

## Antipsychotic drugs increase *N*-acetylaspartate and *N*-acetylaspartylglutamate in SH-SY5Y human neuroblastoma cells

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### Abstract

*N*-Acetylaspartate (NAA) and *N*-acetylaspartylglutamate (NAAG) are related neuronal metabolites associated with the diagnosis and treatment of schizophrenia. NAA is a valuable marker of neuronal viability in magnetic resonance spectroscopy, a technique which has consistently shown NAA levels to be modestly decreased in the brains of schizophrenia patients. However, there are conflicting reports on the changes in brain NAA levels after treatment with antipsychotic drugs, which exert their therapeutic effects in part by blocking dopamine D<sub>2</sub> receptors. NAAG is reported to be an agonist of the metabotropic glutamate 2/3 receptor, which is linked to neurotransmitter release modulation, including glutamate release. Alterations in NAAG metabolism have been implicated in the development of schizophrenia possibly via

dysregulation of glutamate neurotransmission. In the present study we have used high performance liquid chromatography to determine the effects of the antipsychotic drugs haloperidol and clozapine on NAA and NAAG levels in SH-SY5Y human neuroblastoma cells, a model system used to test the responses of dopaminergic neurons *in vitro*. The results indicate that the antipsychotic drugs haloperidol and clozapine increase both NAA and NAAG levels in SH-SY5Y cells in a dose and time dependant manner, providing evidence that NAA and NAAG metabolism in neurons is responsive to antipsychotic drug treatment.

**Keywords:** antipsychotics, clozapine, haloperidol, *N*-acetylaspartate, *N*-acetylaspartylglutamate, schizophrenia.

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The therapeutic efficacy of antipsychotic drugs is generally believed to be due to their ability to block central dopamine D<sub>2</sub> receptors (Seeman *et al.* 1976; Farde *et al.* 1989; Deutch *et al.* 1991; Dixon *et al.* 1995). Haloperidol is one of the most commonly prescribed of the so-called typical antipsychotic drugs, whose actions are accompanied by extrapyramidal side effects, including a Parkinson-like syndrome, and tardive dyskinesia (McEvoy 1983). Clozapine is a commonly prescribed atypical antipsychotic drug which has a lower incidence of extrapyramidal side effects, and appears to be effective for some treatment-resistant positive and negative symptoms of schizophrenia (Deutch *et al.* 1991). The molecular mechanisms underlying the different actions of typical and atypical antipsychotic drugs are not clear.

*N*-Acetylaspartate (NAA) is one of the most abundant amino acid derivatives in the CNS, attaining concentrations between 8 mM (Tanaka *et al.* 2006) and 12 mM (Rigotti *et al.* 2007) in the human brain. Due to its high concentration, the regional levels of NAA can be monitored non-invasively by magnetic resonance spectroscopy (MRS)

(reviewed in Moffett *et al.* 2007). NAA is widely accepted as a marker of neuronal health and viability. MRS studies in schizophrenia patients indicate small reductions in absolute NAA levels, or in the NAA/creatinine ratio, particularly in the prefrontal cortex and hippocampus (Bertolino *et al.* 1996; Callicott *et al.* 2000a; Deicken *et al.* 2000; Abbott and Bustillo 2006).

*N*-Acetylaspartylglutamate (NAAG), the most abundant neuromodulatory peptide in the CNS, is thought to be formed biosynthetically from NAA. Research suggests that

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*Abbreviations used:* DMSO, dimethylsulfoxide; GCP II, glutamate carboxypeptidase II; mGluR2/3, metabotropic glutamate receptor type 2/3; MRS, magnetic resonance spectroscopy; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; PCP, phencyclidine.

NAAG may act to modulate neurotransmitter release through a pre-synaptic metabotropic glutamate receptor (mGluR2/3) agonist mechanism (Zhao *et al.* 2001; Xi *et al.* 2002), and that it has neuroprotectant actions (Thomas *et al.* 2000). NAAG has also been reported to have both weak agonist and antagonist properties at NMDA receptors (Westbrook *et al.* 1986; Burlina *et al.* 1994). HPLC analyses of post-mortem brain samples found NAAG levels to be selectively reduced in the superior temporal cortex of schizophrenia patients (Nudmamud *et al.* 2003). As such, there is evidence that both brain NAA and NAAG levels are reduced in specific brain regions in patients with schizophrenia as compared with age matched control subjects. It has been proposed that subtle alterations in NAAG metabolism may be partly responsible for the etiology of schizophrenia manifested as hypoactivity in prefrontal, temporal and hippocampal glutamatergic neurotransmission (Tsai *et al.* 1995; Flores and Coyle 2003; Tsai 2005; Zhou *et al.* 2005).

