Research Report

Methamphetamine-induced neuronal protein NAT8L is the NAA biosynthetic enzyme: Implications for specialized acetyl coenzyme A metabolism in the CNS

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ABSTRACT

N-acetylaspartate (NAA) is a concentrated, neuron-specific brain metabolite routinely used as a magnetic resonance spectroscopy marker for brain injury and disease. Despite decades of research, the functional roles of NAA remain unclear. Biochemical investigations over several decades have associated NAA with myelin lipid synthesis and energy metabolism. However, studies have been hampered by an inability to identify the gene for the NAA biosynthetic enzyme aspartate N-acetyltransferase (Asp-NAT). A very recent report has identified Nat8l as the gene encoding Asp-NAT and confirmed that the only child diagnosed with a lack of NAA on brain magnetic resonance spectrograms has a 19-bp deletion in this gene. Based on in vitro Nat8l expression studies the researchers concluded that many previous biochemical investigations have been technically flawed and that NAA may not be associated with brain energy or lipid metabolism. In studies done concurrently in our laboratory we have demonstrated via cloning, expression, specificity for acetylation of aspartate, responsiveness to methamphetamine treatment, molecular modeling and comparative immunolocalization that NAT8L is the NAA biosynthetic enzyme Asp-NAT. We conclude that NAA is a major storage and transport form of acetyl coenzyme A specific to the nervous system, thus linking it to both lipid synthesis and energy metabolism.

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1. Introduction

Acetyl coenzyme A (acetyl-CoA) is a central metabolite required for energy derivation and storage, and for acetylation reactions controlling gene expression and protein stability. Acetyl-CoA is the only form of acetate that can be utilized directly for cytoplasmic and nuclear protein acetylation reactions, including histone acetylation. One acetylation reaction involving acetyl-CoA that may be unique to the nervous system is the acetylation of aspartate to produce N-acetylaspartate (NAA). NAA is one of the most concentrated metabolites in the human brain (8-10 mM), and yet more than 50 years after its discovery (Tallan et al., 1954), the physiological functions served by NAA are still a matter of debate and ongoing research. NAA is localized predominantly in neurons and appears as the most prominent peak in proton magnetic resonance spectrograms of the human brain. These characteristics have enabled the use of NAA as a surrogate marker for neuronal loss or dysfunction in a variety of neuropathological conditions (Tsai and Coyle, 1995). NAA levels are decreased in specific brain areas in many neurological disorders, including Alzheimer disease, multiple sclerosis, schizophrenia, stroke and brain injury, but it is not clear if the reductions are due to neuronal cell loss, neuronal dysfunction or altered NAA metabolism (Moffett et al., 2007). Isolation of the NAA biosynthetic enzyme, aspartate N-acetyltransferase (Asp-NAT), has so far been unsuccessful because the enzyme appears to be associated with a large molecular weight protein complex in brain homogenates (Ariyannur et al., 2008; Madhavarao et al., 2003). Therefore, the Asp-NAT gene has remained unidentified, precluding knockout and knockdown studies on the regulation and functions of Asp-NAT and the biosynthesis of NAA under normal and pathological conditions.

The potent CNS stimulant methamphetamine modulates the activity of dopaminergic neurons, including mesolimbic neurons projecting from the ventral tegmental area to the nucleus accumbens (Koob et al., 1998). Methamphetamine acts to increase extracellular dopamine levels in the brain by facilitating dopamine release and by inhibiting reuptake through the dopamine transporter in axonal terminals (Giros et al., 1996). Recently, using the PCR-select cDNA subtraction method, Niwa et al. (2007) have shown that methamphetamine increased expression of a novel protein named “Shati” in the nucleus accumbens of mouse brain, specifically via activation of D1 and D2 dopamine receptors. This protein was identified as N-acetyltransferase-8 like protein (NAT8L) which contains a conserved sequence of the GCN5 superfamily of N-acetyltransferases. Shati was also found to be expressed predominantly in neurons (Niwa et al., 2007) and counteracted the methamphetamine induced inhibition of dopamine reuptake (Niwa et al., 2008). Recently we have shown that antipsychotic medications, which act by blocking dopamine D2 receptors, increase NAA synthesis in SH-SY5Y human neuroblastoma cells (Arun et al., 2008). These findings in conjunction with the identification of Shati/NAT8L as a GCN5 family member that is responsive to dopamine agonists suggested to us that it was an ideal candidate for the NAA biosynthetic enzyme. Therefore we initiated studies to address this question via a number of techniques including cloning and expression of the gene in vitro. Very recently it has been reported that the Nat8l gene is mutated in the single reported case of a patient with no detectable NAA signal in magnetic resonance spectrograms (Wiame et al., 2010). These investigators also determined that NAT8L is highly specific for the acetylation of aspartate, and they concluded that NAT8L is the NAA biosynthetic enzyme Asp-NAT. In research conducted concurrently we confirm and extend these results, and discuss the implications for specialized acetyl-CoA metabolism in the CNS.

2. Results

2.1. Cloning, expression and specificity for aspartate as substrate

We cloned and expressed the mouse Nat8l gene in order to measure Asp-NAT activity. Mouse Nat8l DNA was chemically synthesized, cloned in the mammalian plasmid expression vector pcDNA3.1(-)/myc-His (B) and expressed in HEK-293 cells. Asp-NAT activity was determined using aspartate and glutamate as substrates as described earlier (Ariyannur et al., 2008). Asp-NAT enzyme activity in Nat8l-transfected cells was more than 300 times that of the mock-transfected controls and showed an extraordinarily high enzymatic specificity for aspartate, with less than 1% of enzymatic activity using glutamate as substrate (Fig. 1). Earlier studies have shown that exclusive enzymatic specificity for aspartate is the most characteristic feature of Asp-NAT (Ariyannur et al., 2008;
Madhavarao et al., 2003). Using His tag antibodies, the Nat8l transfected preparation showed a single protein band with a molecular weight of approximately 33–35 kd (Fig. 1, arrow in insert).

