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Evidence for mitochondrial and cytoplasmic N-acetylaspartate synthesis in SH-SY5Y neuroblastoma cells

Peethambaran Arun, John R. Moffett *, Aryan M.A. Namboodiri

Dept of Anatomy, Physiology and Genetics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814, United States

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ABSTRACT

N-acetylaspartate (NAA) is synthesized predominantly in neurons, and brain homogenate subfractionation studies suggest that biosynthesis occurs at both microsomal (cytoplasmic) and mitochondrial sites. We investigated NAA synthesis in SH-SY5Y human neuroblastoma cells using distinct metabolic precursors that are preferentially metabolized in mitochondria and cytoplasm. Incorporation of ^{14}C-aspartate and ^{14}C-malate into NAA was examined in the presence and absence of an inhibitor (aminooxyacetic acid, AOAA) of aspartate aminotransferase (AAT), a central enzyme involved in the biosynthesis of aspartate in mitochondria, and degradation of aspartate in the cytoplasm. AOAA increased the incorporation of ^{14}C-aspartate into NAA, reflecting direct aspartate → NAA synthesis in an extramitochondrial location. As expected, AOAA decreased incorporation of ^{14}C-malate into NAA, reflecting NAA synthesis in mitochondria via the malate → oxaloacetate → aspartate → NAA pathway. When ^{14}C-malate was used as substrate, the ^{14}C-NAA/^{14}C-aspartate ratio was over 20-fold higher than the ratio obtained with ^{14}C-aspartate. Because NAA can only be synthesized from aspartate, the higher ^{14}C-NAA/^{14}C-aspartate (product/substrate) ratio obtained with ^{14}C-malate suggests greater NAA biosynthetic activity. We conclude that NAA biosynthesis occurs in both the cytoplasm and mitochondria of SH-SY5Y cells, and that the contribution from the mitochondrial compartment is quantitatively larger than that in the extramitochondrial compartment.

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1. Introduction

N-acetylaspartate, or NAA, is the second most abundant metabolite in the human central nervous system (CNS), the most abundant being glutamate (Bluml, 1999). The functional significance of the high NAA concentration in the brain remains uncertain, but it confers on NAA a unique clinical significance due to the fact that NAA emits the largest signal in magnetic resonance spectrograms of the human brain. NAA levels measured by magnetic resonance spectroscopy are changed in a wide array of grey and white matter CNS disorders, but it is unknown if the changes are etiological, or secondary. Magnetic resonance studies of human brain disorders have invariably detected decreases in brain NAA concentrations when neuronal loss or dysfunction is involved, with one major exception. The autosomal genetic disease, Canavan disease, involves the accumulation of NAA in the brain due to the lack of degradative enzyme activity (Matalon et al., 1995). Virtually all other neurological disorders involving neuronal loss or dysfunction result in reductions in brain NAA levels including Alzheimer disease, epilepsy, amyotrophic lateral sclerosis, schizophrenia, multiple sclerosis, AIDS, traumatic brain injury, stroke and non-neuronal brain tumors such as glioma (reviewed in Moffett et al., 2007). Although early investigations suggested that the decreases in NAA represented irreversible loss of neurons, more recent evidence indicates that reductions in brain NAA can also result from reversible neuronal damage or mitochondrial dysfunction (Signoretti et al., 2001, 2008).

NAA is primarily localized in neurons (Moffett et al., 1991; Moffett and Namboodiri, 1995; Simmons et al., 1991), but also has been reported to be present in cultured oligodendrocytes (Bhakoo and Pearce, 2000). The biosynthetic enzyme, aspartate-N-acetyltransferase (Asp-NAT; EC 2.3.1.17) is a CNS specific enzyme that catalyzes the transfer of acetate from acetyl-CoA to L-aspartate forming NAA (Goldstein, 1959; Knizley, 1967; Truckenmiller et al., 1985). Asp-NAT-mediated synthesis of NAA has been reported to take place in neuronal mitochondria via an ATP-related mechanism (Patel and Clark, 1979) suggesting that decreased NAA levels could represent mitochondrial dysfunction (Clark, 1998; Signoretti et al., 2001). The first report on the subcellular localization of NAA-synthetic activity found Asp-NAT to occur in both the mitochondrial and microsomal fractions of rat brain homogenates (Gold-

* Corresponding author. Tel.: +1 301 295 9357; fax: +1 301 295 3566.
E-mail address: jmoffett@usuhs.mil (J.R. Moffett).

Abbreviations: ^{14}C-Asp, ^{14}C-aspartate; ^{14}C-Mal, ^{14}C-malate; ^{14}C-NAA, ^{14}C-N-acetylaspartate; AAT, aspartate aminotransferase; AOAA, aminooxyacetic acid; Asp-NAT, aspartate N-acetyltransferase; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; TCA cycle, tricarboxylic acid cycle.

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stein, 1969). Later studies on the subcellular distribution of Asp-NAT in the brain found the enzyme to be localized primarily in mitochondria (Madhavarao et al., 2003). However, a similar recent study showed subcellular localization of Asp-NAT with a predominant presence in microsomes, and lesser expression in the mitochondrial fraction (Lu et al., 2004). Results from studies with aralar1 (−/−) mice also have been interpreted to indicate that NAA synthesis is predominantly microsomal. Aralar1 is a mitochondrial aspartate–glutamate carrier protein that functions as part of the so-called malate–aspartate shuttle to maintain proper mitochondrial substrate levels. An *in vivo* study using Aralar (−/−) mice found greatly reduced levels of both aspartate and NAA in the brains of these mice. The investigators associated reduced aspartate synthesis and export from mitochondria with greatly reduced NAA synthesis, and they postulated that this implied a predominantly extramitochondrial source of NAA synthesis (Jalil et al., 2005). Because of the uncertainty concerning the relative contributions of the mitochondrial and extramitochondrial sources of NAA we have investigated NAA biosynthesis in an intact cell system using a human neuroblastoma cell line.