There are conflicting reports in the literature concerning the effects of antipsychotic drugs on NAA levels in the brain. The majority of studies report that antipsychotic drugs do not result in altered NAA levels (Okumura *et al.* 1959; Lindquist *et al.* 2000; Bustillo *et al.* 2004, 2006, 2007). There are other reports showing that antipsychotic drugs increase (Bertolino *et al.* 2001; Harte *et al.* 2005) or decrease (Fannon *et al.* 2003) NAA levels in the brain. To our knowledge, there are no studies on the effect of antipsychotic drugs on NAAG levels in the brain, although an analysis of cerebrospinal fluid from normal and schizophrenic patients showed no differences in NAA or NAAG concentrations (Faull *et al.* 1999).

Here we determined the effect of the antipsychotic drugs haloperidol and clozapine on NAA and NAAG levels in SH-SY5Y human neuroblastoma cells, a recently developed model for studying the biosynthesis of NAA and NAAG (Arun *et al.* 2004, 2006). SH-SY5Y cells exhibit characteristics of both noradrenergic and dopaminergic neurons, and express the dopamine synthesizing enzyme tyrosine hydroxylase, dopamine transporters, as well as dopamine receptors (Presgraves *et al.* 2004; McMillan *et al.* 2007), including the dopamine D<sub>2</sub> receptor (Dziedzicka-Wasylewska and Solich 2004). Like the dopaminergic cells of the substantia nigra compacta, they are uniquely sensitive to proparkinsonian compound toxicity (Fang *et al.* 1995; Chen *et al.* 2005). For these reasons, we explored the effects of the dopamine D<sub>2</sub> receptor blockers haloperidol and clozapine on NAA and NAAG levels in SH-SY5Y cells. In the present study we used a modified high performance liquid chromatography (HPLC) technique allowing the simultaneous determination of the endogenous levels of both NAA and NAAG in these cells in culture in order to investigate the direct effects of these two antipsychotic drugs in a model dopaminergic system.

## Materials and methods

SH-SY5Y human neuroblastoma cells, Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from American Type Culture Collection (Manassas, VA, USA). NAA, NAAG, dimethylsulfoxide (DMSO), haloperidol and clozapine were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Bromophenacyl-8 reagent (0.1 mmol/mL *p*-bromophenacylbromide and 0.005 mmol/mL crown ether in acetonitrile) was purchased from Pierce Chemical (Rockford, IL). All other chemicals used were of analytical grade and were used without further purification. Deionized and filtered water (SuperQ; Millipore Corporation, Billerica, MA, USA) was used for all experiments.

### Cell culture

SH-SY5Y human neuroblastoma cells were grown in 75 cm<sup>2</sup> treated cell culture flasks in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air. Cells were sub-cultured when flasks reached approximately 90% confluence.

### Treatment of antipsychotic drugs

Effective doses of haloperidol and clozapine were determined empirically. Stock solutions of haloperidol and clozapine (5 mM) were made in DMSO and added to the respective flasks to final concentrations of 20, 40 and 60 μM, and incubated for 24 h. Equal volume of DMSO served as controls. For time course experiments, the cells were grown for varying intervals including 6, 24 and 48 h with 50 μM of each drug. After the incubation period, the medium was removed and the cells were washed twice with phosphate buffered saline. The cells were then harvested using 2 mM EDTA in phosphate buffered saline. The cell suspension obtained was then centrifuged at 40 g for 5 min at 4°C, the supernatant was removed and the sedimented cells were stored at -80°C until used.