2.2. Effect of methamphetamine treatment on NAA synthesis in SH-SY5Y human neuroblastoma cells

Further investigations provided additional evidence that NAT8L is Asp-NAT, including the demonstration that methamphetamine treatment significantly increased Asp-NAT activity in SH-SY5Y cells (Fig. 2). SH-SY5Y human neuroblastoma cells are a model system for studying dopaminergic neurotranschemistry (Brenner-Lavie et al., 2008; Presgraves et al., 2004) and NAA synthesis (Arun et al., 2006, 2008). NAT8L expression has been shown to be increased upon methamphetamine treatment (Niwa et al., 2007) and Asp-NAT activity has been shown to be increased in SH-SY5Y cells after treatment with dopamine receptor blockers (Arun et al., 2008). These findings together link Asp-NAT expression and activity to dopamine receptor activity. In the current study the maximum increase in Asp-NAT activity in SH-SY5Y cells was found at 1 μM methamphetamine at 24 h. At this concentration we detected a significant increase of approximately 2 fold \( (p<0.05) \) in Asp-NAT activity in SH-SY5Y cell homogenates after 24 h of incubation as compared with untreated controls.

2.3. Molecular homology modeling

Molecular homology modeling studies show that only the amino acid aspartate, but not glutamate, can fit into the active site pocket for the transfer of an acetyl group from acetyl-CoA to aspartate (Figs. 3 and 4). Aspartate occupying the binding pocket of NAT8L makes a total of 7 hydrogen bonds with protein groups. The N-terminal nitrogen is stick representation of amino acid residues in the Asp-NAT active site (atom color code: carbon=white; oxygen=red; nitrogen=blue and sulfur=yellow). Aspartate is shown by ball and stick representation in the binding pocket (color coded: carbon=green; oxygen=red and nitrogen=blue). Acetyl-CoA is shown in stick representation with cornflower blue bonds and magenta bonds for the acetate group to be transferred to the N-terminus of aspartate. Critical amino acid residues making hydrogen bonds with aspartate are labeled. Amino acid residues C160 and Y173 are also labeled.

Fig. 2 – Effect of methamphetamine on Asp-NAT activity in SH-SY5Y human neuroblastoma cells. The methamphetamine dose response after 24 h of incubation is shown. The data are means±SEM from four different experiments done in triplicates. Asp-NAT activity is measured as the amount of the product NAA formed and expressed in picomoles of NAA/hr/mg protein. At 1 μM methamphetamine, there is a significant increase in the Asp-NAT activity of approximately 2 times \( (*p<0.05) \).

Fig. 3 – Molecular modeling of the Asp-NAT/NAT8L active site. Stick representation of amino acid residues in the Asp-NAT active site (atom color code: carbon=white; oxygen=red; nitrogen=blue and sulfur=yellow). Aspartate is shown by ball and stick representation in the binding pocket (color coded: carbon=green; oxygen=red and nitrogen=blue). Acetyl-CoA is shown in stick representation with cornflower blue bonds and magenta bonds for the acetate group to be transferred to the N-terminus of aspartate. Critical amino acid residues making hydrogen bonds with aspartate are labeled. Amino acid residues C160 and Y173 are also labeled.

Fig. 4 – A ribbon representation of the homology model of NAT8L (residues from L143 to L283 are shown). The α-helical segments are shown in red, β-strand segments are shown in magenta, and loop regions are shown in gray. Coenzyme A is given in stick representation and the docked aspartate is shown in ball-and-stick representation. The model indicates the restricted pocket size of the Asp-NAT binding site which excludes amino acids larger than aspartate.
positioned at a distance of 4.0 Å from the carbon atom of the acetate group (shown as magenta) bonded to the sulfur residue of coenzyme A in order to transfer the acetate group. R209 makes a stable salt bridge with the carboxyl side chain of Asp, whereas K249 makes a hydrogen bond as well as favorable electrostatic interactions with the C-terminal carboxyl. In order for the catalyzed reaction to occur aspartate must be held in a stable position, and this is accomplished by the hydrogen bonds which hold the substrate in a correct position and orientation for the acetate transfer from acetyl-CoA to the N-terminus of aspartate. R209 is stabilized in position by the stacking interaction between Y173 and the planar part of the side chain of R209. The C160 residue which is located at a distance of 10 Å from aspartate is also labeled in Fig. 4. Minimum energy calculations indicate that only aspartate can fit into the pocket for the transfer of the acetyl group. The longer side chain of glutamate would be blocked by R209 and would be in the wrong orientation for bond formation to occur. The mechanism of the acetate transfer from acetyl-CoA to aspartate is predicted to be the same as proposed from the crystal structure of N-acetyl-L-glutamate synthase from Neisseria gonorrhoeae (Shi et al., 2008) without involving cysteine.

A ribbon model of NAT8L from amino acid residues 143 to 283 is given in Fig. 5. The model demonstrates the narrow reactive site of Asp-NAT, and the orientation of acetyl-CoA and aspartate within it. The binding site is composed of both α-helical (red) and β-sheet (magenta) segments.

Fig. 5 – Comparative immunohistochemical localization of NAT8L and NAA in rat forebrain. Asp-NAT was strongly expressed in neocortical pyramidal neurons (A), which also have strong NAA-immunoreactivity (B). Asp-NAT was moderately to strongly expressed in dentate gyrus granule cells (C), which also exhibited moderate to strong immunoreactivity for NAA (D). Neurons in the supraoptic nucleus and hypothalamus were strongly immunoreactive for Asp-NAT (E) and NAA (F). Bar in f = 120 μm.
2.4. Comparative immunohistochemistry

Asp-NAT/NAT8L and NAA immunoreactivities correlate very well in the rat brain, with primary localization in neurons, including cortical pyramidal neurons and granule cells of the hippocampus (Fig. 5). This pattern of localization in neurons was observed throughout the brain, including in mesencephalic dopaminergic neurons of the substantia nigra and ventral tegmental area (Fig. 6). NAT8L immunoreactivity was also observed in oligodendrocytes, confirming previous studies showing that oligodendrocytes can synthesize NAA (Bhakoo and Pearce, 2000).

