

# Regulation of *N*-acetylaspartate and *N*-acetylaspartylglutamate biosynthesis by protein kinase activators

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## Abstract

The neuronal dipeptide *N*-acetylaspartylglutamate (NAAG) is thought to be synthesized enzymatically from *N*-acetylaspartate (NAA) and glutamate. We used radiolabeled precursors to examine NAA and NAAG biosynthesis in SH-SY5Y human neuroblastoma cells stimulated with activators of protein kinase A (dbcAMP; N6,2'-O-dibutyryl cAMP) and protein kinase C (PMA; phorbol-12-myristate-13-acetate). Differentiation over the course of several days with dbcAMP resulted in increased endogenous NAA levels and NAAG synthesis from L-[<sup>3</sup>H]glutamine, whereas PMA-induced differentiation reduced both. Exogenously applied NAA caused dose dependent increases in intracellular NAA levels, and NAAG biosynthesis from L-[<sup>3</sup>H]glutamine, suggesting precursor-product and mass-action relationships between NAA and

NAAG. Incorporation of L-[<sup>3</sup>H]aspartate into NAA and NAAG occurred sequentially, appearing in NAA by 1 h, but not in NAAG until between 6 and 24 h. Synthesis of NAAG from L-[<sup>3</sup>H]aspartate was increased by dbcAMP and decreased by PMA at 24 h. The effects of PMA on L-[<sup>3</sup>H]aspartate incorporation into NAA were temporally biphasic. Using short incubation times (1 and 6 h), PMA increased L-[<sup>3</sup>H]aspartate incorporation into NAA, but with longer incubation (24 h), incorporation was significantly reduced. These results suggest that, while the neuronal production of NAA and NAAG are biochemically related, significant differences exist in the regulatory mechanisms controlling their biosynthesis.

**Keywords:** *N*-acetylaspartate, *N*-acetylaspartylglutamate, glutamate, glutamine, aspartate, SH-SY5Y cells. *J. Neurochem.* (2006) **98**, 2034–2042.

*N*-Acetylaspartylglutamate (NAAG) was first identified in the mammalian nervous system (Miyamoto *et al.* 1966) and is present at high concentrations in distinct populations of central and peripheral neurons (Cangro *et al.* 1987; Tieman *et al.* 1987; Tieman *et al.* 1991; Moffett *et al.* 1993; Moffett and Namboodiri 1995; Tieman and Tieman 1996; Moffett 2003). NAAG is an acetylated dipeptide comprised of aspartate and glutamate, and is a specific agonist at mGluR3 metabotropic glutamate receptors which modulate neurotransmitter release (Wroblewska *et al.* 1998; Garrido Sanabria *et al.* 2003; Poschel *et al.* 2005; Wroblewska *et al.* 2006). Forty years after its discovery, the biochemical pathway and mechanisms regulating the biosynthesis of NAAG are still unknown. Cangro *et al.* (1987) found that biosynthesis of NAAG from radiolabeled precursors in isolated rat dorsal root ganglia was unaffected by inhibitors of protein synthesis, suggesting that NAAG is synthesized by a non-ribosomal mechanism, most likely by a dipeptide synthetase. However, the level of NAAG synthesis in their model system was low, precluding a detailed analysis of the underlying mechanisms.

NAAG biosynthesis has also been demonstrated in explanted frog retinas (Williamson and Neale 1988), crayfish nerve-cord preparations (Urazaev *et al.* 2001; Buttram *et al.* 2002) and, more recently, in acutely isolated and hemisected rat spinal cord (Gehl *et al.* 2004). The incorporation of radiolabeled precursors into NAAG in these model systems was low, and the studies were hampered by the difficulty in obtaining viable neural tissues. We have recently demonstrated NAAG biosynthesis in a continuous human cell line, SH-SY5Y neuroblastoma cells, with good incorporation rates of radiolabeled precursors into NAAG (Arun *et al.* 2004). SH-SY5Y cells can be differentiated *in vitro* into several

Received March 21, 2006; accepted May 1, 2006.

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**Abbreviations used:** asp-NAT, aspartate *N*-acetyltransferase; dbcAMP, N6,2'-O-dibutyryl cAMP; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; sp. act., specific activity.

different phenotypes, including cells with many of the properties of noradrenergic sympathetic neurons (Pahlman *et al.* 1990). SH-SY5Y cells in culture proved to be a relatively simple model system for studying NAAG biosynthesis, one which provided very reproducible results without the complicating issue of glial cell contamination. In a previous study, we found that differentiation of SH-SY5Y cells with retinoic acid decreased NAAG biosynthesis, whereas differentiation with nerve growth factor had no effect (Arun *et al.* 2004). These results suggested that SH-SY5Y cells have intact NAAG biosynthetic regulatory mechanisms, providing a potential window into the biochemical pathways underlying those mechanisms.