### Simultaneous determination of NAA and NAAG

Both NAA and NAAG were estimated by using a method we have described earlier (Mathew *et al.* 2005), with minor modifications as follows. Cell homogenates were prepared in methanol:water (90/10 vol), by sonicating at 0–4°C in an ultrasonic homogenizer (4710 Series, Cole-Parmer Instruments Co., Chicago, IL, USA) with the microtip probe limit set at 5 and duty cycle set at 30%. The homogenate was then centrifuged at 7800 g for 30 min. The sedimented protein was dissolved in 0.1 N sodium hydroxide and total protein was determined by using a Lowry-based detergent compatible protein assay (Bio-Rad DC assay kit). The supernatant was lyophilized and the residue dissolved in water and passed through a cation-exchange column (AG 50W-X8). The eluate and two column washings were pooled, adjusted to pH between 7–8 with 1 mM KOH and lyophilized again prior to derivatization.

### Preparation of phenacyl esters

*p*-Bromophenacyl ester derivatives of NAA and NAAG were prepared using *p*-bromophenacylbromide and crown ether, a phase transfer catalyst, in acetonitrile. Briefly, 25 μL of methanol was added to the above lyophilized residue and vortex mixed vigorously. Then 100 μL of acetonitrile and 40 μL of *p*-bromophenacylbromide reagent were added, vortex mixed vigorously and kept in a water

bath at 80°C for 1 h with occasional stirring. The vials were then cooled, solvent removed by lyophilization and the residue reconstituted in the HPLC mobile phase solvent mixture and used for HPLC injections.

#### High performance liquid chromatography analysis

High performance liquid chromatography was performed isocratically using a Hewlett Packard HPLC system (Series 1100). Separation of phenacyl derivatives was carried out in a reverse phase column (Bio-Advantage 100 C<sub>18</sub>, 5 µm, 250 × 4.6 mm) with a mobile phase of acetonitrile: water (50/50 v/v) at 25°C. The flow rate used was 1.0 mL/min and detection was at 250 nm, and the signal processed by the integrator. The dried phenacyl esters were dissolved in the mobile phase solvent mixture and 100 µL was injected over the column. The peaks of phenacyl esters of NAA and NAAG from the cell extracts were identified via their retention times in relation to the corresponding standards of NAA and NAAG.

#### Mass spectroscopy

Mass spectroscopic analysis was carried out to confirm the presence of both NAA and NAAG in SH-SY5Y cells. Cells were grown in three 25 cm<sup>2</sup> culture flasks and after reaching confluence, the cells were harvested, pooled, extracted and the extract purified by cation exchange column chromatography as described above. The cation exchange eluate was lyophilized and sent to ITSI Biosciences (Johnstown, PA, USA) for analysis by positive ionization mass spectroscopy according to the methods of Ma *et al.* (Ma *et al.* 1999).

## Results

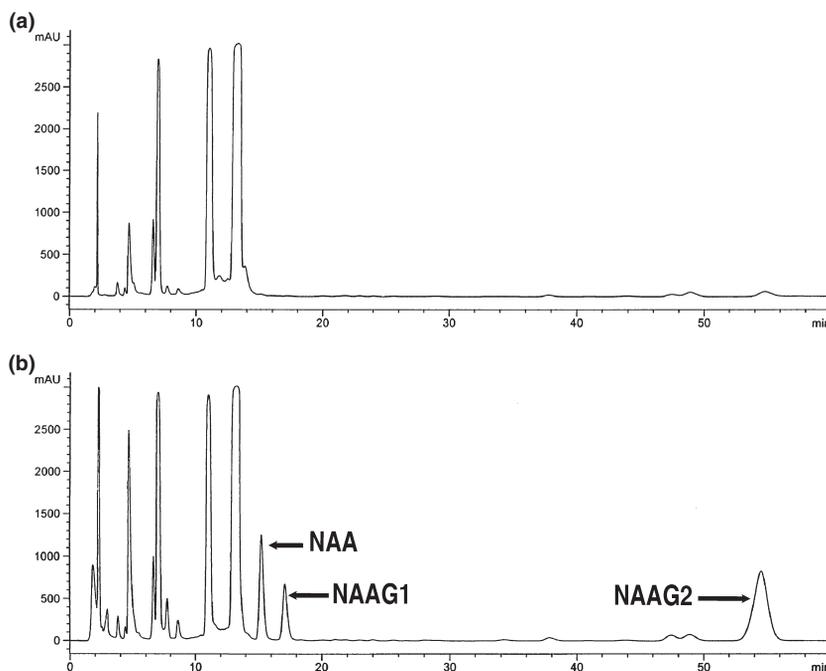
When SH-SY5Y human neuroblastoma cells obtained from ATCC where initially grown in culture, they produced very little NAA and no detectable NAAG. With subsequent

