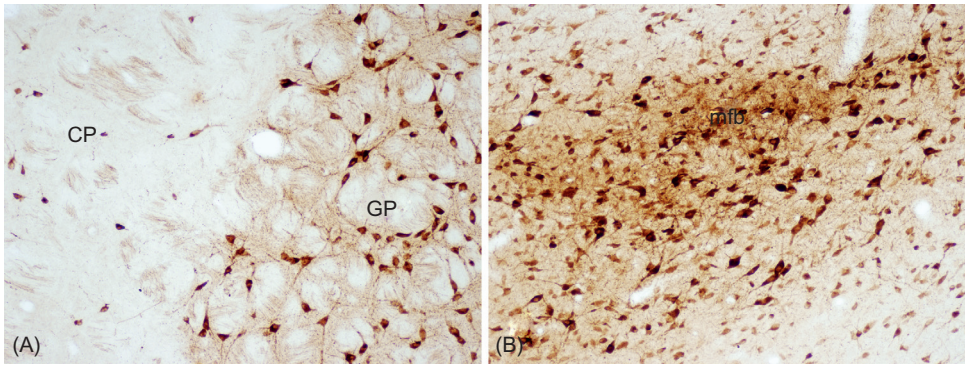

FIGURE 2.1.4 NAAG in rat striatum versus globus pallidus (GP) and in the lateral hypothalamus. NAAG expression was low in the striatum and accumbens, with only scattered neurons and axons of the internal capsule strongly stained (CP in A). In contrast, strong NAAG expression was observed in the majority of neurons in the GP along with strong staining in dendrites. Neurons of the lateral hypothalamus were intensely stained for NAAG, as were axons of the medial forebrain bundle (mfb; B) (10× objective in A and B).

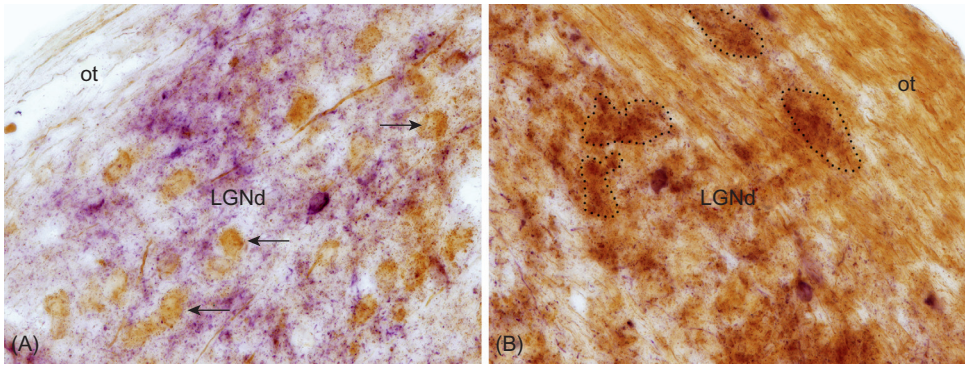

FIGURE 2.1.5 NAAG in the visual system. Some of the highest NAAG expression in the forebrain is associated with the visual pathways from retinal ganglion cells to the lateral geniculate nucleus, including the optic tracts (ot). NAAG-containing glomerular synapses (dotted outlines in B) are present throughout the dorsolateral geniculate nucleus (LGNd), and these contacts are lost upon optic nerve transection (A). The sections are double stained for NAAG (orange) and glutamic acid decarboxylase (purple) the enzyme that synthesizes the inhibitory neurotransmitter GABA. Arrows in A show NAAG expression in principle neurons of the lateral geniculate.