We used SH-SY5Y human neuroblastoma cells because they were found in previous studies to be an excellent model system for studying the regulation of NAA biosynthesis (Arun et al., 2006, 2008). Using this model system we investigated mitochondrial versus extramitochondrial synthesis of NAA in living cells using two radiolabeled precursors, aspartate and malate. These two metabolites have very different fates with respect to mitochondrial transporters. Cytoplasmic malate can be metabolized in the cytoplasm, but the major route of malate metabolism is via uptake into mitochondria, and conversion to oxaloacetate in the mitochondrial matrix. In contrast, aspartate is not normally taken up by mitochondria, but rather is exported from the mitochondrial matrix. Accordingly, the bulk of externally applied malate will be metabolized within mitochondria, whereas the bulk of applied aspartate will be metabolized in the cytoplasm. The differential mitochondrial uptake and metabolism of these two NAA precursors permitted us to estimate the relative contributions of the mitochondrial and extramitochondrial NAA synthetic sites.

Aminooxyacetic acid (AOAA) is a potent inhibitor of transaminases, including the important metabolic enzyme aspartate aminotransferase (AAT). AAT catalyzes the conversion of oxaloacetate and glutamate to aspartate and alpha-ketoglutarate respectively. In the presence of AOAA, AAT activity is blocked in two different intracellular compartments, one in the cytoplasm, and one in mitochondria. The action of AOAA in the cytoplasm prevents aspartate from being converted to oxaloacetic acid, and it will therefore be diverted to NAA production, and protein synthesis. In the presence of AOAA, malate entering mitochondria via the malate–aspartate shuttle cannot be converted to aspartate, and will most likely enter the tricarboxylic acid (TCA) cycle as oxaloacetate. Using radiolabeled aspartate and radiolabeled malate we have examined the synthesis of NAA with and without AOAA in order to differentiate the contributions from cytoplasmic and mitochondrial synthetic sites. By employing radiolabeled substrates that are preferentially utilized in either mitochondria, or the cytoplasm, we have investigated the differential synthesis of NAA in these two subcellular compartments.

2. Materials and methods

2.1. Cell culture

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Manassas, VA, USA) were grown in 12 well treated cell culture plates in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum (American Type Culture Collection, Manassas, VA, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

2.2. Preparation of ^{14}C-NAA

Radiolabeled NAA, for use as a standard, was prepared as described earlier (Madhavarao et al., 2002). Briefly, about 50 μCi of L-[^{14}C]Aspartate (^{14}C-Asp, GE Healthcare, Piscataway, NJ, USA, specific activity of 207 mCi/mmol) was evaporated to dryness in a SpeedVac and the residue dissolved in 10 μl water containing unlabeled L-aspartate. Acetic anhydride (5 μl) was dissolved in pyridine (45 μl) and added to the above solution, in five aliquots of 10 μl with an interval of 2 min incubation on ice after each addition. After addition of the last aliquot, the mixture was kept in ice for 25 min and the ^{14}C-NAA formed was separated from ^{14}C-Asp by TLC using Whatman flexible TLC plates (AL SIL G/UV, 250 μm layer, 20 cm × 20 cm). Methanol:formic acid:chloroform (25:5:70) was used as the mobile solvent system.

2.3. Treatment with AAT inhibitor

Initial studies were done to determine the optimal concentrations of radiolabeled precursors, and the time frame for incubations. A 3-hour incubation period was determined empirically to provide detectable levels of NAA from both radiolabeled substrates. Further, it was found that due to the substantially lower uptake of malate from the culture medium as compared with aspartate, that higher concentrations of externally applied malate were required to achieve intracellular NAA levels that were readily detectable. SH-SY5Y cells were incubated with or without 500 μM AOAA (Sigma–Aldrich, St. Louis, MO, USA) in the above medium for 30 min. Then either ^{14}C-Asp (2 μCi/ml, 207 μCi/μmol) or ^{14}C-malate (^{14}C-Mal, GE Healthcare, Piscataway, NJ,USA, 44 μCi/ml, 49 μCi/μmol) was added to the respective controls (without AOAA) and AOAA treated cells and incubated for 3 h. After incubation, the medium was removed, the cells were washed with phosphate buffered saline (PBS) and then the cells were harvested using 2 mM ethylenediamine-tetraacetate (EDTA) in PBS and stored at −80° until analyzed.

2.4. Analyses of radioactivity in proteins and metabolites

Cell samples were thawed, vortex mixed vigorously with 90% methanol and centrifuged at 7800*g* for 5 min. The sedimented protein was estimated using Bio-Rad DC protein assay kit after dissolving in 0.1N NaOH. The amount of radiolabel incorporated into protein was determined using a Beckman liquid scintillation counter after dissolving 2 μl of the above protein solution in 7 ml of scintillation fluid.

Methanol supernatants containing the metabolites were lyophilized, and each sample was dissolved in 40 μl methanol. Thin layer chromatography (TLC) was carried out on Whatman flexible TLC plates (AL SIL G/UV, 250 μm layer, 20 cm × 20 cm) using the method described earlier (Madhavarao et al., 2002). The mobile phase was chloroform:methanol:formic acid (70:25:5). Following chromatography, ^{14}C-Asp, ^{14}C-Mal and ^{14}C-NAA were detected and identified with phosphor imaging by exposing plastic wrapped TLC plates to phosphor image screens for about 16 h, using radioactive standards that were run in parallel. To quantitate the radioactivity, the phosphor image screens were then scanned in a Fuji Film phosphor image reader (FLA-5100) and spot intensities were quantified using Multi Gauge V3.0 software via comparison to quantitative radioactivity standards.