2.5. Colocalization of Asp-NAT with mitochondrial marker

MitoTracker Red CMXRos is a mildly thiol-reactive chloromethyl moiety for labeling mitochondria, and is retained in mitochondria after fixation. We used this fluorescent mitochondrial probe in conjunction with anti-NAT8L polyclonal antibodies to determine the degree to which the two markers colocalized in SH-SY5Y neuroblastoma cells. SH-SY5Y human neuroblastoma cells have been shown to synthesize NAA, indicating that they express Asp-NAT (Arun et al., 2008, 2009). Figs. 7 and 8 show that the mitochondrial marker and NAT8L antibodies are colocalized in punctate structures that were excluded from the cell nuclei. The immunoreactivity against NAT8L was more widespread in the cytoplasm than that of the mitochondrial marker dye, further confirming that Asp-Nat is present in both cytoplasm and mitochondria. Because SH-SYSY cells natively express Asp-NAT, these results are likely to be more relevant than colocalization studies done in transfected cells that over-express the protein.

3. Discussion

NAA is one of the most concentrated organic molecules in the human brain, but the biological purpose of this exceptionally high concentration has eluded researchers to date. At a concentration of 10 mM or greater in the CNS (Inglese et al., 2008), NAA may be second only to glutamate in abundance. A substantial body of biochemical evidence has suggested that NAA synthesis is linked to both brain energy metabolism, and brain lipid synthesis and myelination (reviewed in Moffett et al., 2007). However, controversies remain over these putative functions, as well as the subcellular localization of NAA synthesis. Facets of NAA synthesis that are not in dispute include the fact that it is synthesized via Asp-NAT predominantly in neurons from the starting substrates aspartate and acetyl-CoA. Aspartate and acetyl-CoA are both produced readily in mitochondria, and the aspartate that is synthesized in the mitochondrial matrix is exported to the cell cytoplasm. Acetyl-CoA can not cross the cell membrane.

Fig. 6 – Asp-NAT (NAT8L) and NAA expression in mesencephalic dopaminergic areas. Asp-NAT was present in neurons in the substantia nigra compact region (SNC; A), and ventral tegmental area (VTA; C). Immunoreactivity for protein-coupled NAA was similar in both the SNC (B) and VTA (D). Abbreviations: SNr = substantia nigra reticular part, mp = mamillary peduncle. Bar in d = 120 μm.
the mitochondrial membrane system, and must be converted to citrate in the tricarboxylic acid cycle in order to be exported to the cytoplasm. If NAA is synthesized in neuronal mitochondria, it would represent an exportable molecule that conveyed both acetate and aspartate to the cytoplasm. Because NAA synthesis requires the utilization of acetyl-CoA on an equimolar basis, it is logical to conclude that NAA is synthesized when acetyl-CoA is in excess of the requirements for other critical physiological functions. This may be analogous to the use of acetyl-CoA for energy storage in the form of lipids such as triglycerides in adipose tissue.

The present studies show that N-acetyltransferase activity for aspartate in Nat8L-transfected cells is over 300 times that of the mock transfected controls, with less than 1% of detectable enzymatic activity using glutamate as substrate. This high degree of specificity toward aspartate is characteristic of Asp-NAT (Truckenmiller et al., 1985), and unique among acetyltransferase enzymes. The amino acid glutamate is only one carbon atom longer than aspartate, and yet enzyme assays in transfected cells demonstrated the strong specificity of Nat8L for aspartate as the acetyl group acceptor. Studies have shown that partially purified Asp-NAT exhibits less than 3% activity against glutamate, glutamine or asparagine (Madhavarao et al., 2003). No other enzyme has been reported to exhibit high N-acetyltransferase activity with acetyl-CoA and aspartate as the preferred substrates.

Additional evidence that indicates that Nat8L is Asp-NAT comes from molecular homology modeling studies showing that only the amino acid aspartate, but not glutamate, can fit into the active site pocket for the transfer of acetyl groups from acetyl-CoA to aspartate (Figs. 3 and 4). The N-terminal nitrogen of aspartate is predicted to approach the carbon atom of the acetate group of acetyl-CoA at a distance of 4.0 Å facilitating the transfer of the acetate group to the N-terminus of aspartate. Minimum energy calculations and molecular

Fig. 7 – Colocalization of Asp-NAT with a mitochondrial marker. Anti-NAT8L antibodies were used to show native Asp-NAT localization in SH-SYSY human neuroblastoma cells, a continuous cell line that has been used to study NAA biosynthesis previously. NAT8L immunoreactivity was generally punctate in nature and was observed predominantly in the cytoplasm of SH-SYSY cells (pointers in A). The mitochondrial marker MitoTracker Red CMXRos also exhibited a punctate staining pattern in the cytoplasm (pointers in B). Cell nuclei are shown stained for DAPI in (C). In the merged image, substantial colocalization was seen between NAT8L immunoreactivity and the mitochondrial marker (yellow to orange staining; pointers in D).
modeling studies show that among amino acids, only aspartate can fit into the pocket for the transfer of the acetyl group. The longer side chain of glutamate would be blocked by R209, and would be in the wrong orientation for bond formation to occur.

Methamphetamine is chemically similar to dopamine, and acts as both a dopamine release stimulant and an uptake blocker that prolongs the actions of dopamine in the synaptic cleft (Fleckenstein et al., 2007). Previous work with NAT8L showed strong upregulation in the nucleus accumbens following methamphetamine treatment (Niwa et al., 2007). Antipsychotic drugs that act as dopamine receptor blockers increase NAA synthesis in SH-SYSY human neuroblastoma cells (Arun et al., 2008). SH-SYSY cells have characteristics of dopaminergic neurons, and have been shown to synthesize NAA from labeled aspartate or malate (Arun et al., 2009). In the current study methamphetamine treatment significantly increased Asp-NAT activity in SH-SYSY cells by approximately 2 fold at a dose of 1 μM, providing further evidence that NAT8L is Asp-NAT, and that the expression or activity of Asp-NAT is regulated by dopamine modulating agents.