rounds of cell harvesting using EDTA and culturing under the same conditions, NAA levels increased and NAAG levels became detectable, and increased with further passages (data not shown). The reason for this phenomenon is unclear, but we have found that differentiating SH-SY5Y cells in culture with various differentiating agents significantly alters their biosynthetic production of NAA and NAAG (Arun *et al.* 2004, 2006). As such, it seems likely that some aspect of serial passage of the cells by sub-culturing, for example, the low Ca<sup>++</sup> conditions experienced during cell harvesting with EDTA, act to induce a more differentiated state in which NAA and NAAG syntheses are increased.

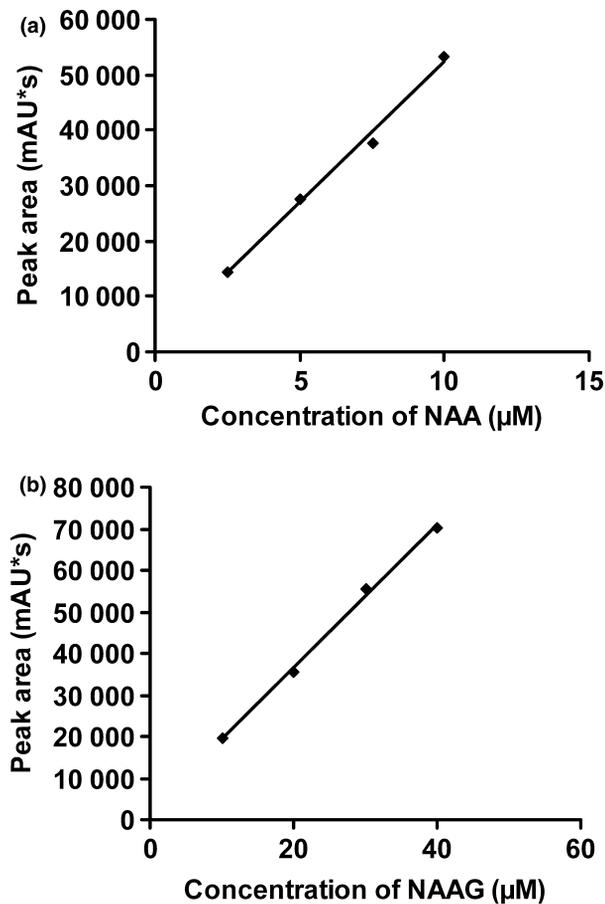
Figure 1 is a representative HPLC chromatogram showing the separation of NAA and NAAG under the HPLC conditions used. NAA shows a single peak at 15.2 min and NAAG shows two peaks, one at 17.0 min and the second at 54.2 min. Since the ratio of the peak area of both peaks from NAAG remains the same with varying concentrations, the peak at 54.2 min, which is well separated from NAA and other peaks, was used for measurements. Figure 2 shows the standard curves for NAA (a) and NAAG (b) plotted using concentrations of the pure standards against the peak area. The concentrations of NAA and NAAG in the cells were calculated using these standard curves.

#### Effect of methanol on peak area of NAA and NAAG

Preliminary studies showed that taking up the lyophilized sample in methanol increased the efficiency of derivatization and hence the corresponding peak areas. So it was of interest



**Fig. 1** Representative HPLC chromatograms showing (a) reagent blank and (b) mixture of NAA (5 µg) and NAAG (40 µg) standards. NAAG1 and NAAG2 are the two peaks obtained for NAAG and NAA shows only one peak. Peak heights are expressed in milli-absorption unit (mAU).