*N*-acetylaspartate (NAA, or *N*-acetyl-L-aspartate) is one of the most abundant compounds in the CNS (Tallan *et al.* 1956; Tallan 1957). The functions served by NAA in the CNS remain matters of inquiry, but one hypothesis is that NAA is an immediate precursor for NAAG biosynthesis. NAAG is thought to be synthesized enzymatically by the ligation of NAA and glutamate, but this has never been demonstrated directly in cell-free homogenates. Rather, it has been inferred from studies using explanted neural tissues. SH-SY5Y cells offer a relatively simple system for studying the relationship between NAA and NAAG biosynthesis and the underlying mechanisms that regulate the enzymes involved. In the present report, we have studied the effects of protein kinase A (PKA) and protein kinase C (PKC) activators on the biosynthesis of NAA and NAAG in SH-SY5Y cells to determine if these intracellular signaling pathways are involved in regulating NAA and NAAG biosynthesis.

## Materials and methods

### Materials

SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium/Ham's F12 (50 : 50 vol : vol) medium with and without 2 mM glutamine were purchased from Cambrex Corporation (Baltimore, MD, USA). L-[<sup>3</sup>H]Glutamine (specific activity [sp. act.] 52.0 Ci/mmol) and L-[<sup>3</sup>H]aspartate (sp. act. 36.0 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). B-27 serum-free supplement was from Invitrogen (Carlsbad, CA, USA). L-glutamate, L-glutamine, NAA, NAAG, N6,2'-O-dibutyl cAMP (dbcAMP) and N6,2'-O-dibutyl cAMP, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St Louis, MO, USA). The p-bromophenazyl-8 reagent was obtained from Pierce (Rockford, IL, USA). Deionized and purified water (SuperQ; Millipore Corporation, Bedford, MA, USA) was used for all experiments.

### Cell culture

SH-SY5Y human neuroblastoma cells were grown in 12.5-cm<sup>2</sup>-treated cell culture flasks in Dulbecco's modified Eagle's medium/

F12 supplemented with 2 mM glutamine and B-27 serum-free supplement. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air. PMA (5–50 nM) or dbcAMP (0.5–10 μM) were added to the medium on the day of subculturing the cells into different flasks, and the cells were allowed to grow for various time periods (1–11 days). Preliminary studies indicated that dbcAMP and PMA had maximal effects at concentrations of 1 μM and 10 nM, respectively. However, the concentration of 1 μM dbcAMP led to some degree of cell mortality with long-term treatments, so, for differentiation studies, a concentration of 500 μM dbcAMP was used. Twenty-four hours before each fixed time point, the above medium was replaced with fresh medium plus L-[<sup>3</sup>H]glutamine (0.5 mM; sp. act. 20 μCi/μmol) and the cells were incubated for 24 h. The cells were harvested by incubating with 2 mM EDTA in phosphate-buffered saline (2 min, 37°C) followed by centrifugation (50 g, 5 min, 4°C). The cell pellets were kept frozen at –80°C until used.

### Determination of radiolabeled NAAG

Radiolabeled NAAG levels in the cell pellets were determined as described earlier (Arun *et al.* 2004). Briefly, the frozen cells were sonicated for 30 s in 1 mL of 90% methanol in water and centrifuged (800 g for 5 min at 4°C). All extracts were first purified on a cation-exchange column after lyophilization to remove positively charge molecules, followed by separation via anion exchange HPLC using an isocratic elution with 200 mM potassium phosphate buffer (pH 4.9). The NAAG eluate was detected by monitoring the optical density at 231 nm using unlabeled NAAG as a standard. The HPLC fractions corresponding to the NAAG peak were collected and the radioactivity was measured in a Beckmann liquid scintillation counter. The identity of radiolabeled NAAG was confirmed as described earlier (Arun *et al.* 2004).

### Determination of radiolabeled glutamine and glutamate levels

To quantitate radiolabeled glutamine and glutamate, the frozen cells were sonicated for 30 s in 1 mL of 90% methanol in water and centrifuged (800 g for 5 min at 4°C). Total protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) after solubilizing the sedimented pellets in 0.1 N NaOH. The supernatants were lyophilized and the residue was dissolved in 250 μL water containing 50 μg non-radioactive glutamine and glutamate and the HPLC was carried out as in the case of NAAG, except that 50 mM potassium phosphate buffer was used. The levels of radioactivity associated with the non-radioactive glutamine (retention time, 4.5 min) and glutamate (retention time, 6.5 min) peaks were measured.

### Incubation of exogenous NAA with SH-SY5Y cells

In order to test whether intracellular NAA can be increased, SH-SY5Y neuroblastoma cells were incubated with 10 mM NAA for 24 h and the cells were harvested as described above after thorough washing. The endogenous levels of NAA in the cells were then estimated as described earlier (Mathew *et al.* 2005). Briefly, the cells were homogenized in 90% methanol in water and centrifuged at 800 g for 5 min to separate the clear supernatant. The total protein in the precipitate was determined using the Bio-Rad DC protein assay kit after solubilizing in 0.1 N NaOH. The supernatant was lyophilized, purified by cation exchange column chromatography

and the NAA was derivatized using the p-bromophenacyl-8 reagent. The phenacyl ester of NAA was then separated and determined by reverse-phase HPLC using pure NAA as a standard.

#### Effect of external NAA concentration on NAAG biosynthesis from [<sup>3</sup>H]glutamine

SH-SY5Y cells were grown as described above and, upon reaching about 90% confluence, their medium was replaced with fresh medium plus L-[<sup>3</sup>H]glutamine (0.5 mM; sp. act. 20 μCi/μmol) containing varying concentrations (0, 1, 5 and 10 mM) of NAA and incubated for 24 h. The [<sup>3</sup>H]NAAG formed after 24 h of treatment was determined as described above.