One additional piece of indirect evidence indicating that NAT8L is Asp-NAT comes from immunohistochemical localization studies that show NAT8L and NAA immunoreactivities correlate very well in the rat brain (Figs. 5 and 6). Using polyclonal antibodies to an NAT8L-specific peptide sequence and highly purified antibodies to protein-coupled NAA, it was observed that the biosynthetic enzyme and its product are localized primarily in neurons throughout the brain, including cortical pyramidal neurons, hippocampal granule cells, and mesencephalic dopaminergic neurons of the substantia nigra and ventral tegmental area. In addition to neurons, NAT8L was also observed in oligodendrocytes, supporting previous in vitro observations that oligodendrocytes can produce NAA (Bhakoo and Pearce, 2000).

The subcellular localization of NAT8L was examined by immunofluorescence colocalization studies using the anti-NAT8L antibodies in SH-SYSY cells. These human neuroblastoma cells have been shown to synthesize NAA, and in the current studies we have confirmed that they express Asp-NAT. By using the SH-SYSY cell system we could image the localization of NAT8L without the need to transfect cells with the Nat8l gene, possibly providing a more biologically relevant expression pattern. Supporting the findings in a number of previous investigations we observed that the enzyme is expressed both in mitochondria and in the cytoplasm of SH-

Fig. 8 – High magnification colocalization of Asp-NAT and mitochondrial marker. Higher magnification images of NAT8L colocalization with the mitochondrial marker MitoTracker Red in SH-SYSY cells. The NAT8L immunoreactivity was present in both cytoplasm and mitochondria. Colocalization between the mitochondrial marker and NAT8L is indicated by a yellow to orange color in the merged image (D). Asp-NAT localization in the cytoplasm is indicated by green in the merged image.
SY5Y cells (Figs. 7 and 8). This dual compartment localization pattern is consistent with all previous reports on the subcellular localization of Asp-NAT which have shown enzyme activity in mitochondrial and microsomal fractions. A recent report by Wiame et al. (2010) showed an exclusive endoplasmic reticulum localization for transfected Asp-NAT. The methods employed by Wiame et al. to determine the subcellular localization of Asp-NAT involved expression in HEK293T cells and primary neurons in culture by viral transfection of the gene. This technique is very useful to ascertain enzymatic activity, but it is not the most reliable method of determining the native subcellular localization of endogenously encoded and translated proteins. It is possible that this artificial expression system resulted in the translated protein remaining in the endoplasmic reticulum, rather than being transported through the mitochondrial protein import system (TOM/TIM complexes). Further investigations will be required to resolve this issue.

Our initial identification of NAT8L as Asp-NAT was published online as an abstract for the annual meeting of the Society for Neuroscience (Ariyannur et al., 2009), and shortly thereafter the article by Wiame et al. (2010) appeared as an advance online publication in Biochemical Journal. In transfusion studies Wiame and colleagues found that expression of NAT8L protein in HEK293T cells conferred the ability to synthesize NAA from acetyl-CoA and aspartate, with high specificity for aspartate as the acetate acceptor. Importantly, these investigators showed that a 19-bp deletion in the Nat8l gene was present in the case of the only known patient with hypoaacetylaspartia, i.e., a lack of detectable NAA in brain spectrograms (Burlina et al., 2006; Wiame et al., 2010). Based on their transfusion studies in HEK293T cells and primary neuronal cultures, and noting the lack of a mitochondrial targeting sequence in the Nat8l gene, Wiame and colleagues concluded that the expressed protein was present almost exclusively in the endoplasmic reticulum, and was not present in mitochondria. This conclusion is novel and contradicts virtually all previous biosynthetic and fractionation studies on this topic which have consistently shown dual localization in microsomal and mitochondrial fractions (Ariyannur et al., 2008; Arun et al., 2009; Goldstein, 1969; Lu et al., 2004; Madhavarao et al., 2003). Rather than trying to reconcile their findings with the previous body of work, these authors concluded instead that prior investigations into the subcellular localization of Asp-NAT from multiple laboratories are suspect for technical reasons.

Therefore, it is of interest to examine the arguments presented by Wiame and colleagues in some detail. Asp-NAT was originally reported to be present in a particulate fraction from brain homogenates, with activity being present both in crude mitochondrial and microsomal fractions (Goldstein, 1969). These conclusions were confirmed in other laboratories, but results were somewhat conflicting as to the predominant localization being within the cytoplasm (Lu et al., 2004), or within mitochondria (Ariyannur et al., 2008; Madhavarao et al., 2003). Based on the methods used to purify the different fractions by centrifugation, some results indicated predominant cytoplasmic synthesis, whereas others indicated predominant mitochondrial synthesis. Wiame et al., discounted the fractionation data pointing to mitochondrial localization, despite the fact that all investigators involved in the work concluded that Asp-NAT was found to some extent in both subcellular compartments.