2.5. Anion exchange HPLC analysis to confirm ^{14}C-NAA and ^{14}C-Asp TLC spots derived from ^{14}C-Mal

In order to confirm the identity of the spots corresponding to NAA and aspartate formed from radiolabeled malate, the spots corresponding to NAA and aspartate from the TLC plates were scrapped and incubated with 1N NaOH for about 30 min with vigorous mixing. After neutralizing the pH using 1N HCl, the precipitated silica gel particles were separated by centrifugation at 7800*g* for 5 min. The supernatants were then mixed with 1 mM each of non-labeled NAA or aspartate and then injected into a Gilson HPLC system connected with an anion exchange column (SAX 100A, 5 μm, 250 mm × 4.6 mm, Thomson Instrument Company, Clear Brook, VA). The solvent system used was 200 mM potassium phosphate buffer (pH 4.9) at a flow rate of 1 ml/min, and the optical density was set at 231 nm. Under these conditions, aspartate had a retention time of 5 min and NAA had a retention time of 13 min. HPLC fractions were collected and the radioactivity associated with each fraction was determined by liquid scintillation counting.

3. Results

3.1. Formation of ^{14}C-NAA from ^{14}C-Asp and ^{14}C-Mal in SH-SY5Y cells

Results showed that ^{14}C-NAA is formed from both ^{14}C-Asp and ^{14}C-Mal in SH-SY5Y cells under the described experimental conditions. Fig. 1 shows a typical phosphor image of a thin layer chromatogram showing separation of aspartate, malate and NAA. Fig. 1A shows the chromatographically separated aspartate, malate and NAA when ^{14}C-Asp was used as substrate, whereas Fig. 1B

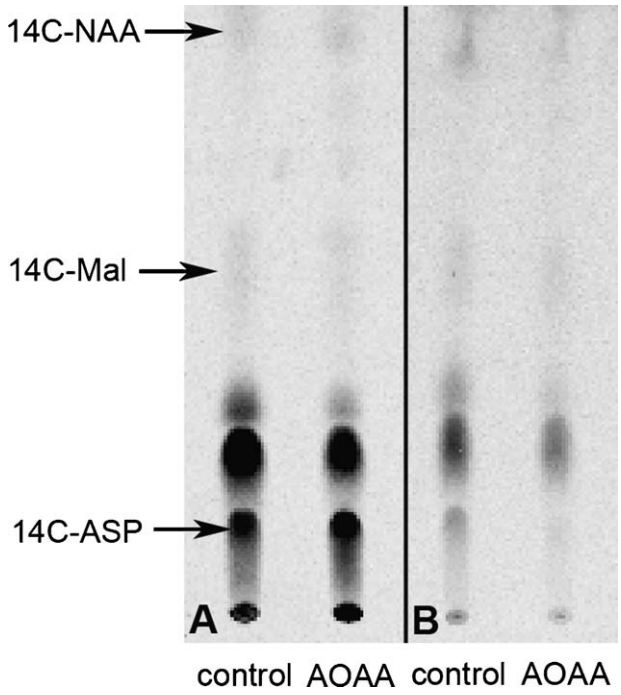

Fig. 1. Separation of aspartate, malate and NAA via TLC when ^{14}C-Asp (A) and ^{14}C-Mal (B) were used as substrates in the presence and absence of AOAA.

shows the pattern when ^{14}C-Mal was used. Under the chromatographic conditions described, aspartate, malate and NAA showed Rf values of 0.3, 0.5 and 0.8 respectively.

Fig. 2 provides confirmation of the identity of ^{14}C-NAA and ^{14}C-Asp formed from ^{14}C-Mal. Fig. 2A is a representative high performance liquid chromatograph showing the retention times of both aspartate and NAA using pure standards. Fig. 2B shows the HPLC elution profile of a mixture of aspartate and NAA spots obtained from the TLC plate when ^{14}C-Mal was used as the precursor. Radioactivity associated with the spots corresponding to aspartate and NAA on the TLC plates co-migrated in the anion-exchange HPLC with respective pure standards, confirming their identity.