#### Effects of PMA and dbcAMP on endogenous NAA levels in SH-SY5Y cells

The cells were grown as described above and, when they reached about 90% confluence, the cells were treated with dbcAMP (1 mM) or PMA (10 nM) for 24 h. Control flasks received no treatment. For cultures receiving 5 days of treatment, a concentration of 500 μM dbcAMP was used rather than 1 mM in order to prevent toxicity. After treatment, the cells were removed from the flasks by the addition of 2 mM EDTA in phosphate-buffered saline, and the endogenous levels of NAA in the cells were determined as described above.

#### NAAG biosynthesis with short incubation times

The cells were grown as described and, after they reached 90% confluence, the medium was replaced with fresh medium plus L-[<sup>3</sup>H]glutamine (26.0 μM; sp. act. 1926 μCi/μmol) in the presence and absence of dbcAMP (1 mM) or PMA (10 nM) and incubated further for varying intervals (1, 3 and 6 h). [<sup>3</sup>H]NAAG levels were determined as described above.

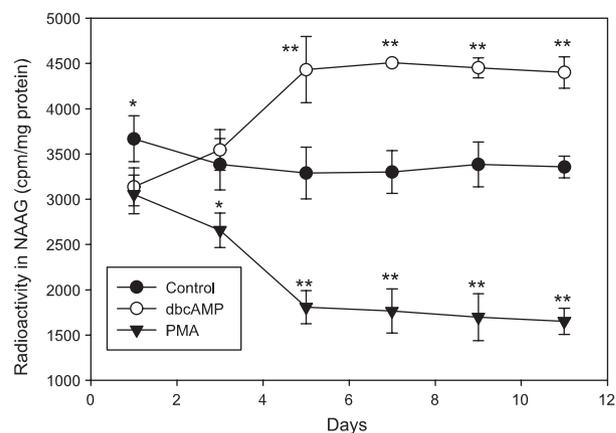
#### NAA and NAAG biosynthesis from [<sup>3</sup>H]aspartate

SH-SY5Y cells were grown as described above and, after they reached about 90% confluence, the medium was replaced with fresh medium plus L-[<sup>3</sup>H]aspartate (20.0 μM; sp. act. 450 μCi/μmol) in the presence or absence of dbcAMP (1 mM) or PMA (10 nM) and incubated further for varying intervals (1, 6 and 24 h). The [<sup>3</sup>H]NAAG levels were determined as described above. The [<sup>3</sup>H]NAA formed was measured using the same methods, and had a retention time of 12 min on anion exchange HPLC. Samples were re-chromatographed by reverse-phase HPLC as described previously (Arun *et al.* 2004) to determine what percentage of radiolabel co-migrating with NAA and NAAG by anion exchange would also co-migrate on a reverse-phase column. Under those conditions, NAA had a retention time of 8 min and NAAG had a retention time of 22 min. In order to confirm that the radioactivity was associated with NAAG, the reverse-phase HPLC fractions corresponding to NAAG were collected separately and subjected to acid hydrolysis followed by re-chromatography as described earlier (Arun *et al.* 2004). In order to further confirm the identity of radiolabeled NAA, the reverse-phase HPLC fractions corresponding to NAA were collected, pH adjusted to 11.0 by adding 5.0 N NaOH, kept overnight at room temperature (21°C), pH adjusted back to 7.0 and applied again to reverse-phase HPLC to determine if the radiolabel co-migrated with non-radiolabeled aspartate having a retention time of 4.5 min.

## Results

SH-SY5Y cells in culture were exposed for 6 days to varying concentrations of dbcAMP or PMA to induce neuronal differentiation, and then the medium was removed and replaced with fresh medium containing L-[<sup>3</sup>H]glutamine for 24 h. Incorporation of L-[<sup>3</sup>H]glutamine into NAAG was measured to determine the optimal doses of dbcAMP and PMA for further experiments. Long-term treatment (≥ 24 h) with PMA reduced L-[<sup>3</sup>H]glutamine incorporation into NAAG, whereas dbcAMP increased incorporation. PMA-induced differentiation of SH-SY5Y cells had a maximal effect on NAAG biosynthesis at 10 nM, and dbcAMP had a maximal effect at increasing glutamine incorporation into NAAG at a dose of 1 mM. Figure 1 shows the time course for the effect of 500 μM dbcAMP or 10 nM PMA on the biosynthesis of NAAG from L-[<sup>3</sup>H]glutamine. NAAG synthesis was increased approximately 35% by the 5th day of treatment with dbcAMP, and remained elevated for the remainder of the time course. Treatment with PMA decreased NAAG biosynthesis to nearly 50% of control levels by the 5th day, and the biosynthesis remained depressed through the 11th day of treatment. The slight but significant decreases in incorporation observed in the treated groups on day 1 may have been because the treatments with dbcAMP and PMA in this series of experiments were begun at the time the cells were subcultured into different flasks. This may have delayed their adherence and maturation slightly on the first day relative to untreated control cells.