neurons express NAAG in the rat striatum, whereas expression is very strong in the adjacent globus pallidus (Fig. 2.1.4). Other regions with notably high expression levels of NAAG in the rat forebrain include the hypothalamus (Fig. 2.1.4), the visual pathways and retinal target areas (Fig. 2.1.5), and the thalamic reticular nucleus. Therefore NAAG is present in both excitatory (visual pathways) and inhibitory neurons (globus pallidus and thalamic reticular nucleus). In fact NAAG has also been found in cholinergic, noradrenergic, and serotonergic neuronal groups (Forloni et al., 1987; Moffett & Namboodiri, 2006). These findings place NAAG in most neuronal types in the CNS, which is in good agreement with its proposed role as a neuromodulatory agent involved in neurotransmitter release regulation. NAAG is expressed at exceptionally high levels in the medulla and spinal cord in the rat, suggesting an important role in somatosensory and motor functions, possibly including regulation of neurotransmitter release. NAAG expression also exhibits some species specificity. For example, large pyramidal neurons in neocortex typically express low levels of NAAG in the rat, but high levels of NAAG in the rhesus monkey (Fig. 2.1.6).

NAAG Catabolism

In 1987, Robinson and colleagues (1987) demonstrated NAAG hydrolyzing enzymatic activity by measuring hydrolysis of several radiolabeled NAAG substrates in lysed synaptosomal membrane preparations from rat forebrain. They assigned this activity the name *N*-acetylated α-linked acidic dipeptidase, or

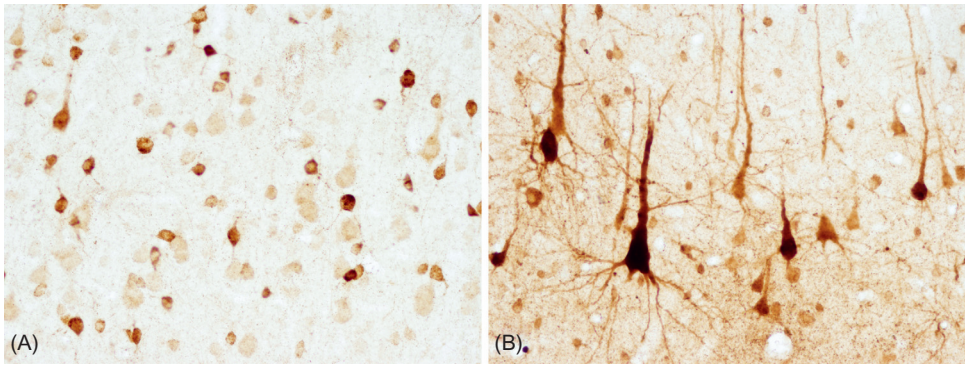

FIGURE 2.1.6 NAAG expression in layer V of neocortex. NAAG expression in rat neocortex was strong in interneurons and weak or absent in large pyramidal neurons (A). In contrast, in rhesus monkey large pyramidal neurons and smaller interneurons were immunoreactive for NAAG (B) (20× objective A and B).

NAALADase. The enzyme activity was found to require mono and divalent anions and was inhibited by the excitatory amino acid agonist, L-quisqualic acid, as well as polyvalent anions such as phosphate and sulfate. They demonstrated that the enzyme hydrolyzes the aspartate-glutamate peptide bond but does not remove the NH_2-terminal acetate group. The enzyme was subsequently identified as identical to glutamate carboxypeptidase II (GCPII; Blakely et al., 1988), as well as the prostate cancer marker prostate-specific membrane antigen (Carter et al., 1996). Using a cloned GCPII, Luthi-Carter and coworkers (1998) reported that NAAG is hydrolyzed by GCPII. Inhibitors of GCPII such as β-NAAG, quisqualic acid, and 2-(phosphonomethyl) pentanedioic acid (PMPA) significantly inhibited the hydrolysis of NAAG. Subsequent investigations using mice lacking GCPII indicated that there is another enzyme in the brain designated GCPIII, which can carry out the hydrolysis of NAAG in the absence of GCPII (Bacich et al., 2002; Bzdega et al., 2004). GCPII knockout mice did not show any neurological abnormalities indicating that GCPIII in the brain was sufficient for metabolizing NAAG. A comparative study showed that several inhibitors of GCPII also inhibited GCPIII, and the two enzymes showed different pH and salt concentration dependence and substrate specificities indicating that these homologs might play distinct biological roles in the CNS (Hlouchova et al., 2007). Studies using recombinant GCPII and GCPIII indicated that GCPII is responsible for the majority of NAAG hydrolysis in the human brain (Sacha et al., 2007). Immunohistochemical studies indicate that the cellular localization of GCPII in the brain is exclusively in astrocytes, but that it is not expressed in all astrocytes (Sacha et al., 2007). In addition to the CNS, GCPII is also expressed in peripheral organs including prostate, kidney, and intestine among others.