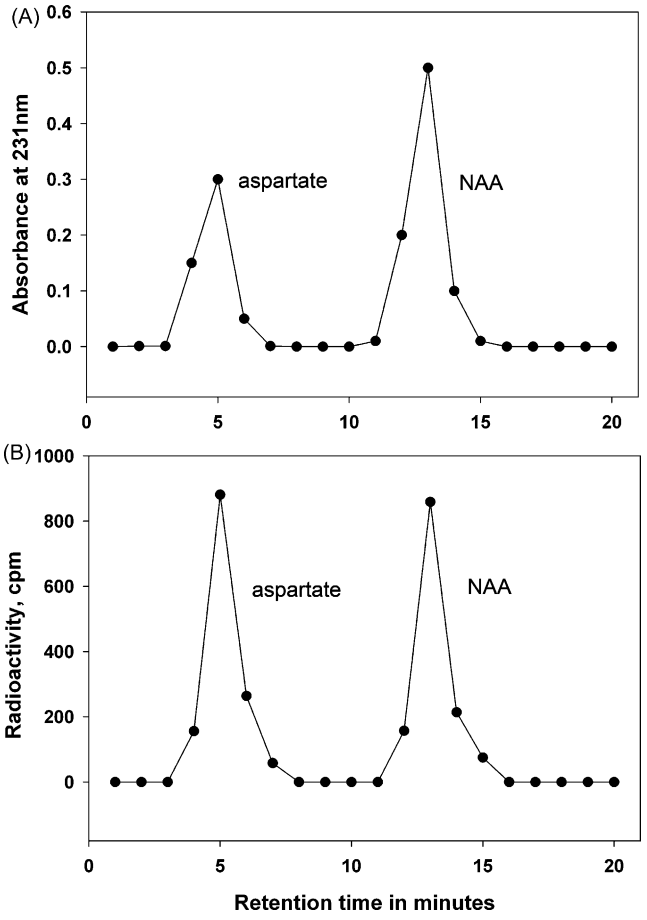

Fig. 2. Confirmation of the identity of aspartate and NAA obtained from malate. (A) Representative HPLC pattern showing the retention times of aspartate and NAA using pure standard mixture. (B) HPLC elution of radioactivity of the mixture of aspartate and NAA spots obtained from the TLC when malate was used as the substrate.

3.2. Differential effects of AAT inhibitor on the incorporation of radiolabel from aspartate and malate into NAA

Table 1 shows the incorporation of radiolabel from ^{14}C-Asp into ^{14}C-NAA in the presence and absence of the AAT inhibitor, AOAA. When radiolabeled aspartate was used as substrate, both ^{14}C-Asp and ^{14}C-NAA levels were significantly higher in the AOAA treated cells compared to untreated cells. The increase in ^{14}C-Asp presumably reflects decreased conversion of aspartate to oxaloacetate in the cytoplasm, which would result in increased specific activity, and hence a higher level of radioactivity in the immediate product, NAA. With aspartate as substrate, AOAA resulted in a significant drop in radiolabel incorporation into malate, as expected from inhibition of AAT, and reduced production of ^{14}C-oxaloacetate, the immediate precursor for malate production.

In contrast, inhibition of AAT by AOAA significantly decreased the incorporation of radiolabel from ^{14}C-Mal into ^{14}C-NAA, most likely due to reduced conversion of oxaloacetate to aspartate in mitochondria (Table 1). When ^{14}C-Mal was used as substrate, the level of radiolabeled malate in cells was unaffected by treatment with the AAT inhibitor, which shows that the uptake of malate into cells is unaffected by AOAA. Based on the malate–aspartate shuttle mechanism, conversion of malate to aspartate takes place in mitochondria, and AOAA inhibits this conversion. As such, ^{14}C-NAA formation from ^{14}C-Mal will be inhibited in the presence of AOAA. The opposing effects of AOAA on NAA synthesis from ^{14}C-Asp versus ^{14}C-Mal are in agreement with the mechanisms of the

Table 1
Effects of AOAA on the incorporation of ^{14}C-aspartate into ^{14}C-NAA and ^{14}C-malate (top two rows) and on the incorporation ^{14}C-malate into ^{14}C-NAA and ^{14}C-aspartate (bottom two rows). Values are means ± S.D. of 6 independent experiments. Values of AOAA treated groups in each case were compared to that of control. Statistical analysis was carried out using Student's "*t*" test.

	^{14}C-Asp (cpm/mg protein)	^{14}C-NAA (cpm/mg protein)	^{14}C-Mal (cpm/mg protein)
Control ^{14}C-Asp	54,686 ± 11,168	2,147 ± 661	3,388 ± 275
AOAA ^{14}C-Asp	245,216 ± 127,692**	3,107 ± 401*	2,650 ± 401**
Control ^{14}C-Mal	7,106 ± 392	6,600 ± 564	10,030 ± 715
AOAA ^{14}C-Mal	4,001 ± 550**	5,248 ± 440**	10,255 ± 447

* $p < 0.05$.
** $p < 0.01$.

Table 2
Effect of AOAA on the incorporation of radiolabel in to protein from ^{14}C-Asp and ^{14}C-Mal. Values are means ± S.D. of 6 independent experiments. Values of AOAA treated groups in each case were compared to that of control. Statistical analysis was carried out using Student's "*t*" test.

Radiolabel used	Radiolabel incorporated into protein (cpm/mg protein)	
	Control	AOAA
^{14}C-Asp	114,507 ± 15,808	362,284 ± 79,147**
^{14}C-Mal	40,984 ± 6,011	26,426 ± 3,620**

** $p < 0.01$.

malate–aspartate shuttle, and provide supporting evidence that NAA synthesis takes place in two subcellular locations, one mitochondrial and the other cytoplasmic.

3.3. Effect of AAT inhibitor on the incorporation of radiolabel from aspartate and malate into total protein

Results presented in Table 2 show that AAT inhibition significantly increased the incorporation of radiolabel from aspartate into total protein most likely due to decreased conversion of ^{14}C-Asp to ^{14}C-oxaloacetate, and the resultant increase in specific activity of radiolabeled aspartate. In contrast, incorporation of radiolabel from ^{14}C-Mal into protein was significantly decreased by the AAT inhibitor due to the decreased formation of radiolabeled aspartate in mitochondria, and decreased release into the cytoplasm, resulting in decreased specific activity for protein synthesis in the cytoplasm.