Table 1 shows the levels of radiolabeled glutamine, glutamate and NAAG following 24-h incubation with L-[<sup>3</sup>H]glutamine after 6 days of neuronal differentiation with dbcAMP or PMA. SH-SY5Y cells converted most of the



**Fig. 1** Time course of the effects of dbcAMP (500 μM) and PMA (10 nM) on the incorporation of radiolabel from L-[<sup>3</sup>H]glutamine into NAAG. Values are means ± SD of three different experiments at each time point. Values of dbcAMP- and PMA-treated groups at each time point were compared with that of control. Using Student's *t*-test, \**p* < 0.05, \*\**p* < 0.01.

**Table 1** Effect of differentiation of SH-SY5Y cells for 7 days with dbcAMP (500  $\mu$ M) or PMA (10 nM) on the levels of radiolabeled glutamine, glutamate and NAAG after 24-h exposure to L-[ $^3$ H]glutamine.

| Compound  | Retention time <sup>a</sup><br>(minutes) | Radioactivity (cpm/mg protein) |                    |                    |
|-----------|--|--------------------------------|--------------------|--------------------|
|           |  | Control                        | cAMP               | PMA                |
| Glutamine | ~4.5                                     | 84618 $\pm$ 9456               | 86878 $\pm$ 17246  | 77711 $\pm$ 6511   |
| Glutamate | ~6.5                                     | 320826 $\pm$ 19062             | 324554 $\pm$ 23947 | 312493 $\pm$ 17560 |
| NAAG      | ~42.0                                    | 3301 $\pm$ 237                 | 4508 $\pm$ 28*     | 1765 $\pm$ 242*    |

<sup>a</sup>Potassium phosphate buffer (50 mM; pH 4.9) was used for the separation of both glutamine and glutamate, whereas 200 mM potassium phosphate buffer (pH 4.9) was used for the separation of NAAG. Values are means  $\pm$  SD for three different experiments. Using Student's *t*-test, the values of dbcAMP- and PMA-treated groups were compared with those of control; \**p* < 0.01.

L-[ $^3$ H]glutamine to L-[ $^3$ H]glutamate during the course of the 24-h treatment period, with approximately 78% of the radiolabel recovered as glutamate. There were no significant changes in the levels of radiolabeled glutamine or the formation of radiolabeled glutamate after treatment with either dbcAMP or PMA. However, NAAG biosynthesis was affected; dbcAMP increased synthesis, and PMA decreased it. Approximately 1% of L-[ $^3$ H]glutamine was converted to NAAG in the case of control cultures, whereas approximately 1.35% was converted to NAAG after neuronal differentiation with dbcAMP, and approximately 0.5% was converted into NAAG after neuronal differentiation with PMA.

Table 2 shows the levels of radiolabeled glutamine, glutamate and NAAG in cells treated for 24 h with dbcAMP or PMA. Here, the cells were grown without treatment for 4 days, and on the 4th day the cells were incubated with L-[ $^3$ H]glutamine with and without dbcAMP (1 mM) or PMA (10 nM) for 24 h. Under these conditions, there were no significant changes in the levels of radiolabeled glutamine or the formation of radiolabeled glutamate after treatment with either compound. dbcAMP significantly increased the level of radiolabeled NAAG, but with PMA there was no significant change. As with the case of long-term treatment with dbcAMP and PMA, most of the L-[ $^3$ H]glutamine applied to the cells was recovered in the form of L-[ $^3$ H]glu-

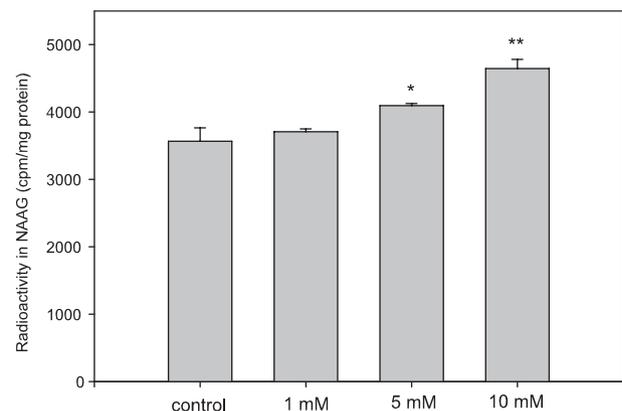
**Table 2** Comparison of radiolabeled glutamine, glutamate and NAAG levels in mature SH-SY5Y cells after 24 h of treatment with and without dbcAMP (1 mM) or PMA (10 nM) in the presence of L-[ $^3$ H]glutamine

| Compound  | Radioactivity (cpm/mg protein) |                    |                    |
|-----------|--------------------------------|--------------------|--------------------|
|           | Control                        | cAMP               | PMA                |
| Glutamine | 87284 $\pm$ 7154               | 83587 $\pm$ 11205  | 85711 $\pm$ 10674  |
| Glutamate | 325980 $\pm$ 19062             | 358754 $\pm$ 23947 | 342584 $\pm$ 17560 |
| NAAG      | 3227 $\pm$ 142                 | 3908 $\pm$ 173*    | 2877 $\pm$ 296     |

Values are means  $\pm$  SD for five different experiments. Using Student's *t*-test, the values of dbcAMP- and PMA-treated groups were compared with those of control; \**p* < 0.01.

tamate after 24-h treatment. Based on these findings, approximately 1% of the radiolabeled glutamine that is converted to glutamate is further metabolized to NAAG over a 24-h period.