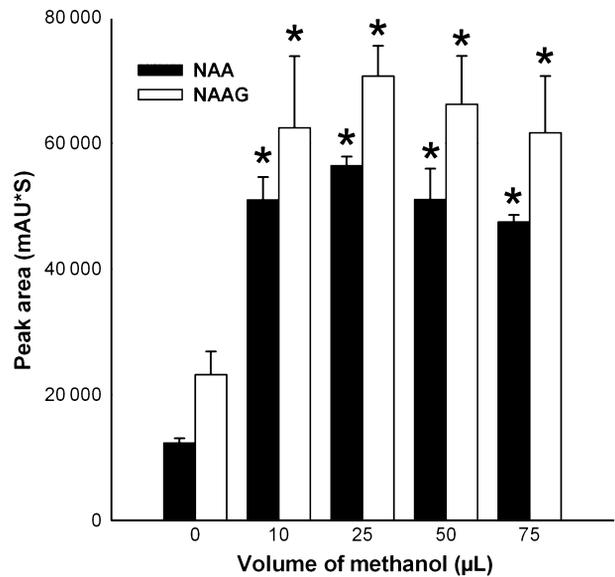


**Fig. 2** Standard curves for NAA (a) and NAAG (b) plotted using their concentrations against their respective peak area. Data points are the average of three replicates.

to test the concentration-dependence of this effect from methanol. Figure 3 shows the effect of methanol on the peak area of both NAA and NAAG ( $n = 3$  per data point). The results obtained clearly show that addition of between 10  $\mu\text{L}$  and 25  $\mu\text{L}$  methanol to the samples before derivatization with *p*-bromophenacylbromide increases the peak area and hence the sensitivity of measurement of both NAA and NAAG. However, increasing the methanol concentration further did not have any added effect.

#### Dose response of haloperidol and clozapine on NAA and NAAG in SH-SY5Y cells

Figure 4 shows the dose response of haloperidol and clozapine on NAA (a) and NAAG (b) synthesis in SH-SY5Y cells after 24 h of treatment ( $n = 5 \pm \text{SD}$ ). The results indicate that both haloperidol and clozapine increase NAA and NAAG levels in a dose-dependent manner. Neither compound increased NAA or NAAG levels at a dose of 20  $\mu\text{M}$ . At a dose of 40  $\mu\text{M}$ , haloperidol increased NAA levels by  $150 \pm 8\%$  and NAAG levels by  $176 \pm 5\%$ , whereas clozapine at this dose increased NAA levels by



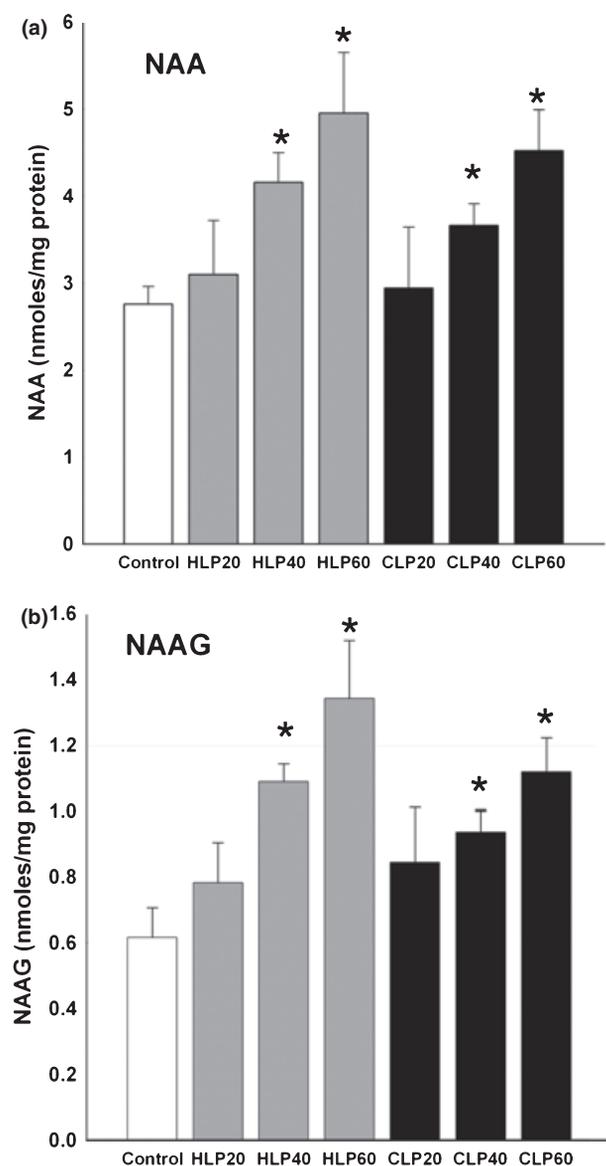
**Fig. 3** Effect of methanol solubilization on the peak area of NAA and NAAG. Lyophilized samples were dissolved in methanol before derivatization with *p*-bromophenacylbromide. Using Student's *t* test the peak area without methanol was compared to those after addition of varying volumes of methanol ( $n = 3$  per data point  $\pm \text{SD}$ ,  $*p < 0.001$ ).