3.4. Evidence that rate of NAA synthesis in mitochondria is several fold higher than that in the cytoplasm

Several aspects of the current data support the conclusion that the mitochondrial component of NAA synthesis is quantitatively larger than that in the cytoplasm. The radiolabeled malate used in these experiments had a specific activity of only about 1/4 that of labeled aspartate (^{14}C-Asp = 207 μCi/μmol vs. ^{14}C-Mal = 49 μCi/μmol), and there is further isotopic dilution at each enzymatic step. Therefore it would be expected that less ^{14}C-Mal would be incorporated into NAA due to the fact that there are 3 enzymatic steps from malate to NAA, but only one from aspartate to NAA. Despite these issues, the level of radiolabel incorporation into NAA from malate was 3 times the level incorporated from aspartate (ratio of 3.1:1). Because the malate–aspartate shuttle moves malate into mitochondria, and aspartate out, these two substrates should differentiate the mitochondrial from the cytoplasmic enzyme pools.

Due to the fact that aspartate is the immediate precursor for NAA in mitochondria and cytoplasm, we used the ^{14}C-NAA/^{14}C-Asp (product/substrate) ratio as a general measure of the relative contributions of Asp-NAT activity in the two subcellular compartments in SH-SY5Y cells. Fig. 3 shows the ^{14}C-NAA/^{14}C-Asp ratio obtained on incubation of SH-SY5Y cells with ^{14}C-Asp and ^{14}C-Mal. The ^{14}C-NAA/^{14}C-Asp ratio is approximately 20-fold higher (23.3:1) when ^{14}C-Mal is used as the precursor as opposed to when ^{14}C-Asp is used as the precursor. This higher ratio of NAA to aspartate obtained when ^{14}C-Mal was used indicates that the level of aspartate N-acetyltransferase activity (rate of NAA synthesis) in mitochondria is quantitatively greater than that in the cytoplasm.

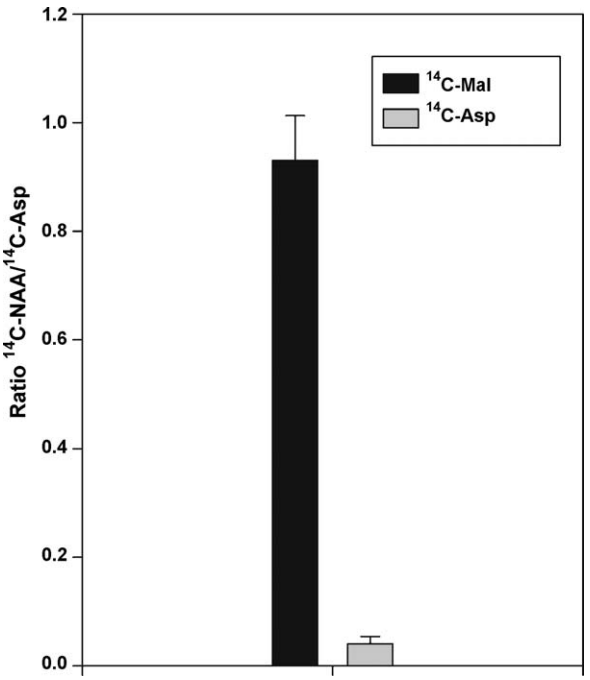

Fig. 3. NAA/aspartate ratios obtained when ^{14}C-Asp and ^{14}C-Mal were used as the substrates (data from Table 1). Despite the facts that much more radiolabeled aspartate is present when ^{14}C-Asp is supplied as substrate, and that more isotopic dilution occurs when ^{14}C-Mal is used as substrate, a much higher ratio of NAA/aspartate is obtained when ^{14}C-Mal is applied to SH-SY5Y cells.

4. Discussion

NAA is present primarily in neurons, but the subcellular location of its synthesis remains controversial. The biosynthetic enzyme for NAA, Asp-NAT, has not been well investigated, and little is known of its structure or cellular distribution. The gene(s) encoding Asp-NAT have not been identified, making its study difficult. Several previous investigations on the subcellular distribution of Asp-NAT in the brain have suggested that it is localized primarily in mitochondria (Goldstein, 1969; Madhavarao et al., 2003; Ariyannur et al., 2008). However, a similar study concluded that Asp-NAT has a subcellular localization with greater presence in microsomes, which are predominantly of cytoplasmic origin (Lu et al., 2004). Aralar1 is a mitochondrial aspartate–glutamate carrier protein that is a part of the malate–aspartate shuttle in mitochondrial membranes. An *in vivo* study using aralar1 (−/−) mice found greatly reduced aspartate and NAA levels in the brain, and the authors proposed that this implied a predominant extramitochondrial site of NAA synthesis (Jalil et al., 2005).

In light of these discrepancies, and a significant body of biochemical evidence that indicates the biosynthesis of NAA is affected by mitochondrial uncoupling or damage (Benuck and D'Adamo, 1968; Bates et al., 1996; Clark, 1998; Madhavarao et al., 2003; Patel and Clark, 1979), it is important to determine the relative contributions from these two sources in order to understand the biochemical functions of NAA in different subcellular locations. In the present studies using a different approach with an intact cell system we provide further evidence that (1) there are two sites of NAA synthesis in neurons, most likely mitochondrial and cytoplasmic, and (2) that the rate of NAA synthesis in the mitochondrial compartment is quantitatively greater than that in the cytoplasmic compartment. These data offer the first experimental support for multiple subcellular sites of NAA synthesis using living neural cells, rather than fractionated brain homogenates.

Previous studies suggesting dual subcellular sites for NAA synthesis were done *in vitro* using subcellular fractionation centrifugation techniques followed by assays of Asp-NAT enzyme activity. The limitations that confound gradient density centrifugation studies include heterogeneous subcellular fractions with

contributions from both neurons and glia, and contamination of subcellular fractions with material from neighboring fractions. The first limitation cannot be avoided with brain homogenates, but the second can be minimized and controlled for. The SH-SY5Y human neuroblastoma cell line can be differentiated into cells that have characteristics of dopaminergic neurons, and these cells have been shown to synthesize NAA (Arun et al., 2006, 2008). The SH-SY5Y cell culture system provides a number of advantages over brain homogenate sub-fractionation methods, including the maintenance of normal cellular physiology and biochemical pathways, and the lack of glial cells, whose mitochondria do not synthesize NAA. Additionally, SH-SY5Y cells do not have any detectable activity for the NAA degrading enzyme aspartoacylase (unpublished observations), and therefore most or all of the NAA synthesized during the 3-hour incubation period remains unmetabolized.

