SH-SY5Y neuroblastoma cells: a model system for studying biosynthesis of NAAG

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N-Acetylaspartylglutamate (NAAG) is a neuropeptide that is thought to modulate neurotransmitter release through pre-synaptic mGluR3 receptors. Despite years of research into NAAG biochemistry, almost nothing is known about NAAG biosynthesis. To date, NAAG biosynthesis has only been demonstrated conclusively in explanted animal neural tissues, including frog retina, rat dorsal root ganglia and crayfish nerve cord, but not in human cells or tissues. We show here that a human neuroblastoma cell line, SH-SY5Y, provides a good model system for the study of NAAG biosynthesis. Radiolabled NAAG synthesis occurred using both L-[^{3}H]glutamic acid and L-[^{3}H]glutamine as precursors, with glutamine being the preferred substrate. Differentiation of SH-SY5Y cells with retinoic acid resulted in decreased radiolabel incorporation into NAAG, whereas differentiation with nerve growth factor did not affect radiolabel incorporation. *NeuroReport* 15:1167–1170 © 2004 Lippincott Williams & Wilkins.

Key words: N-Acetylaspartylglutamate; Cell culture; Glutamate; Glutamate carboxypeptidase II; Glutamine; Neuronal differentiation; SH-SY5Y neuroblastoma cells

INTRODUCTION

N-Acetylaspartylglutamate (NAAG), the most abundant peptide in the mammalian CNS, was first identified almost 40 years ago [1]. NAAG is present in subpopulations of CNS neurons [2,3], and is released upon neuronal depolarization by a Ca^{2+}-dependent process [4,5]. NAAG is an endogenous agonist at mGluR3 receptors [6] and might also act as an agonist or antagonist at NMDA receptors at higher concentrations [7]. One important action of NAAG is the presynaptic inhibition of neurotransmitter release [8]. The enzyme, glutamate carboxypeptidase II (GCP-II), also called NAALADase, is localized primarily on astrocytes and hydrolyses NAAG to N-acetylaspartate and glutamate [9]. Inhibition of this enzyme decreases neurotoxicity in a number of model systems [10,11].

Despite decades of research, the biochemical pathway and mechanisms regulating synthesis of NAAG are mostly unknown. NAAG biosynthesis from glutamine and/or glutamate has been demonstrated in a limited number of tissue explant systems. For example, synthesis has been demonstrated in isolated rat dorsal root ganglia, but the level of biosynthesis was very low [12]. Protein synthesis inhibitors had no effect on NAAG synthesis in this model system, suggesting that NAAG is synthesized by a non-ribosomal mechanism. NAAG biosynthesis has also been shown in explanted frog retinas [13], and more recently, in a crayfish nerve cord preparation [14,15]. However, to date NAAG biosynthesis has not been demonstrated in any human tissue, or in any continuous cell line. We show here that the human neuroblastoma cell line, SH-SY5Y, provides a useful model system for studying the biosynthesis of NAAG.

MATERIALS AND METHODS

The human neuroblastoma cell line (SH-SY5Y) was purchased from American Type culture collection (Manassas, VA, USA). Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12) with and without 2 mM glutamine was obtained from Cambrex Corporation (Baltimore, MD, USA). Characterized fetal bovine serum was from Hyclone (Logan, UT, USA). L-Gutamate, L-glutamine, NAAG, all-*trans*-retinoic acid and nerve growth factor (NGF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-[^{3}H]glutamine (sp. act. 50.0 Ci/mmol) and L-[^{3}H]glutamic acid (sp. act. 56.0 Ci/mmol) were from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were of analytical grade and were used without further purification. Deionized and filtered water (super Q: Millipore Corporation, Billerica, MA, USA) was used for all studies.

Cell culture: SH-SY5Y human neuroblastoma cells were grown in treated cell culture flasks (25 cm^2) in DMEM/F12 supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After reaching confluence, the above medium was replaced with the same medium except for L-[^{3}H]-glutamine (0.5 mM; sp. act. 20 mCi/mmol)

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or L-[^{3}H]-glutamate (0.05 mM; sp. act. 200 mCi/mmol) or both and grown for 24 h. After 24 h, the cells were harvested by treating with 2 mM EDTA in phosphate buffered saline (2 min, 37°C) followed by centrifugation (700 r.p.m., 5 min, 4°C). The cell pellets were kept frozen at −80°C until used.