In addition to the fractionation studies on Asp-NAT, Wiame and colleagues also discounted many investigations into the biochemistry of NAA synthesis that point to mitochondrial synthesis of NAA, and connections to brain energy metabolism (reviewed in Moffett et al., 2007). Among the many studies linking mitochondrial function to NAA synthesis, the pioneering studies of Patel and Clark (1979, 1980) were instrumental in demonstrating NAA synthesis in partially purified rat brain mitochondria. Among their many findings one is of particular importance for the current discussion; that NAA efflux from isolated brain mitochondria was linked to a source of acetyl-CoA, namely pyruvate. In the absence of pyruvate only aspartate efflux from mitochondria was observed (see Fig. 4 in Patel and Clark, 1979). However, with increasing concentrations of the acetyl-CoA precursor pyruvate, NAA efflux was increased in a dose-dependent manner, and aspartate efflux was concomitantly diminished. Wiame et al., suggested that this data is suspect because the NAA assay employed by Patel and Clark involved the use of a relatively crude enzyme preparation to degrade NAA into acetate and aspartate. They suggested that Patel and Clark may have been observing a different reaction in their mitochondrial preparations, for example a reaction with asparagine rather than NAA. However, the controls used by Patel and Clark, including the omission of pyruvate from the in vitro mitochondrial system, were carefully done and convincing. Wiame and colleagues also failed to note that the Clark laboratory continued this work in the 1990s and used HPLC to determine NAA levels (Bates et al., 1996; Clark, 1998). In these studies they found that inhibitors of each of the respiratory chain complexes reduced oxygen consumption, ATP levels and NAA synthesis, confirming their results with other methods.

Another line of evidence suggesting that some proportion of NAA synthesis occurs within mitochondria comes from radiolabeled metabolite studies in SH-SY5Y cells. Arun et al. (2009) found that when these cells are incubated with either radiolabeled aspartate or radiolabeled malate, labeled NAA can be recovered from the cells. The results with the two precursors of NAA, in conjunction with results obtained using an inhibitor of the enzyme aspartate aminotransferase, suggested that a substantial proportion of NAA synthesis occurred intra-mitochondrially. Wiame and colleagues argued that these biochemical results were also suspect because Arun et al. only identified the radioactive product by means of thin layer chromatography (TLC), a method that is not considered definitive. However, this is a misreading of the results from Arun et al. who performed TLC separation followed by high-performance liquid chromatography to confirm the identity of the product as radiolabeled NAA.

The final point that led Wiame and colleagues to conclude that Asp-NAT is exclusively localized in the endoplasmic reticulum was the lack of an N-terminal mitochondrial targeting sequence associated with the Nat8l gene. However, the two targeting prediction programs they employed (TargetP and PSORT II) to determine the presence of potential mitochondrial targeting sequences (MTS) are not capable of identifying
many such sequences that are not located at the N-terminus of the protein; so-called internal targeting sequences (Neupert and Herrmann, 2007). Many mitochondrial membrane-associated proteins that are encoded in the nuclear genome and destined for insertion into the mitochondrial inner membrane do not contain N-terminal targeting sequences, but instead, have internal targeting signals distributed throughout the protein that are not cleaved upon import, and are difficult to identify (reviewed in Truscott et al., 2003). This type of mitochondrial targeting does not involve a specific peptide sequence, but instead is based on the overall amino acid sequence and physicochemical properties of the nascent protein. Subcellular targeting prediction programs that look at the entire amino acid sequence come to different conclusions concerning the probable subcellular localization of NAT8L. For example, the prediction program SubLoc (Chen et al., 2006) predicts a mitochondrial localization for NAT8L with an expected accuracy of 97% and reliability index (RI) of 6. Additionally, the prediction program HSLPred (Garg et al., 2005) predicts a mitochondrial localization with an expected accuracy of 67% and reliability index of 3. Another prediction program, CELLO (Yu et al., 2006) uses 4 types of sequence coding schemes including the amino acid composition, the di-peptide composition, the partitioned amino acid composition and the sequence composition based on the physicochemical properties of amino acids to predict subcellular localization. CELLO predicts that the highest probability subcellular localization for NAT8L is mitochondrial, with the second most probable location being plasma membrane. Combined with the available data on NAA synthesis in purified mitochondria, subfractionation studies, radiolabel incorporation studies, and our current localization results (Figs. 7 and 8) it is likely that some proportion of Asp-NAT is imported into the mitochondrial inner membrane where it forms part of a larger protein complex.

Wiame et al. also called into question the connections between NAA degradation and lipid synthesis in the brain. They noted that the genetic defect in the Nat8l gene (hypoacetylaspartia) which has so far only been reported in a single patient (Burlina et al., 2006) results in a mild hypomyelination as compared with that associated with Canavan disease, which results from mutations in the NAA-degrading enzyme aspartoacylase (Matalon et al., 1995). This led Wiame et al. to speculate that NAA may not have any role in myelination, despite numerous lines of evidence that indicate a lipogenic role for NAA, particularly during postnatal myelination in the CNS (D’Adamo et al., 1968; D’Adamo and Yatsu, 1966; Madhavarao et al., 2005; Patel and Clark, 1980; Wang et al., 2009). For example, it has been shown that the acetate moiety of NAA is converted to acetyl-CoA, and then incorporated into lipids in the brain (Mehta and Namboodiri, 1995). Further, it has been shown that radiolabeled NAA is transported between neurons and oligodendrocytes, where the acetate moiety is incorporated into myelin lipids (Chakroborty et al., 2001). Recent gene knockout studies on the mitochondrial aspartate-glutamate carrier (Aralar1) have indicated a dramatic reduction in brain NAA levels, and concomitant hypomyelination (Jalil et al., 2005), highlighting the connection between brain NAA levels and myelin lipid synthesis. A report of a single case of a child that has a genetic defect in Aralar1 also points to reduced brain NAA levels and hypomyelination as etiological aspects of the disorder (Wibom et al., 2009). Some investigators have questioned if the hypomyelination associated with Aralar1 deficiency is only secondary to neuronal loss (Wolf and van der Knaap, 2009), but it is likely that the lack of NAA synthesis and therefore lack of NAA transport to oligodendrocytes is a contributing factor in the pathophysiology of Aralar1 deficiency (Jalil et al., 2005). So why doesn’t hypoacetylaspartia, which results from a lack of Asp-NAT activity, and therefore a lack of brain NAA, result in a more severe hypomyelination than Canavan disease, which results from an inability to de-acetylate NAA?