Aminooxyacetic acid (AOAA) is not a specific inhibitor for the enzyme aspartate aminotransferase (AAT). At high concentrations (above 1 mM), AOAA can inhibit all pyridoxyl phosphate-dependent enzymes to some extent. The enzymes most sensitive to AOAA inhibition are those involved in GABA metabolism (glutamate decarboxylase and GABA-aminotransferase), but inhibiting these enzyme activities would not affect the results presented here (McKenna et al., 2006). Further, AOAA does not inhibit AAT completely at lower doses. For example, in MRS studies of hearts perfused with ^{13}C-acetate, AOAA was found to inhibit AAT activity by approximately 60% at 0.5 mM (Sherry et al., 1998), the concentration used in the present series of experiments. Despite these limitations, AOAA does provide a method for studying mitochondrial vs. extramitochondrial substrate flux when using different radiolabeled precursors due to its ability to reduce AAT activity. AAT is expressed in mitochondria and in the cytoplasm, and the inhibitor AOAA blocks activity in both locations, preventing the transamination reaction of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate. An heuristic model of the biosynthesis of NAA in neurons is given in Fig. 4. According to this model, NAA is made by neurons in at least two subcellular locations, one in mitochondria and the other most likely in endoplasmic reticulum, and the two radiolabeled precursors can be used to differentiate the biosynthetic capacity in these two sites.

When ^{14}C-malate is provided as substrate in the absence of AOAA, it will be transported into mitochondria via the malate–aspartate shuttle, which moves malate in, and aspartate out of the mitochondrial matrix when cytoplasmic malate levels are adequate. Therefore, the most direct route for conversion of externally applied ^{14}C-malate is for it to enter mitochondria where it would be converted to ^{14}C-oxaloacetate, and then by the action of AAT, into ^{14}C-aspartate before being converted to ^{14}C-NAA by Asp-NAT (Fig. 4, blue pathway). In the presence of AOAA, intramitochondrial ^{14}C-malate conversion to ^{14}C-NAA is blocked at the AAT enzymatic step (^{14}C-oxaloacetate to ^{14}C-aspartate). In the current study, as expected, the addition of AOAA to the cell cultures during incubation with ^{14}C-malate caused a significant reduction in the synthesis of NAA (20% ± 9%). It also resulted in a significant drop in ^{14}C-labeled aspartate (Table 1) and ^{14}C-labeled proteins (Table 2), because in the presence of the AAT inhibitor, less radiolabeled aspartate was formed in the mitochondrial matrix, and less was exported to the cytoplasm for protein synthesis.

When ^{14}C-aspartate is provided as substrate in the absence of AOAA, the most direct route for the synthesis of ^{14}C-NAA is acetylation in the cell cytoplasm by the action of cytoplasmic Asp-NAT (Fig. 4, red pathway). However, an indirect route also exists if cytoplasmic AAT converts the ^{14}C-aspartate into ^{14}C-oxaloacetate, which then can be converted to ^{14}C-malate, which will enter mitochondria via the malate–aspartate shuttle. AOAA blocks the indirect, mitochondrial pathway that requires cytoplasmic AAT,

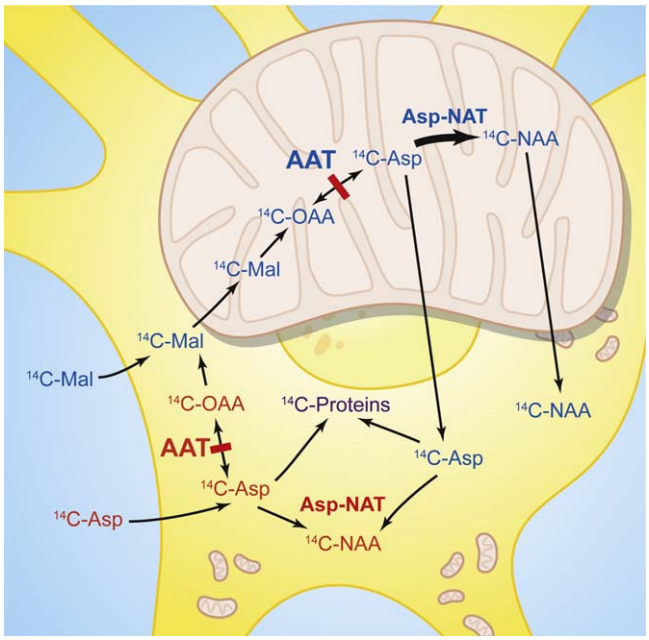

Fig. 4. Model schematic of NAA synthesis in mitochondria and cytoplasm of neurons. ^{14}C-Malate (^{14}C-Mal) predominantly enters mitochondria via the malate–aspartate shuttle, where it is converted to ^{14}C-oxaloacetate (^{14}C-OAA), and then to ^{14}C-aspartate (^{14}C-Asp) by the action of aspartate aminotransferase (AAT). Intramitochondrial ^{14}C-Asp can then be converted to ^{14}C-NAA by mitochondrial aspartate N-acetyltransferase (Asp-NAT). AOAA inhibits AAT, as depicted by the red bars in both mitochondria and cytoplasm. With ^{14}C-Mal as substrate, the major inhibition of NAA synthesis by AOAA will be mitochondrial, reducing NAA synthesis. As expected, AOAA inhibits the formation of ^{14}C-Asp in mitochondria, leading to less ^{14}C-Asp export to the cytoplasm, and a reduction of aspartate incorporation into proteins as well. When ^{14}C-Asp is used as substrate, AOAA inhibits cytoplasmic conversion to ^{14}C-OAA, thus increasing ^{14}C-NAA synthesis, as well as increasing ^{14}C-Asp incorporation into proteins.