$133 \pm 7\%$  and NAAG levels by  $150 \pm 8\%$ . At a dose 60  $\mu\text{M}$  haloperidol raised NAA levels by  $179 \pm 14\%$  and NAAG levels by  $216 \pm 13\%$ . Clozapine at 60  $\mu\text{M}$  increased NAA levels by  $164 \pm 10\%$ , and NAAG levels by  $181 \pm 9\%$ .

Figure 5 is a representative chromatogram showing NAA and NAAG peaks after treatment with and without 50  $\mu\text{M}$  each of haloperidol or clozapine for 48 h. The peak heights and peak areas for NAA in the treated groups were significantly higher than the control. In the case of NAAG, even though the peak at 54.2 min. showed only a slight change in height, the broader peak gave an area for the treated groups that was two to three times greater than the control. The responses of SH-SY5Y cells to 50  $\mu\text{M}$  of either haloperidol or clozapine after 48 h of treatment are given below. The changes in peak area values for NAA and NAAG are an average ( $\pm \text{SD}$ ) of three separate experiments. NAA levels at 48 h after treatment with 50  $\mu\text{M}$  haloperidol increased approximately  $324 \pm 4\%$  relative to control, and in clozapine-treated cultures NAA was increased approximately  $345 \pm 23\%$ . NAAG levels at 48 h after treatments increased by approximately  $217 \pm 25\%$  (50  $\mu\text{M}$  haloperidol) and  $240 \pm 19\%$  (50  $\mu\text{M}$  clozapine) respectively.

#### Time course of haloperidol and clozapine treatment on NAA and NAAG levels in SH-SY5Y cells

Figure 6 shows the time course of haloperidol and clozapine treatments on NAA and NAAG levels in SH-SY5Y cells. The results show that the drug treatments increase both NAA



**Fig. 4** Dose (0, 20, 40 and 60  $\mu$ M) response of haloperidol (HLP) and clozapine (CLP) on the levels of NAA (a) and NAAG (b) in SH-SY5Y cells after 24 h of treatment. Statistical analysis was carried out using Student's *t* test. Mean values of the treated groups were compared to that of control. Values are the means of five independent experiments ( $n = 5$ ), and error bars represent standard deviation (\* $p < 0.01$ ).

and NAAG content in a time dependant manner, and that NAAG levels were increased substantially more at 24 h than NAA levels. Neither drug affected NAA or NAAG levels with 6 h of treatment, but both drugs increased NAA and NAAG concentrations with 24 and 48 h of treatment ( $n = 3 \pm$  SD). After 24 h of treatment, NAA concentrations were increased  $30 \pm 10\%$  with clozapine treatment, and  $37 \pm 3\%$  with haloperidol treatment. In contrast, similar treatments increased NAAG levels by  $425 \pm 11\%$  and  $350 \pm 37\%$ , respectively at 24 h. After 48 h, NAA levels

were increased  $388 \pm 9\%$  by haloperidol treatment and  $507 \pm 4\%$  by clozapine treatment, whereas NAAG levels were increased  $408 \pm 23\%$  and  $358 \pm 17\%$ , respectively.