One potential explanation for the difference in severity of hypomyelination between Canavan disease and hypoacetylaspartia is that acetyl-CoA is not utilized to make NAA in hypoacetylaspartia. In Canavan disease, a portion of brain acetyl-CoA is converted into NAA, but the acetate then becomes trapped in a substrate that can not be metabolized due to a lack of the degrading enzyme, and NAA is instead excreted in the urine (Kelley and Stamas, 1992). As such, oligodendrocytes become deficient in acetyl-CoA, which is critical for myelination and other essential developmental functions. This acetyl-CoA is lost, and can not be recovered leading to a more severe dysmyelination syndrome in Canavan disease patients. In hypoacetylaspartia, functional Asp-NAT is not expressed, and the acetyl-CoA required for myelin lipid synthesis is not sequestered in a compound that can not be further metabolized. This increased availability of acetyl-CoA may explain the difference in phenotypic severity between hypoacetylaspartia and Canavan disease. The generation of a hypoacetylaspartia mouse model (Asp-NAT−/−) will help answer these questions.

The link between acetyl-CoA availability and NAA synthesis may provide a clue to the primary function of NAA in the nervous system as a method for storing excess acetyl-CoA for later use, and for transporting the acetate moiety between neurons and oligodendrocytes. Because acetyl-CoA is critically involved in energy metabolism, lipid synthesis and other key cellular functions, we propose that the high levels of NAA in the brain represent a storage and transport form of this key metabolite. At a concentration of approximately 10 to 14 mM in the brain (Inglese et al., 2008), NAA represents a rich source of both acetate and aspartate for CNS metabolism. In conjunction with the exceptionally high concentration of the non-synaptic glutamate pool, the nervous system stores several critical precursors for energy metabolism. Aspartate can be converted to oxaloacetate, and glutamate to alpha keto glutarate by the action of the enzyme aspartate aminotransferase. Together with the acetate moiety of NAA, oligodendrocytes would have access to several key tricarboxylic acid cycle intermediates that can be used for energy derivation or lipid synthesis. The brain has a relatively low concentration of glycogen, which is stored predominantly in astrocytes. The level of glycogen in astrocytes is insufficient to supply metabolic energy for extended periods of time (Brown et al., 2004; Brown and Ransom, 2007). The high energy demands of the central nervous system must be met by some other energy storage system, and it is possible that NAA in combination with glutamate could provide an important local reservoir of stored energy. However, because many
neurons lack the NAA degrading enzyme aspartoacylase (Madhavarao et al., 2004) a substantial portion of NAA must be transported to oligodendrocytes before it can be metabolized. Neuronally derived NAA provides metabolically active oligodendrocytes with an acetyl-CoA precursor that can be used for many metabolic functions including myelin lipid synthesis.

The discovery of the gene for the NAA biosynthetic enzyme will have a major impact on Canavan disease research. Canavan disease is an autosomal genetic disorder that results in progressive leukodystrophy, paralysis and death, usually between 3 and 10 years of age. Canavan disease is caused by mutations in the gene that codes for the enzyme aspartoacylase (Le Coq et al., 2006; Matalon et al., 1993) (ASPA; EC 3.5.1.15), which hydrolyzes NAA to acetate and aspartate. Currently there are no effective treatments for this fatal disorder. The two primary metabolic effects of mutations in the gene for ASPA in Canavan disease patients are (1) buildup of NAA in the brain (Matalon et al., 1988) and (2) decrease of acetate availability in the brain (Madhavarao et al., 2005). Which of these two metabolic effects plays the critical role in the pathogenesis of CD still remains unclear. Nat8l gene knockout studies will be valuable for resolving this and other questions on the functional roles of NAA in the CNS.

In conclusion, the identification of NAT8L as the NAA biosynthetic enzyme will greatly accelerate research into the functions served by NAA in the brain, and will provide the tools necessary to study the connections between NAA and brain acetyl-CoA and energy metabolism. Evidence points strongly to NAA as a storage and transport form of acetyl-CoA that is specific to the nervous system, an energy demanding organ system that lacks substantial glycogen reserves. Based on recent results with dopamine blockers and methamphetamine, the identification of NAT8L as Asp-NAT is also likely to have profound implications for the study of dopaminergic neurotransmission and related neurological disorders. Both clinical and non-clinical applications of this gene will be essential for directly studying the functional roles of NAA biosynthesis in a wide range of neuropsychiatric disorders, as well as possible roles in methamphetamine actions and addiction. The identification of Nat8l as the gene for Asp-NAT will herald an exciting new era in the neurobiology of NAA with potential impact on diagnosis and treatment of a host of neurological and neuropsychiatric disorders.

4. **Experimental procedures**

4.1. **Gene synthesis, cloning, transfection and specificity for aspartate**

The mouse Nat8l gene (Genbank Accession: NM_001001985) was synthesized commercially (GenScript Inc.). The gene was cloned into the pcDNA 3.1(−)/myc-His (B) expression vector with hexa-histidine tag (His-tag) oriented to be on the C-terminus. An additional sequence was added to the open reading frame at the 5’ end (CTCGAG), replacing the stop codon at the 3’ end (TAAGCTT) in-frame, to facilitate incorporation into the plasmid expression vector. The synthesized mouse Nat8l open reading frame (900 bp) was inserted between Xhol and HindIII restriction sites of pcDNA 3.1 vector (CloneEZ Recombination Procedure). HEK 293 cells were grown in growth medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 Units/ml of penicillin, and 100 μg/ml of streptomycin) at 37 °C in 5% CO2. Approximately 1.5×10^9 cells were plated in 25 cm² flasks 24 h before transfection, by which time they reached 50-70% confluence. Cells were washed once with 3 ml of OptiMEM (Invitrogen, Carlsbad, CA), then 3 ml of OptiMEM was added to each flask and the flasks returned to the CO2 incubator for 20-30 min before transfection. Four micrograms of recombinant Nat8l plasmid DNA was used for transfection of each 25 cm² flask. A transfection mix was prepared by adding 4 μg of recombinant plasmid DNA (for mock transfection controls, 4 μg of vector DNA was used) and 20 μl of Lipofectamine 2000 (Invitrogen) to 500 μl of OptiMEM according to the manufacturer’s instructions. After incubation at room temperature for 20 min, the transfection mix was added to the cells. After 4–6 h at 37 °C, the media containing the transfection mix was removed and 6 ml of growth medium was added. Cells were harvested 48 h after transfection.