NAAG, GCPII Inhibitors, and Metabotropic Glutamate Receptors

Unlike classical neurotransmitters, application of NAAG to various regions of the brain often elicits no electrophysiological response, or mixed responses that include slow depolarization or slow hyperpolarization (Henderson & Salt, 1988; Riveros & Orrego, 1984; Jones & Sillito, 1992). Studying the actions of NAAG in the nervous system is confounded by the fact that the NAAG-hydrolyzing enzyme GCPII does not generate inert substances, but instead generates glutamate, the most prevalent excitatory neurotransmitter in the brain. This is very distinct from most extracellular neurotransmitter-inactivating enzymes such as acetylcholine esterase, which generate less active metabolites that can be taken up and recycled. Synaptically released NAAG is rapidly converted to NAA and glutamate, but it is still uncertain if the glutamate generated by GCPII localized on astrocytes is taken up by the astrocytes immediately, or if it is available for binding to cell-surface ionotropic or metabotropic glutamate receptors. This uncertainty has made the study of NAAG actions in the nervous system difficult, and this difficulty is exacerbated by the fact that it has been extremely difficult to determine what receptors NAAG acts on. Initially it was thought that NAAG acted on ionotropic glutamate receptors including the *N*-methyl-D-aspartate (NMDA) receptor (Westbrook et al., 1986), but the high levels required for activation called into question the physiological relevance. Subsequently NAAG was reported to act through metabotropic glutamate receptors (mGluR) to inhibit cyclic AMP production (Wroblewska et al., 1993). Over the ensuing years the connection to mGluR, particularly the mGluR3 subtype of metabotropic receptor, has been the primary focus of the majority of work on NAAG actions in the CNS. However, this connection has not been without controversy, again because NAAG

hydrolysis generates glutamate, and also because commercially available NAAG preparations have been found to be contaminated with low levels of glutamate that could be responsible for some of the reported actions (Chopra et al., 2009). For a review of the physiology of metabotropic glutamate receptors see Niswender and Conn (2010).

Because of the confounding issue of the high potency of glutamate at all types of metabotropic and ionotropic glutamate receptors, it has become standard procedure to study the actions of NAAG by inhibition of the hydrolytic enzyme GCPII. Limiting hydrolysis of NAAG theoretically leads to prolonged NAAG occupation of mGluR and reduced production of NAAG-derived glutamate (Wozniak et al., 2012). This approach, using GCPII inhibitors in conjunction with mGluR 2/3 receptor antagonists to block NAAG action at these receptors, has led to many interesting findings about possible NAAG actions. For example, inhibition of GCPII has shown significant protection of motor neurons in both *in vitro* and *in vivo* models of familial amyotrophic lateral sclerosis, and it was proposed that the decrease in extracellular glutamate is the mechanism involved in these observations (Ghadge et al., 2003; Thomas et al., 2003). It has also been hypothesized that altered glutamate neurotransmission in the brainstem is involved in pain perception in different animal models of pain (Burchiel et al., 1985; Kawamata & Omote, 1996) and GCPII inhibitors have shown protection against neuropathic pain supporting the above notion (Zhang et al., 2002, 2006; Yamada et al., 2012). Inhibition of GCPII has also shown promising results in the treatment of brain injury. Post-injury administration of GCPII inhibitor significantly reduced neuronal and astrocytic cell death in the hippocampus in a rat model of traumatic brain injury combined with a secondary hypoxic insult (Feng et al., 2012). Moreover, mice lacking GCPII showed significant protection against peripheral neuropathy and reduced infarct volume in an animal model of middle cerebral artery occlusion indicating the role of NAAG hydrolysis in peripheral neuropathy and stroke (Bacich et al., 2005).