To determine the extent to which SH-SY5Y cells can take up external NAA, the cells were exposed to 10 mM unlabeled NAA in the culture media for 24 h, and intracellular NAA concentrations were determined by HPLC after derivatization with p-bromophenazyl-8. In control cultures, intracellular NAA levels were 823.7 ng/mg protein (*n* = 3, SD  $\pm$  98.8). These levels increased to 1869.2 ng/mg protein (*n* = 3, SD  $\pm$  144.4, *p* < 0.01) in cells exposed to 10 mM external NAA, an approximately 2.2-fold increase in intracellular NAA. In order to investigate whether or not increasing the internal NAA concentrations could increase the incorporation of L-[ $^3$ H]glutamine into NAAG, we incubated SH-SY5Y cell cultures with increasing concentrations of unlabeled NAA. It was found that extracellular NAA increased L-[ $^3$ H]glutamine incorporation into NAAG in a dose-dependent manner, but the increases were relatively modest (Fig. 2). For example, application of 10 mM NAA to the cell culture media resulted



**Fig. 2** Effect of external NAA concentration on NAAG biosynthesis in SH-SY5Y cells. The cells were incubated with L-[ $^3$ H]glutamine and with increasing concentrations of NAA for 24 h. Values are means  $\pm$  SD from three different experiments. Values of different doses of NAA are compared with that of control, using Student's *t*-test, \**p* < 0.05, \*\**p* < 0.01.

in an approximately 30% increase in tritiated glutamine incorporation into NAAG during a 24-h incubation period.

NAA is presumed to be a direct precursor for NAAG biosynthesis, and so it was of interest to determine what endogenous levels of NAA occurred in SH-SY5Y cells in culture, and whether these levels were affected by dbcAMP or PMA. Table 3 shows the endogenous levels of NAA in the cells after 24 h and 5 days treatment with and without dbcAMP (500  $\mu$ M) or PMA (10 nM). Treatment with dbcAMP for 24 h or 5 days significantly increased the endogenous levels of NAA, whereas PMA caused a significant decrease in NAA levels after 5 days of treatment.

In order to explore whether NAAG biosynthesis could be detected at earlier time points after exposure to radiolabeled precursors, we incubated SH-SY5Y cells with  $\sim$ 96-fold higher specific activity L-[ $^3$ H]glutamine (26.0  $\mu$ M; sp. act. 1926  $\mu$ Ci/ $\mu$ mol) for 1, 3 and 6 h with and without dbcAMP or PMA (Fig. 3). No radiolabeled NAAG was

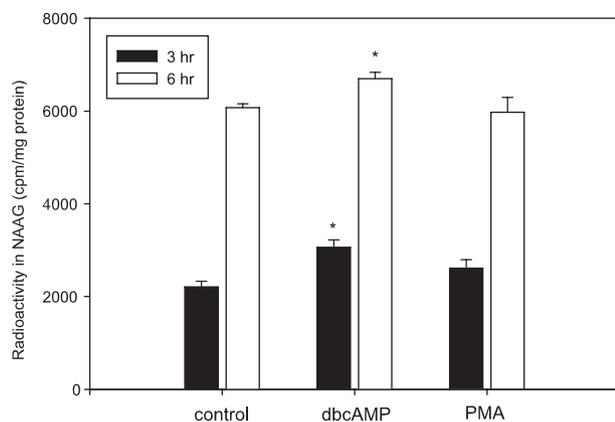
detected at 1 h under any tested conditions. Incorporation of radiolabel into NAAG was substantial at 3 h, and dbcAMP treatment for 3 h slightly increased incorporation. PMA treatment for 3 h had no effect on glutamine incorporation into NAAG. A similar pattern of radiolabel incorporation was observed with 6 h of exposure, but substantially more radioactivity was incorporated into NAAG at 6 h than at 3 h.

Because aspartate acts as a direct precursor for NAA synthesis via the L-aspartate *N*-acetyltransferase reaction, we investigated the effects of 1-, 6- and 24-h incubation with dbcAMP and PMA on the incorporation of L-[ $^3$ H]aspartate into both NAA and NAAG in SH-SY5Y cells (Table 4). dbcAMP had no effect on L-[ $^3$ H]aspartate incorporation into NAA at 1 h, but incorporation was significantly increased at both 6 and 24 h. In contrast, the effect of PMA was dependent on the length of treatment, wherein PMA substantially increased L-[ $^3$ H]aspartate incorporation into NAA with 1 and 6 h of treatment, but resulted in a significant drop in radiolabeled aspartate incorporation into NAA with 24 h of exposure. Radiolabeled aspartate was not incorporated detectably into NAAG after either 1 or 6 h of exposure to L-[ $^3$ H]aspartate, but incorporation was detectable at 24 h of exposure, and the incorporation was increased with dbcAMP and decreased with PMA treatment. Radioactivity was confirmed to be present in NAA and NAAG by re-chromatography using a reverse-phase column, wherein  $\geq$ 90% of the radiolabel associated with NAA, and  $\geq$ 80% of the radiolabel associated with NAAG on anion exchange HPLC were found to co-migrate with the corresponding compounds on reverse-phase HPLC. Hydrolyzed samples of radiolabeled NAAG and NAA