Ion exchange chromatography: To identify radiolabeled NAAG, frozen cells were sonicated for 30 s in 1 ml 90% methanol and centrifuged at 2500 r.p.m. for 5 min at 4°C. Total protein was determined using the Bio-Rad DC protein assay kit after solubilizing the sedimented cells in 0.1 N NaOH. The supernatants were lyophilized in a Speed Vac, and the residue was dissolved in 1 ml distilled water and passed through a cation exchange column (0.5 × 1 cm, 50 W-X8 cation-exchange resin, Bio-Rad Laboratories, Hercules, CA, USA) to remove radiolabeled glutamine or glutamate. The column eluate and two 1 ml column washes were pooled and lyophilized in a Speed Vac. The residue was dissolved in 250 µl water containing 50 µg non-radioactive NAAG and injected into a Gilson HPLC system with an anion exchange column (BioAdvantage SAX 100A 5 µm 250 × 4.6 mm, Thompson Instrument Company, VA, USA). Isocratic elution (1 ml/min) was carried out using 200 mM potassium phosphate buffer (pH 4.9) as the mobile phase with the detection set at 231 nm for NAAG, which had a retention time of 42–45 min. Fractions (1 ml) corresponding to the eluted non-radioactive NAAG were collected and radioactivity was detected (Beckman liquid scintillation counter) in 500 µl of each fraction.

Reverse phase chromatography: To confirm identity of the NAAG, HPLC fractions corresponding to NAAG elution were pooled, lyophilized, and the residue dissolved in 250 µl water containing 25 µg non-radioactive NAAG. HPLC was carried out as above using a reverse phase column (BioAdvantage 100 C18 5 µm 250 × 4.6 mm) and 0.1% trifluoroacetic acid in water as the mobile phase. Under these conditions, NAAG has a retention time of about 26 min. Radioactivity was determined as described above.

Acid hydrolysis: HPLC fractions corresponding to NAAG elution using anion exchange HPLC were pooled, lyophilized, the residue dissolved in 250 µl water containing 25 µg non-radioactive NAAG and reverse phase HPLC was carried out as above. The reverse phase column fractions (1 ml) corresponding to NAAG elution were pooled and lyophilized by Speed Vac. The tube containing the lyophilized residue was kept in a hard glass tube containing 100 µl 6 N HCl and capped with a Mininert valve (Pierce, Rockford, IL, USA). Nitrogen gas was passed through the valve to create an inert atmosphere inside the tube, which was incubated at 100°C overnight. The residue was then dissolved in 250 µl water containing 25 µg non-radioactive NAAG and 5 µg non-radioactive L-glutamic acid. Reverse phase HPLC was carried out as above. Under these conditions, L-glutamic acid had a retention time of about 4 min. Radioactivity was determined as above.

NAAG synthesis in differentiated SH-SY5Y cells: SH-SY5Y cells were grown in DMEM/F12 containing 2 mM L-glutamine with B-27 serum free supplement (Invitrogen Corporation, Carlsbad, CA, USA) in 25 cm^2 cell culture treated flasks. The serum-free supplement was used in place of fetal bovine serum to eliminate the effects of growth factors present in fetal serum. To induce differentiation, cells were treated for different times (1,3,5 and 7 days) with and without 10 µM all-*trans*-retinoic acid or 50 ng/ml NGF. After each time point, the medium was replaced with the same medium except for L-[^{3}H]-glutamine (0.5 mM; sp. act. 20 mCi/mmol) and incubated at 37°C for 24 h. The cells were then processed and analyzed as described above. Total protein was determined using the Bio-Rad DC protein assay kit after solubilizing the sedimented cells in 0.1 N NaOH.

RESULTS

Figure 1 shows the radioactivity associated with anion exchange HPLC fractions corresponding to the co-eluted unlabeled NAAG. Incorporation of radiolabel from [^{3}H]glutamine was ~2-fold higher than that from [^{3}H]glutamate, despite the fact that the specific activity of the [^{3}H]glutamine was 10-fold lower. A proportionate increase in [^{3}H]NAAG was not observed when [^{3}H]glutamine and [^{3}H]glutamate were co-applied to the cells.

When the HPLC fraction corresponding to NAAG obtained from anion exchange chromatography was then subjected to reverse phase chromatography, the radioactivity co-migrated with non-radioactive NAAG, confirming its identity as NAAG.