There are a number of controversies that surround the above observations (reviewed in Johnson, 2011; Neale, 2011). The first involves a debate over whether or not the effects of GCPII inhibition involve NAAG, or the inhibitors themselves. Another controversy centers on whether NAAG actually acts through mGluR3 receptors, or works via mGluR2 receptors, or some other type of receptor. A widely accepted conclusion is that GCPII inhibitors increase NAAG levels in the brain and most or all of the observed effects of NAAG peptidase inhibition involve NAAG binding to the mGluR3 receptor subtype (Olszewski et al., 2012a; Neale, 2011). The evidence supporting this conclusion is substantial; however, this view has been challenged by a number of studies. For example, a microdialysis study testing the effect of 2-PMPA, a GCPII inhibitor, in a model of chronic pain (Nagel et al., 2006) concluded that while the anti-allodynic effect of 2-PMPA was observed, the NAAG concentration in dialysate did not reach a sufficient concentration to have any impact on metabotropic glutamate receptors. Two research groups have questioned the validity of the experimental results involving direct effects of NAAG based on their observation that the effect of NAAG in *in vitro* systems disappears when glutamate contamination in the commercial NAAG preparation is removed by further purification (Chopra et al., 2009; Fricker et al., 2009; Johnson, 2011). According to these investigators the observed effects are due to glutamate contamination, as well as contamination with other unknown compounds in commercially available NAAG preparations, and are not related to NAAG activity at any type of glutamate receptor. A review of these controversies has been published noting that many of the studies on NAAG actions at mGluR3 utilized re-purified NAAG, and the review concludes that the majority of studies indicate that NAAG is active at these receptors (Neale, 2011). Now that these controversies have been better delineated, and the genes for NAAG synthase have been identified, it is likely that studies in the near future will be able to come to more definitive conclusions on the actions of NAAG in the CNS, and possible activity at the mGluR3 receptor.

NAAG, mGluR, and Neurotransmitter Release Modulation

Metabotropic glutamate receptors are a diverse class of G-protein-coupled neuromodulatory receptors found throughout the CNS that regulate various processes including neuronal activity and neurotransmitter release (Niswender & Conn, 2010). mGluR3 receptors are present on presynaptic endings where they are in a position to modulate neurotransmitter release. Because NAAG is colocalized with virtually all major neurotransmitters including glutamate, GABA, dopamine, serotonin, norepinephrine, and acetylcholine, it is also in a position to regulate their release from neurons. In fact, several studies based on inhibition of GCPII and exogenous NAAG application have indicated that NAAG can act by inhibiting the release of neurotransmitters including glutamate and GABA from presynaptic terminals, most likely by an mGluR3-mediated mechanism (Zhao et al., 2001; Zhong et al., 2006). It is noteworthy that NAAG inhibits both excitatory and inhibitory neurotransmitter

release, and further that NAAG localization in GABAergic and glutamatergic neurons is distinct. In excitatory systems such as the visual pathways NAAG is present in neuronal cell bodies, dendrites, axons, and synaptic terminals, whereas in GABAergic systems such as the thalamic reticular nucleus and globus pallidus, NAAG is only present in neuronal cell bodies and dendrites, but not in axonal projections or synaptic terminals (Moffett, 2003). These findings suggest that NAAG is coreleased with glutamate from synaptic endings, but that it may only be released from somatic and dendritic compartments in GABAergic neurons. While the concentrations of NAAG used in earlier studies ranged from low to high micromolar, a recent study has shown that NAAG is extraordinarily potent in inhibiting KCl induced release of glycine from spinal cord synaptosomal preparations with a dose response in the 0.01 to 1.0 pmol range (Romei et al., 2012). Furthermore, this effect of NAAG was reversed by LY341495, an mGluR2/3 antagonist and also by β-NAAG, a structural analog of NAAG that blocks GCPII enzymatic activity. Also, glutamate was not very effective under the experimental conditions ruling out the possibility that glutamate contamination in the NAAG preparation was responsible for the effect. The extraordinary potency of NAAG in this system is difficult to explain. The authors are of the opinion that the relatively higher concentrations required in earlier studies have to do with the degradation of NAAG by GCPII located on astrocytes. However, the effective concentration of NAAG is so low in this system that this explanation is not entirely convincing. The authors further suggested that the NAAG receptors involved are most likely located in non-synaptic transmission areas where receptors generally exhibit higher affinity than receptors expressed in the areas of synaptic transmission. Clearly, future studies are required to establish this extraordinarily potent receptor mediated action of NAAG.