**Table 3** Endogenous NAA levels in SH-SY5Y cells after 1 and 5 days of treatment with dbcAMP (500  $\mu$ M) or PMA (1 nM)

| Days  | NAA (ng/mg protein) |                 |                |
|-------|---------------------|-----------------|----------------|
|       | Control             | cAMP            | PMA            |
| 1day  | 963 $\pm$ 141       | 1351 $\pm$ 96*  | 893 $\pm$ 101  |
| 5days | 1045 $\pm$ 65       | 1290 $\pm$ 54** | 892 $\pm$ 45** |

NAA concentrations were determined by HPLC (see Materials and methods). Values are means  $\pm$  SD of five different experiments in each case. Values of cAMP- and PMA-treated groups were compared with those of controls, using Student's *t*-test, \**p* < 0.05, \*\**p* < 0.01.



**Fig. 3** Effects of short-term treatment with dbcAMP (1 mM) or PMA (10 nM) on the biosynthesis of NAAG in SH-SY5Y cells using L-[ $^3$ H]glutamine with much higher specific activity. Values are means  $\pm$  SD from three different experiments in each case. Values of dbcAMP- and PMA-treated groups were compared with those of controls, using Student's *t*-test; \**p* < 0.01.

**Table 4** Simultaneous determination of L-[ $^3$ H]aspartate incorporation into NAA and NAAG with dbcAMP (1 mM) or PMA (10 nM) treatment of SH-SY5Y cells

|         | NAA (cpm/mg protein) | NAAG (cpm/mg protein) |
|---------|----------------------|-----------------------|
| Control |                      |                       |
| 1hr     | 5417 $\pm$ 119       | ND                    |
| 6hrs    | 6818 $\pm$ 149       | ND                    |
| 24hrs   | 6272 $\pm$ 181       | 844 $\pm$ 122         |
| cAMP    |                      |                       |
| 1hr     | 5754 $\pm$ 265       | ND                    |
| 6hrs    | 7535 $\pm$ 290*      | ND                    |
| 24hrs   | 6929 $\pm$ 212*      | 1421 $\pm$ 164**      |
| PMA     |                      |                       |
| 1hr     | 9410 $\pm$ 464**     | ND                    |
| 6hrs    | 9034 $\pm$ 377**     | ND                    |
| 24hrs   | 5469 $\pm$ 410*      | 476 $\pm$ 33**        |

Values are means  $\pm$  SD of three different experiments in each case. Values of cAMP- and PMA-treated groups at each time point were compared with those of controls, using Student's *t*-test, \**p* < 0.05, \*\**p* < 0.01. ND, non-detectable.

obtained from the reverse-phase column were re-chromatographed by reverse-phase HPLC, and the radioactivity from both samples co-migrated with non-radioactive aspartate (retention time, 4.5 min).

## Discussion

Little is known about the mechanisms of NAAG biosynthesis, or how that synthesis is regulated, primarily as a result of a paucity of simple model systems that can be used for such studies. Although early reports described the synthesis of *N*-acetyl-peptides, including NAAG, in brain homogenates (Reichelt *et al.* 1971; Reichelt and Kvamme 1973; Reichelt *et al.* 1976; Lahdesmaki and Timonen 1982), these results have not been reproducible in other laboratories. In contrast to those unconfirmed reports of NAAG biosynthesis in homogenates, NAAG synthesis from radiolabeled precursors has been reliably demonstrated predominantly in explanted neuronal tissues such as rat dorsal root ganglia (Cangro *et al.* 1987), frog retina (Williamson and Neale 1988), crayfish nerve cord (Buttram *et al.* 2002) and hemisected rat spinal cord (Gehl *et al.* 2004). The capacity to synthesize NAAG is lost upon neural tissue homogenization, but the reason remains unknown. This has hampered research into NAAG biosynthesis for almost 20 years. The SH-SY5Y human neuroblastoma cell line provides an improved model system for studying the mechanisms of NAAG biosynthesis. This cell line can be differentiated into different neuronal phenotypes in culture, and the cells readily incorporate radiolabeled precursors into NAA and NAAG. Furthermore, this model system does not suffer from contamination by other cell types, including astrocytes which express the NAAG-degrading enzyme carboxypeptidase II (Berger *et al.* 1999). The current investigations were greatly facilitated by the use of this model system.

NAAG biosynthesis has also been reported to occur in two types of glial cell; primary cultures of rat cortical astrocytes (Gehl *et al.* 2004) and crayfish nerve-cord glial cells (Buttram *et al.* 2002). These systems were reported to display robust synthesis of a radiolabeled compound that co-migrated with NAAG chromatographically. In both of these investigations, the glial cells were reported to synthesize much more NAAG from radiolabeled precursors than did neuronal explants. However, the co-migrating radiolabeled compound produced by glia in both of these studies was not confirmed to be NAAG by other techniques, such as re-chromatography by reverse-phase HPLC, or acid hydrolysis, as has been carried out in previous reports on NAAG biosynthesis (Cangro *et al.* 1987; Arun *et al.* 2004). Immunohistochemical studies have failed to show NAA or NAAG expression by astrocytes in the brain (Moffett *et al.* 1991, 1993; Moffett and Namboodiri 1995), so the issue of NAAG biosynthesis by glial cells remains an open question requiring further research.