Figure 2 shows the radioactivity obtained from different reverse phase HPLC fractions before and after acid hydrolysis. The results indicate that, after hydrolysis, about 70% of the radioactivity co-migrated with non-radioactive L-Glutamate. This further confirms the identity of the radioactive molecules as NAAG. As expected, no significant radioactivity was observed in the HPLC fraction corresponding to NAAG after hydrolysis, but two minor peaks with retention times of 10 min and 19 min were detected.

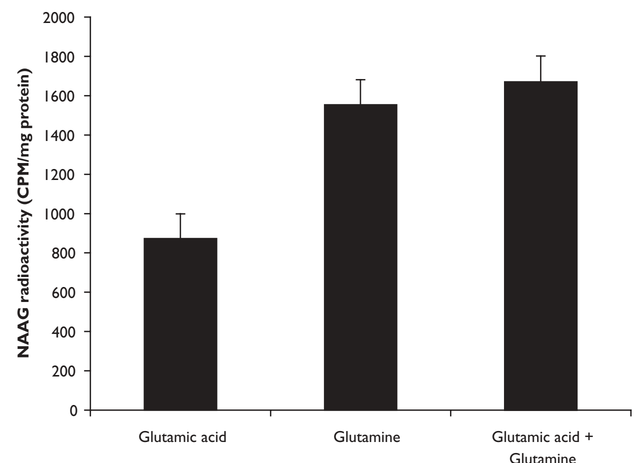

Fig. 1. SH-SY5Y cells were incubated with L-[^{3}H]-glutamine (0.5 mM; sp. act. 20 mCi/mmol) or L-[^{3}H]-glutamate (0.05 mM; sp. act. 200 mCi/mmol), or both, for 24 h. The radioactivity associated with the anion exchange HPLC fractions corresponding to co-eluted unlabeled NAAG was counted. The values are the average of 3 different experiments in each group. Statistical analyses were carried out using Student's *t*-test. The [^{3}H]-glutamine and the [^{3}H]-glutamine + [^{3}H]-glutamate values were significantly different from those where [^{3}H]-glutamate was used alone ($p < 0.005$).

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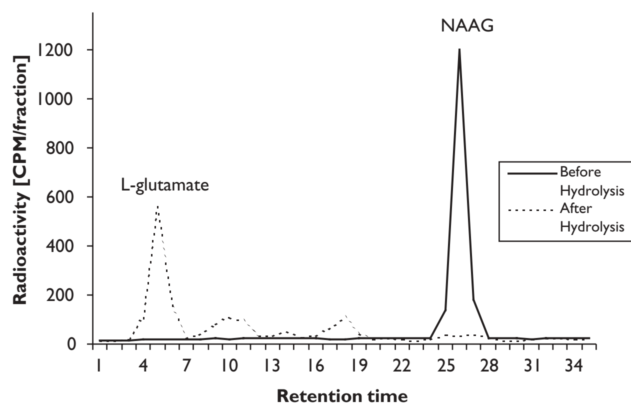

Fig. 2. The radioactivity profile using reverse phase HPLC corresponding to NAAG elution before (solid line) and after (broken line) acid hydrolysis. The solid line shows the radioactivity profile (CPM/fraction) of different HPLC fractions obtained using reverse phase HPLC after injecting the HPLC fractions corresponding to NAAG obtained from the anion exchange column. The dashed line shows the radioactivity profile (CPM/fraction) of different HPLC fractions obtained using reverse phase HPLC after acid hydrolysis of the HPLC fractions corresponding to NAAG obtained from the anion exchange column. The pattern shown is a representative of two similar experiments.

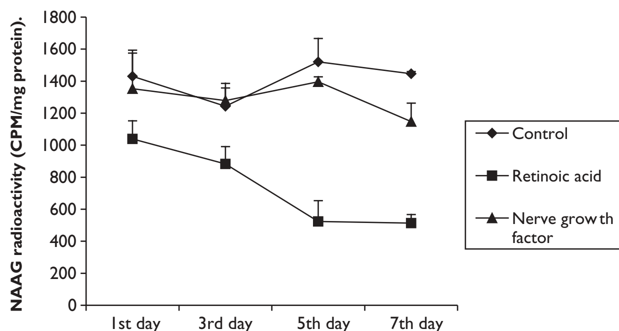

Fig. 3. The effect of two differentiating agents on NAAG synthesis over time. All data points are the average of two values. At each time point, the radioactivity of NAAG in cells (CPM/mg protein) with and without differentiation agents is shown. Statistical analysis was carried out using Student's *t*-test, and the retinoic acid values were significantly different from those in the control and NGF cultures after 5 days ($p < 0.05$).