Transfected cell pellets (approximately 20 μl) were washed twice with 1 ml of phosphate buffered saline (PBS), resuspended in 100 μl of homogenization buffer containing 1 mM dithiothreitol and 1 mM CHAPS (Calbiochem) and Protease Inhibitor Cocktail (Sigma-Aldrich) and homogenized in an ultrasound cell homogenizer, 20 cycles/s, 1 s pulse X 10. Asp-NAT and Glu-NAT assays were done separately in mock transfected cell homogenates and Nat8l-transfected cell homogenates using a radiometric assay as described previously (Ariyanur et al., 2008; Madhavarao et al., 2003) with minor modifications. Briefly, 30 μl of the total assay volume contained a final concentration of ~0.4 mM L-aspartate/L-glutamate (specific activity ≈44 μCi/μmol) and 0.67 mM acetyl-CoA. Assays were done at 37 °C for 1 h and stopped by adding an equal amount of ethanol containing 3 mM NAA or NAG (ice cold). The reaction mix was centrifuged and supernatant subjected to thin layer chromatography. The reaction products were detected using phosphor-imaging and expressed in pmol of NAA/hr/mg protein. Protein estimation was done using a modified Lowry method (DC protein assay, BioRad).

4.2. **Western blotting**

SDS PAGE was done using 10% Tris-glycine pre-cast gels (Invitrogen, Carlsbad CA) with equal protein concentrations of transfected HEK 293 cell homogenates in PBS containing 1 mM dithiothreitol, 1 mM CHAPS (Calbiochem) and Protease Inhibitor Cocktail (Sigma-Aldrich). The gel was electro-blotted onto PVDF membrane for 90 min. The blot was blocked against non-specific binding, and treated overnight with rabbit anti-His-tag antibody (1:1000 dilution) to detect histidine-tagged NAT8L protein. After washing, the membrane was treated with goat anti-rabbit secondary antibody coupled to HRP (KPL, Gaithersburg, MD), and developed using Sigma-Fast DAB reagent tables (Sigma-Aldrich). The blot was dried and imaged using a Fuji Image Reader (LAS 3000).
4.3. **Effect of methamphetamine treatment on NAA synthesis in SH-SY5Y human neuroblastoma cells**

Briefly, SH-SY5Y human neuroblastoma cells were grown in 25 cm²-treated cell culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂/95% air. Cells were sub-cultured when flasks reached approximately 90% confluence.

In order to study the effect of methamphetamine treatment, we used SH-SY5Y human neuroblastoma cells. SH-SY5Y cells exhibit characteristics of both noradrenergic and dopaminergic neurons, and express the dopamine synthesizing enzyme tyrosine hydroxylase, dopamine transporters, as well as dopamine receptors (Presgraves et al., 2004), including the dopamine D2 receptor (Dziedzicka-Wasylewska and Solich, 2004). Moreover, SH-SY5Y cells have been employed as an in-vitro model system for studying the biosynthesis of NAA and the related dipeptide N-acetylaspartylglutamate (NAAG) (Arun et al., 2006, 2008). Methamphetamine concentrations ranging from 0.3 μM to 3 μM were incubated with SH-SY5Y cells (70% confluence or greater) for varying time periods (6 to 24 hrs). For studying the effect of methamphetamine, we measured Asp-NAT enzyme activity in cell homogenates via radiometric enzyme assay using 14C-aspartate as an in-vitro model system for studying the biosynthesis of NAA and the related dipeptide N-acetylaspartylglutamate (NAAG) (Arun et al., 2006, 2008). Methamphetamine concentrations ranging from 0.3 μM to 3 μM were incubated with SH-SY5Y cells (70% confluence or greater) for varying time periods (6 to 24 hrs). For studying the effect of methamphetamine, we measured Asp-NAT enzyme activity in cell homogenates via radiometric enzyme assay using 14C-aspartate as described earlier for mammalian brain preparations (Ariyannur et al., 2008; Madhavarao et al., 2003) with minor modifications.

After washing twice with phosphate buffered saline (10 μM; PBS), a 10% homogenate of the SH-SY5Y cells was prepared in PBS (pH 7.2) containing 1 mM DTT, 1 mM CHAPS (10 mM; PBS), a 10% homogenate of the SH-SY5Y cells was prepared in PBS (pH 7.2) containing 1 mM DTT, 1 mM CHAPS and protease inhibitor cocktail, using an ultrasonic cell homogenizer (20 cycles/s, 1 s pulses ten times). Briefly, 50 μl of the total assay volume contained a final concentration of ~0.2 mM l-aspartate (specific activity ~217 μCi/μmol) and 0.67 mM acetyl-CoA. Assays were done at 37 °C for 1 h and stopped by adding an equal amount of ethanol containing 3 mM NAA (ice cold). The reaction mixture was centrifuged and supernatant was subjected to thin layer chromatography. The reaction products were detected using phosphor-imaging, and expressed in pmoles of NAA/hr/mg protein. Protein estimation was done using a modified Lowry method (DC protein assay, BioRad).