For reasons that are not fully understood, radiolabeled glutamine is a better precursor for NAAG biosynthesis than either aspartate or glutamate, despite the fact that glutamate is thought to be a direct precursor for NAAG. It has been postulated that the preference for applied glutamine as a precursor for NAAG biosynthesis is because neurons have a strong uptake system for glutamine, but not for glutamate (Cangro *et al.* 1987). In the present study, we observed lower incorporation of radiolabeled aspartate into NAAG, as has been reported in other studies (Gehl *et al.* 2004). This may again relate to less efficient uptake systems for aspartate than glutamine in neurons, but other mechanisms, including isotopic dilution of the immediate precursor (radiolabeled NAA), or other complicating factors, may also be involved (see below).

The synthesis of NAA has been better characterized than that of NAAG. NAA synthesis occurs in neuronal mitochondria via the action of the nervous system-specific enzyme *L*-aspartate *N*-acetyltransferase (EC 2.3.1.17; asp-NAT). Early studies showed that brain tissue contained an enzyme that synthesized NAA, and the enzyme was found to be highly specific to aspartate as the amino acid substrate, with activity detectable only in the nervous system (Benuck and D'Adamo. 1968). This highly specific neuronal enzyme is membrane bound, and utilizes the substrates aspartate and acetyl CoA to synthesize NAA (Truckenmiller *et al.* 1985). Recently, asp-NAT was partially purified from rat brain using multiple chromatographic techniques, and the active enzyme was reported to be a high molecular weight protein complex (~670 kDa; Madhavarao *et al.* 2003). Subcellular fractionation experiments suggest that asp-NAT activity is distributed in two subcellular fractions, with approximately 3-fold higher total activity in the microsomal fraction than in highly purified mitochondria (Lu *et al.* 2004). Little is known about how asp-NAT expression and NAA synthesis are regulated in the CNS, or how they are related to NAAG biosynthesis.

Factors that affect or regulate NAAG biosynthesis have been only cursorily studied. Using the SH-SY5Y neuroblastoma model system, we previously demonstrated that NAAG biosynthesis is decreased by retinoic acid-induced neuronal differentiation, whereas nerve growth factor-induced neuronal differentiation had no significant effect (Arun *et al.* 2004). Gehl and colleagues showed that potassium-induced depolarization increased radiolabeled precursor incorporation into NAAG in spinal cord explants, and that increasing the external NAA concentration enhanced NAAG synthesis in cultured cortical astrocytes (Gehl *et al.* 2004). In the present report, we also found that exogenously applied NAA led to increased NAAG synthesis in SH-SY5Y cells, and that treatment with dbcAMP and PMA for 24 h or longer caused the level of unlabeled NAA in the cells to change in the same manner as NAAG synthesis (see below). These results support the earlier observations that NAAG is

synthesized enzymatically from NAA, and that increases in NAA levels lead to increased NAAG synthesis (Gehl *et al.* 2004).

Differentiation of SH-SY5Y cells with an activator of PKA had opposite effects on the biosynthesis of both NAA and NAAG as compared with differentiation using a PKC activator. Biosynthesis of NAA and NAAG were both increased after differentiation with dbcAMP (a PKA activator), but were decreased after differentiation with the phorbol ester PMA (a PKC activator). These results suggest that the expression or activity of the NAA and the NAAG biosynthetic systems in SH-SY5Y cells can be enhanced or repressed by differentiating the cells toward different neuronal phenotypes. SH-SY5Y cells are thought to provide a useful *in vitro* model for neuronal differentiation (Encinas *et al.* 2000), and can be induced to differentiate along different pathways using different culturing conditions. For example, phorbol esters such as TPA or PMA raise tyrosine hydroxylase and noradrenaline levels, whereas retinoic acid slightly increases choline acetyltransferase activity (Pahlman *et al.* 1995). Thus, phorbol esters such as TPA and PMA induce noradrenergic differentiation occurring via PKC activation (Goodall *et al.* 1997). In the present study, we have shown that differentiation of SH-SY5Y cells with PMA reduces NAAG biosynthesis substantially, suggesting that differentiation toward a noradrenergic neuronal phenotype may be associated with reduced levels of NAAG synthesis. dbcAMP is known to induce SH-SY5Y neuronal differentiation and neurite elongation by rapidly increasing PKA activity (Sanchez *et al.* 2004). We found that dbcAMP slightly but significantly increased NAAG biosynthesis in as little as 3 h after application (see Fig. 3), suggesting some connection between early PKA activation and increased NAAG biosynthesis. However, the effect was relatively small, and it was slower than the effect of PKC activation on NAA synthesis from aspartate.