These appear to be degradation products of glutamate formed during extreme conditions of acid hydrolysis.

Figure 3 shows NAAG synthesis after different days of treatment with two differentiating agents, retinoic acid and nerve growth factor (NGF). For these differentiation studies, cells were grown in the same media except that fetal bovine serum was replaced with B27 serum-free supplement to avoid the effects of growth factors in fetal serum. Radiolabel incorporation into NAAG was similar using both media conditions (see Fig. 1, Fig. 3). Visually, differentiation of SH-SY5Y cells under both conditions was similar, but neurite length was greater with retinoic acid treatment as compared with NGF treatment. Retinoic acid treatment resulted in a significant decrease in the synthesis of NAAG, reaching a minimum around the 5th day of differentiation. There was no significant effect of NGF on the synthesis of NAAG compared to the corresponding controls.

DISCUSSION

Recent developments in NAAG research have advanced our knowledge about this neuropeptide considerably with regard to receptor systems, second messengers [6], and enzymatic hydrolysis [9]. However, our knowledge about how NAAG is synthesized, and how that synthesis is regulated, is limited. The lack of progress has been due, in part, to the lack of simple model systems with which to study the biochemical mechanisms involved. To date, NAAG biosynthesis has only been demonstrated in explanted animal neural tissues, and the incorporation rates of radiolabeled glutamate or glutamine are low [12,14,15]. Homogenization of these tissues reduces the incorporation of radiolabeled precursors into NAAG, hampering subsequent enrichment of the activity. Little is known about how NAAG is synthesized from the proposed precursors, NAA and glutamate, but because synthesis was not blocked by protein synthesis inhibitors, it has been hypothesized that NAAG is synthesized by a dipeptide synthetase [12].

It would be advantageous to develop an *in vitro* model system for the study of NAAG biosynthesis in which the level of precursor incorporation is relatively high, and where the tissue does not have to be surgically harvested from numerous animals to provide sufficient quantities for the subsequent enrichment of activity. As such, the demonstration that NAAG biosynthesis occurs in a continuous neuroblastoma cell line that is simple to grow in culture should provide researchers a substantial advantage over explanted neural tissues for the study of NAAG biosynthesis. Here we show that a human neuroblastoma cell line, designated SH-SY5Y, can synthesize NAAG starting from glutamine or glutamate, with glutamine being the preferred substrate, possibly due to its greater uptake into neural cells. Because this cell line is of human origin, the results should be directly relevant to the study of human health and disease.

Retinoic acid and NGF are standard agents for inducing differentiation of neuronal cells in tissue culture, and have been used to induce differentiation in SH-SY5Y cells [16]. We studied the biosynthesis of NAAG in the SH-SY5Y cells differentiated using these agents to determine whether these two well-characterized differentiating agents, which are often used with this cell line, had any effect on NAAG biosynthesis. Differentiation of SH-SY5Y cells after 5 days with NGF did not affect their capacity to synthesize NAAG. However, differentiation for the same period with retinoic acid significantly reduced NAAG synthesis, suggesting that expression of the NAAG biosynthetic machinery is regulated during retinoic acid-induced neuronal differentiation. This finding may be useful for future studies on how NAAG synthesis is regulated in neurons. Examples of dipeptide synthesis by non-ribosomal means are known, for example, carnosine is a prevalent dipeptide (β-alanyl-L-histidine) that is synthesized by the enzyme carnosine synthase [17]. Efforts are currently underway to characterize the enzyme or enzymes responsible for NAAG biosynthesis in SH-SY5Y cells.

CONCLUSIONS

SH-SYSY human neuroblastoma cells synthesize NAAG from both glutamine and glutamate and can be used as a model system for characterizing the NAAG biosynthetic pathway and its regulation. Neuronal differentiation using

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retinoic acid significantly decreased NAAG biosynthesis, and therefore, this cell culture system should be useful for studying molecular mechanisms that regulate NAAG biosynthesis.

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