thus favoring NAA synthesis in the cytoplasm, and this is reflected by the fact that AOAA significantly enhanced the synthesis of ^{14}C-NAA from ^{14}C-aspartate by 45% ± 31% (Table 1). Because AOAA inhibits the conversion of ^{14}C-aspartate to ^{14}C-oxaloacetate in the cytoplasm, more ^{14}C-aspartate remains unconverted, thus increasing cytoplasmic ^{14}C-aspartate levels by 448% ± 52% (Table 1). Additionally, when cytoplasmic AAT is inhibited, more ^{14}C-aspartate is available in the cytoplasm for ^{14}C-labeled protein synthesis, and this is clearly the case with a 316% ± 22% increase in radiolabel found in protein in the presence of AOAA with ^{14}C-aspartate as substrate (Table 2).

The total radiolabel incorporation into NAA from the two substrates, and the NAA to aspartate (product/substrate) ratio obtained with the two precursors, provide evidence that the mitochondrial (malate) synthetic site is quantitatively larger than the cytoplasmic (aspartate) synthetic site. As noted in section 3.4, the radiolabeled malate used in these experiments had a specific activity of only about 1/4 that of the labeled aspartate. Further, malate requires 3 enzymatic steps to produce NAA, whereas aspartate only requires 1 enzymatic step (Fig. 4). There is substantially greater isotopic dilution during the conversion of malate to NAA due to the greater number of enzymatic steps. For these reasons, if both synthetic sites were equal in capacity it would be expected that less radiolabel would be incorporated into NAA from ^{14}C-Mal. Despite these issues, the level of radiolabel incorporation into NAA from malate in the absence of inhibitor was 3 times the level incorporated from aspartate (ratio of 3.1:1, Table 1). More importantly, we compared the ^{14}C-NAA/^{14}C-Asp ratio obtained with the two starting substrates as a general measure of the relative contributions of Asp-NAT activity in the

cytoplasm and mitochondria. Fig. 3 shows the ^{14}C-NAA/^{14}C-Asp ratios when SH-SY5Y cells were incubated with ^{14}C-Asp and ^{14}C-Mal. The ^{14}C-NAA/^{14}C-Asp (product/substrate) ratio was over 20-fold higher (23.3:1) when ^{14}C-Mal was applied to the cells as opposed to when ^{14}C-Asp is used as the biosynthetic precursor. This higher ratio of NAA to aspartate obtained when ^{14}C-Mal was used provides strong evidence that the level of aspartate N-acetyltransferase activity (rate of NAA synthesis) in mitochondria is quantitatively greater than that in the extramitochondrial compartment.

A note of caution may be appropriate in these interpretations. *In vitro* data from SH-SY5Y cells may not be directly applicable to neuronal metabolism *in vivo*. For example, we have found that the concentration of NAA in differentiated human SH-SY5Y neuroblastoma cells is about 10–20 times lower than that in neurons in the human brain (Arun et al., 2006). Additionally, enzymes such as malic enzyme have been shown to have much higher activity in mitochondria isolated from the brain as compared with those isolated from primary neuronal cell culture (McKenna et al., 2000). Neuronal metabolism in cell cultures is likely to be quantitatively different from neuronal metabolism *in vivo*, so additional work in other model systems, and *in vivo*, will be required to fully elucidate the mechanisms of NAA synthesis in the brain.

Pyruvate is the primary source of acetyl-CoA for the TCA cycle when glucose is available (Fig. 5). The malate–aspartate shuttle acts to move reducing equivalents into the mitochondrial matrix in the form of malate to sustain respiration, and aspartate is one of the mitochondrial byproducts of malate import and conversion to oxaloacetate and reduced NAD+(NADH). The mitochondrial malate–aspartate shuttle complex in neurons incorporates the glutamate–aspartate exchanger aralar1, which is important for removing excess aspartate from the mitochondrial matrix. Neurons have very high cytoplasmic levels of glutamate, so there is a ready supply of the counter-transport ion needed to exchange with intramitochondrial aspartate. It has previously been shown in isolated brain mitochondrial preparations that the efflux of NAA increases and that of aspartate decreases with increasing concentrations of pyruvate in the presence of glutamate and malate as substrates (Patel and Clark, 1979). These results suggest that the rate of NAA synthesis from aspartate in the mitochondrial matrix is proportional to the substrate flux through the TCA cycle. This is due to the fact that acetyl CoA is one of the primary products of pyruvate oxidation in the TCA cycle, and is also probably the rate limiting substrate in NAA synthesis. This fits well with the finding that ATP synthesis and NAA synthesis are directly correlated with oxygen consumption in studies involving respiratory chain inhibitors (Bates et al., 1996). Further, investigations utilizing brain synaptosomes demonstrated that inhibition of AAT activity by β-methylene-aspartate also led to decreased oxygen consumption in the presence of glutamate and malate as substrates (Cheeseman and Clark, 1988). Taken together, these results suggest that NAA is a byproduct of energy metabolism in neuronal mitochondria that must be removed to the cytoplasm to maintain TCA cycle throughput (reviewed in Moffett et al., 2007).