Incorporation of L-[<sup>3</sup>H]aspartate into NAAG was delayed relative to the incorporation of L-[<sup>3</sup>H]glutamine by over 3 h. NAAG biosynthesis from radiolabeled glutamine could not be detected at 1 h, but was detected at 3 h, whereas NAAG biosynthesis from radiolabeled aspartate was undetectable at 6 h, but was observed by 24 h. The reason for the slow incorporation of aspartate as opposed to glutamine is not likely to be as a result of the number of enzymatic steps involved, because both substrates would require a minimum of two enzyme steps to produce NAAG. The discrepancy may be in part because glutamine is taken up much more avidly by neurons, and therefore enters the metabolic pool more quickly. However, substantial NAA synthesis was observed within 1 h of L-[<sup>3</sup>H]aspartate application, indicating that aspartate uptake and metabolism was robust in SH-SY5Y cells. It is also possible that newly synthesized [<sup>3</sup>H]NAA formed from L-[<sup>3</sup>H]aspartate becomes diluted in the unlabeled pool of intracellular NAA. However, a similar

isotopic dilution effect should also occur when L-[<sup>3</sup>H]glutamine is converted to L-[<sup>3</sup>H]glutamate, prior to incorporation into NAAG. In the current study, no changes in intracellular glutamine or glutamate levels were observed with dbcAMP or PMA treatments, indicating that neither glutamine uptake, nor conversion of glutamine to glutamate were affected by these treatments. The delayed incorporation of radiolabeled aspartate into NAAG as compared with the more rapid incorporation of radiolabeled glutamine is difficult to explain based on the present data, and requires further investigation.

NAA synthesis from radiolabeled aspartate was temporally biphasic in response to PMA in the sense that incorporation into NAA was increased by 74% after 1 h and by 33% after 6 h of treatment, but was reduced by 11% with 24 h of treatment (see Table 4). The later effect of PMA on NAA synthesis was similar to the effect on NAAG biosynthesis, that is, a significant reduction in the incorporation of radiolabeled aspartate. It is possible that the early stimulating effect of PMA is mediated by PKC regulation of NAA biosynthesis at the level of asp-NAT phosphorylation. In contrast, long-term treatment of SH-SY5Y cells with PMA results in neuronal differentiation into a phenotype which synthesizes less NAA and less NAAG than untreated cells.

The present findings are the first to demonstrate sequential NAA and NAAG biosynthesis from one radiolabeled precursor in a single cell type. Several lines of evidence presented here suggest that NAA is a direct precursor for NAAG biosynthesis, and that increased NAA levels lead to increased NAAG synthesis by a mass action mechanism. First, both of the protein kinase activators used in the current study resulted in differentiated neuronal phenotypes where NAA levels and NAAG synthesis changed in similar fashion. PMA-induced differentiation resulted in reduced NAA and NAAG synthesis, whereas dbcAMP-induced differentiation resulted in greater NAA and NAAG synthesis. Further, greatly increasing the extracellular concentration of NAA, which more than doubled the intracellular concentration, was found to increase NAAG biosynthesis by approximately 30%. These findings argue in favor of regulation of NAA and NAAG biosynthesis at the initial enzymatic step at asp-NAT. However, there are several facts that argue against simple NAA mass action regulation of NAAG synthesis. It is well documented that NAA and glutamate are constitutively expressed in most neurons at very high concentrations, making mass action control of a NAAG synthetic enzyme problematic. Also, in the current study, NAA synthesis was increased within 1 h of incubation with PMA and L-[<sup>3</sup>H]aspartate, but NAAG synthesis from labeled aspartate in the same cultures was not seen until between 6 and 24 h after introduction of the radiolabel, suggesting some type of bottleneck between the two synthetic steps. Perhaps the strongest evidence arguing against a mass action effect is that PMA treatment for 3 and 6 h did not increase NAAG biosynthesis (Fig. 3), despite the fact that similar treatment

substantially increased NAA synthesis (Table 4). It should be noted that dbcAMP treatment increased NAAG biosynthesis from L-[<sup>3</sup>H]glutamine at 3 and 6 h, indicating that SH-SY5Y cells are able to up-regulate NAAG biosynthesis in that time frame. Therefore, the lack of an early effect of PMA on NAAG biosynthesis argues against a mass action control of NAAG biosynthesis by increasing NAA biosynthesis.

The current results demonstrate the opposing effects of PKA- and PKC-mediated neuronal differentiation on the synthesis of NAA and NAAG. PKA-induced differentiation of SH-SY5Y cells enhanced NAA and NAAG biosynthesis, and PKC-induced differentiation inhibited them. Short-term (hours) versus long-term (days) treatment of neuroblastoma cells with protein kinase activators alters cellular behavior in distinct ways. The more rapid effects of protein kinase activators are mediated by protein phosphorylation, with subsequent enhancement or diminution of protein function. These early effects can be contrasted with the changes induced in SH-SY5Y cells by long-term treatment with protein kinase activators, which result in altered phenotypes and altered protein expression in the differentiated cells. The rapid and potent effect of PMA on increasing L-[<sup>3</sup>H]aspartate incorporation into NAA suggests a role for PKC-mediated regulation of asp-NAT activity and the synthesis of NAA in SH-SY5Y cells. This finding in human neuroblastoma cells raises the possibility that NAA synthesis in neurons may be regulated *in vivo* by PKC phosphorylation of asp-NAT. In comparison, the regulation of NAAG biosynthesis was found to be more complex, wherein the effects of protein kinase activators were relatively slow. This could imply that the activity of the NAAG synthesizing enzyme is not regulated directly by PKA- or PKC-mediated phosphorylation, but by other downstream factors requiring hours to affect changes in biosynthesis.

## Acknowledgements

We wish to thank Michael Doh for his excellent technical assistance in the initial phase of these studies. This work was supported by the USUHS internal grant (C070PB).

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