NAAG and Schizophrenia

One theory of schizophrenia involves dysregulated glutamate signaling in certain brain regions including prefrontal cortex. This theory was predicated on observations that so-called open-channel NMDA receptor antagonists including PCP, MK801, and ketamine, produce schizophrenia-like symptoms in animals, and exacerbate those symptoms in schizophrenia patients. Open-channel NMDA antagonists block the channel only when it has opened because they bind within the ion channel itself. It has been found that inhibition of NAAG hydrolysis using GCPII inhibitors has the ability to reverse the effects of PCP in experimental animals, and therefore it is thought that this may represent a novel method of treating schizophrenia. GCPII inhibition has shown promise in reducing both positive and negative symptoms in the rat and mouse (Olszewski et al., 2004; Takatsu et al., 2011; Zuo et al., 2012). More recently, this was found to be true for the dopamine-based model of schizophrenia in mice as well (Olszewski et al., 2012b). There is currently no consensus on the underlying mechanisms involved with one theory pointing to agonistic activity on mGluR3 receptors that can inhibit glutamate release (Zhao et al., 2001; Flores & Coyle, 2003; Zhong et al., 2006), and another that the decrease in free glutamate formation from NAAG after inhibition of GCPII plays a significant role in properly regulating glutamate release in the prefrontal cortex (Ghadge et al., 2003; Cavaletti & Slusher, 2012). In a more recent study testing the effect two GCP inhibitors in an animal model of schizophrenia, there was little or no correlation between the observed effect on motor activation and inhibition of GCPII activity *in vivo*. Currently there is also no general consensus on which subtype of mGluR is involved in the effects of GCPII inhibitors, with some evidence pointing to mGluR3, and other evidence pointing to mGluR2 (Fell et al., 2008; Woolley et al., 2008).Therefore, future studies in different model systems are needed to establish the mechanisms by which GCPII inhibition can reverse the effects of open-channel NMDA antagonists.

NAAG as a "Pro-transmitter"

In light of the diverse and contradictory findings in multiple model systems, it is difficult to come to a consensus on the controversies surrounding the actions of NAAG. This is indicated by only a very brief mention of NAAG in a recent comprehensive review on metabotropic glutamate receptors (Nicoletti et al., 2011). It is possible that part of the controversy is due to the diverse nature of the model systems used and partly due to the complex but closely related nature of the different members of the metabotropic glutamate receptor family. However, it is also possible that the current views of NAAG and metabotropic receptor activation are incomplete. There is one view of NAAG that has the potential to explain some of the reported discrepancies; namely that a major function of NAAG is to generate glutamate (Tsukamoto et al., 2007). If NAAG is viewed as a "pro-transmitter" rather than a neurotransmitter modulator, then the release of NAAG would generate local increases in extracellular glutamate in specific brain systems where GCPII was active, for example, at presynaptic, glial, or extrasynaptic glutamate receptors. In this view GCPII is the

activating enzyme, glutamate is the active agent, and NAAG is acting as a pro-transmitter. Therefore, blocking GCPII blocks a specific type of glutamate release, perhaps at specialized synapses that contain GCPII, or at extrasynaptic sites where synaptically released glutamate is excluded. Because glutamate is more potent than NAAG at all types of ionotropic and metabotropic glutamate receptors, this view has some logical appeal. This interpretation could explain how blocking NAAG hydrolysis could prevent pain transmission in the brainstem, and why preventing NAAG breakdown would reduce brain injury due to ischemia or motor neuron disease. Future studies will clarify these issues and advance our understanding of the functional roles of NAAG in the CNS. Availability of mice in which the biosynthetic enzyme for NAAG is knocked out will be instrumental in this regard. It is safe to say that research into NAAG actions in the CNS is still in its early stages, and that major discoveries await future studies.

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