Studies with aralar1 (−/−) mice have also provided support for a connection between NAA synthesis and TCA cycle throughput (15). Aralar1 is the major mitochondrial aspartate–glutamate carrier in the brain, and an integral part of the malate–aspartate shuttle (Fig. 5) (Del Arco and Satrustegui, 1998). Jalil and colleagues have shown that aralar1 (−/−) mice lacking glutamate–aspartate exchange activity have dramatically reduced brain NAA levels, and show reduced neuronal respiration on glutamate (Jalil et al., 2005). The authors of this study concluded that the drastic reductions in NAA were due to the lack of aspartate export

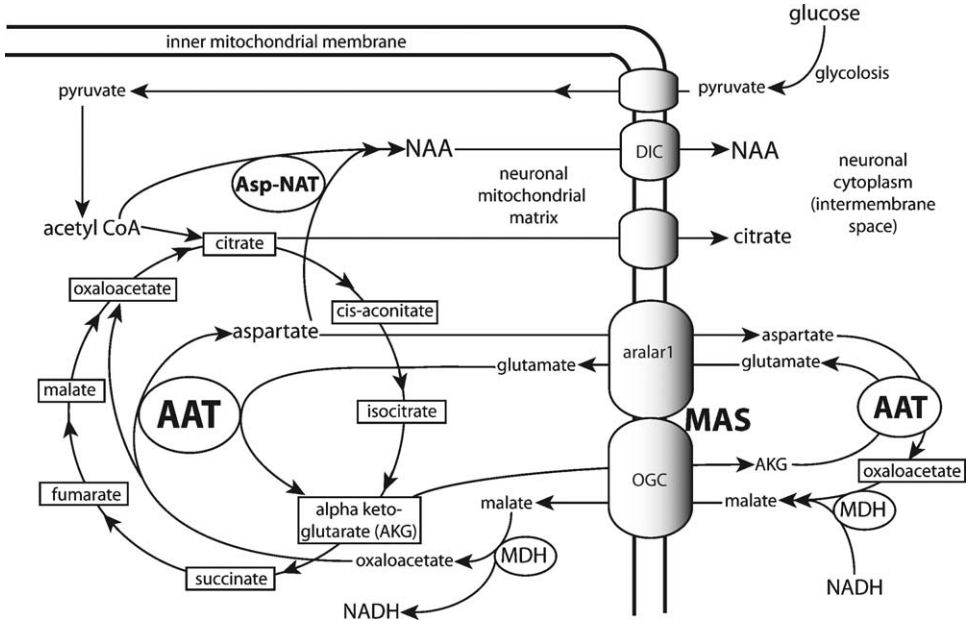

Fig. 5. The tricarboxylic acid (TCA) cycle in neuronal mitochondria, and its connections to the malate–aspartate shuttle (MAS) and Asp-NAT. Glycolysis provides the pyruvate needed to produce acetyl CoA, which then enters the TCA cycle by combining with oxaloacetate to form citrate. Reducing equivalents are moved into the mitochondrial matrix as malate, in exchange for alpha-ketoglutarate by the malate–alpha-ketoglutarate exchanger (oxoglutarate carrier; OGC). The malate which enters the mitochondrial matrix is converted to oxaloacetate and NADH by the action of the enzyme malate dehydrogenase (MDH). Oxaloacetate and glutamate are converted to aspartate and alpha-ketoglutarate in the matrix by the action of aspartate aminotransferase (AAT), and this aspartate is exported from the mitochondrial matrix by aralar1, the aspartate–glutamate exchanger that is part of the malate–aspartate shuttle. The aspartate which is exported to the intermembrane space can be converted to oxaloacetate by AAT, and then malate again by the action of MDH, and the malate is recycled back to the mitochondrial matrix. When both acetyl CoA and aspartate are plentiful, they can be converted to NAA by the action of aspartate N-acetyltransferase (Asp-NAT). However, once acetyl CoA and aspartate are converted to NAA by Asp-NAT, further hydrolysis in neurons is not thought to occur, and the NAA is exported to the cytoplasm, possibly via the mitochondrial dicarboxylate carrier (DIC) (Patel and Clark, 1979). In this way, products of both pyruvate catabolism (acetyl CoA) and malate metabolism (aspartate) are combined into a single export product which is removed from the MAS recycling shuttle that maintains the proper levels of TCA cycle intermediates.

from mitochondria to cytoplasm, and cited the work by Lu and colleagues (Lu et al., 2004) as their reason for suggesting a predominant cytoplasmic locus for NAA synthesis. We suggest that an alternative explanation for the drop in NAA in aralar1 (−/−) mice is that aspartate cannot be synthesized from oxaloacetate in mitochondria if there is not a constant supply of glutamate for the enzyme AAT to use as co-substrate (Fig. 5). Without aralar1 activity, mitochondria are starved of the glutamate needed for AAT activity (Ariyannur et al., 2008). It is likely that the lack of glutamate import into mitochondria, and the lack of intramitochondrial aspartate synthesis via AAT, is responsible for the precipitous drop in NAA in the brains of aralar1 (−/−) mice.

The current data provide additional evidence that NAA is synthesized predominantly in neural mitochondria, but they are far from conclusive on this matter. Further research will be required to elucidate the mechanisms of NAA biosynthesis. Understanding the precise roles played by NAA in CNS metabolism is critical to determine if altered NAA metabolism is etiological in CNS disorders, or simply a secondary effect of those disorders. Determining the mechanisms of NAA metabolism in the CNS will be crucial to developing treatment strategies for many of these nervous system disorders if impaired NAA metabolism is found to be involved in their pathophysiology.

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