4.4. **Homology modeling**

The protein sequence of N-acetyltransferase 8-like [Nat8l, Mus musculus] (NCBI Reference Sequence: NP_001001985.3 (Niwa et al., 2007)) was searched against the sequence of the protein structures in the Protein Data Bank (http://www.rcsb.org) using sequence alignment in Molecular Operating Environment, Molecular Information Simulation (MOE; Chemical Computing Group). Amino acid residues from 143 to 283 of Nat8l matched with amino acid residues from 17 to 159 of the crystal structure of acetyltransferase from Streptococcus Agalactiae (PDB ID: 2Q7B; [http://www.rcsb.org]). Using this sequence alignment and the crystal structure as a template (residues 143 to 283), a 3D homology model for NAT8L was built and energy minimized using MOE software. By structure alignment of the homology model with the crystal structure of ribosomal s18 n-alpha-protein acetyltransferase in complex with Coenzyme A (PDB ID: 2CN7; [http://www.rcsb.org]) (Vetting et al., 2008), the coordinates for the bound structure of CoA were transferred to the homology model. Because residue C160 In the homology model is located at a distance of 10 Å from the acetyl group of CoA, the catalytic mechanism does not involve C160 as suggested by the ping-pong catalytic mechanism. However, from the reported crystal structure of N-acetylglutamate synthase (Shi et al., 2008), a one-step catalysis is proposed in which the a-amino nitrogen atom of l-glutamate is 2.5 Å from the carbon atom of the acetyl group, positioned to attack the acetyl group directly. We assumed single step catalysis in docking the substrate (aspartate) into the binding site of the homology model for NAT8L. In the reported crystal structure (2Q7B), a citrate anion was bound to the active site. The position of the citrate anion was used to manually dock the substrate, aspartate. The orientation of the aspartate was such that the amino terminal will be at an attacking distance of 4.0 Å from the carbon atom in acetyl-CoA containing the acetyl group to be transferred to the N-terminal of the substrate. In addition, the side chain containing the carboxyl group of aspartate was oriented to form a hydrogen bond with R209. The coordinates for the whole complex (NAT8L:CoA:Asp) were energy minimized using MOE software. An implicit solvent method was used in calculating the electrostatic interactions.

4.5. **Immunohistochemistry of NAT8L and NAA**

Adult Sprague Dawley rats were anesthetized and perfused transcardially with 4% paraformaldehyde. Brains were post-fixed in paraformaldehyde before being passed through a series of 10%, 20% and 30% sucrose solutions. Tissue was fixed in paraformaldehyde before being passed through a series of 10%, 20% and 30% sucrose solutions. Tissue was frozen and sections were cut at a thickness of 20 μm in the coronal plane at a temperature of –18 °C. Free floating tissue sections were collected and washed in PBS, and then incubated for 30 min with 1:1 methanol/water containing 1% H₂O₂ to block endogenous peroxidase. Tissue sections were blocked against non-specific antibody binding by incubating with PBS containing 2% normal goat serum (NGS) and 0.1% sodium azide. The sections were then processed for immunoperoxidase histochemistry using the avidin–biotin complex (ABC) method with horseradish peroxidase as the enzyme marker (Vectastatin Elite, Vector Labs).

A polyclonal antibody to an 18 mer NAT8L peptide sequence (CMSVDSRFRGKGIAKALG) was used for the immunohistochemical and immunofluorescence studies. This antibody has been described and characterized previously (Niwa et al., 2007). Tissue sections were incubated with anti-NAT8L peptide antibodies (diluted 1:4,000 to 1:5,000) in PBS containing 2% NGS plus 0.1% sodium azide for 12 to 16 h. Tissue sections were washed in PBS and treated with biotinylated secondary antibody in 2% NGS/PBS for 90 min. Sections were washed again in PBS and treated with the avidin–peroxidase complex solution containing 0.5% bovine serum albumin for 90 min. After thorough washing in PBS, tissue sections were developed using a nickel and cobalt enhanced diaminobenzidine chromogen (Pierce Chemical Co.). After final wash in distilled water with 0.01% bovine serum albumin, the tissue sections...
were transferred to treated slides (Superfrost plus, Thermo Fisher Scientific), dried at 50 °C, dehydrated in an ethanol series, cleared in xylene, and mounted with coverslips using cytoseal-60 (Richard Allen Scientific Inc.).

Carbodiimide-fixed rat brain tissue sectioned at a thickness of 20 μm was stained with highly purified polyclonal antibodies to protein-coupled NAA. The procedures and characterization of the antibodies have been described in detail previously (Moffett et al., 1993; Moffett and Namboodiri, 1995). Micrographic images were acquired using a DIC-equipped Olympus BX51 microscope and Olympus DP71 camera, and were adjusted for brightness and contrast using PC based imaging software (Media Cybernetics and Adobe Systems Inc.).

4.6. Colocalization of Asp-NAT with mitochondrial marker

SH-SYSY cells were incubated in Nunc Lab-Tek® Chamber Slides (Thermo Fisher Scientific). 0.2-0.4 ml of Dulbecco’s modified Eagle’s medium (DMEM) (ATCC) containing harvested SH-SYSY cells (obtained from a confluent 25 cm² treated cell culture flask using trypsin EDTA) was added in to each chamber and cultured for two days at 37 °C in a humified incubator chamber containing 5% CO₂/95% air. The media were removed and the cells incubated with a 200-nM working solution (in DMEM) of MitoTracker® Red CMXRos (Invitrogen) for 30 min. The chamber slides were washed with pre-warmed PBS, and the cells were fixed using 3.7% formaldehyde in culture media (DMEM) that was freshly pre-warmed PBS, and the cells were fixed with fixed and permeabilized with the cells at 37 °C for 15 min. Cells were washed 3 times in pre-warmed PBS, and were permeabilized by washing twice with PBS containing 0.2% Triton X100 for 10 min each. Anti NAT8L antibodies (1:100 dilution in PBS containing 5% normal goat serum and 0.2% Triton X100) were incubated overnight with fixed, permeabilized SH-SYSY cells. Cells were washed twice with PBS containing 0.2% Triton X-100, and goat anti-rabbit secondary antibody (1:1,000) coupled to Alexa Fluor 488® (Invitrogen) was added to the chamber slides and incubated for 1 h at room temperature. After two final washes in PBS the chambers were removed and slides mounted with cover slips using DAPI (Vector labs). The immunofluorescence was visualized on an Olympus BX51 microscope equipped with a cooled high sensitivity camera, and were adjusted for brightness and contrast using PC based imaging software (Media Cybernetics Image Browser Version 5).

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