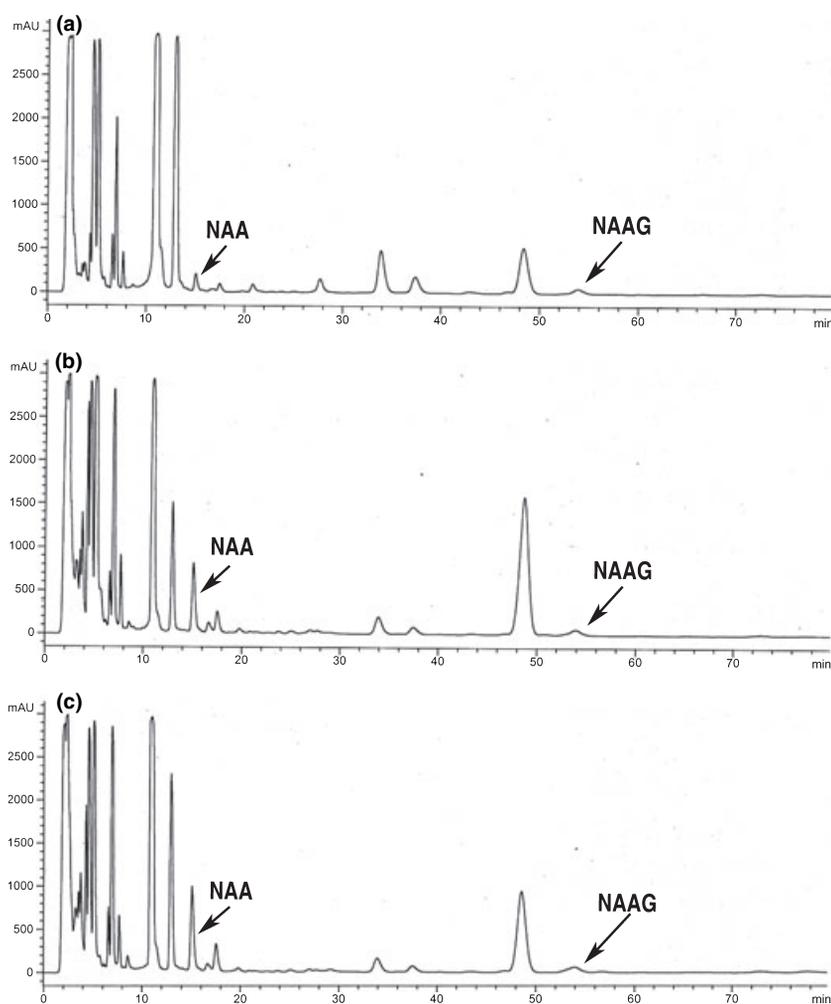
#### Mass spectroscopy

*N*-acetylaspartate and NAAG chemical standards showed mass peaks of 176 and 305  $m/z$  (mass/charge ratio) respectively (Fig. 7a and b). The lyophilized cation exchange eluates from cell homogenates showed peaks on mass spectrograms at 176 and 305  $m/z$  units corresponding to NAA (arrow in Fig. 7c) and NAAG (arrow in Fig. 7d).

#### Discussion

The addition of methanol to cell extract samples before derivatization significantly increased the sensitivity of the assay over the previously described method (Mathew *et al.* 2005), probably by increasing the solubility of NAA and NAAG. This additional sensitivity facilitated the simultaneous measurement of these two related neurometabolites. Mass spectroscopy was used to confirm that the HPLC peaks were associated with NAA and NAAG. Positive ionization mass spectroscopy showed a mass peak at 176  $m/z$  in the cell extracts corresponding to NAA, and a mass peak at 305  $m/z$  in the cell extracts corresponding to NAAG.

The results presented here establish that the antipsychotic drugs haloperidol and clozapine increase the concentrations of NAA and NAAG in SH-SY5Y human neuroblastoma cells *in vitro* in a time and dose dependent manner. The increases were several fold for both compounds, but the large elevation of NAA was delayed relative to that of NAAG (Fig. 6). It is unlikely that the responses of SH-SY5Y cells to antipsychotic drug treatment *in vitro* can be extrapolated directly to *in vivo* neurobiology. Despite the fact that differentiated SH-SY5Y cells in culture have many characteristics of dopaminergic neurons, they are not phenotypically identical to normal dopaminergic neurons *in vivo*. Further, the responses of SH-SY5Y cells to drug treatment are non-physiological due to the fact that only a single neuronal cell type is responding in isolation, without the active participation of glial cells. Because astrocytes are the primary cellular locus of the major NAAG hydrolyzing enzyme, glutamate carboxypeptidase II (GCP II) (Berger *et al.* 1999; Sacha *et al.* 2007), it is probable that NAAG catabolism is minimal in SH-SY5Y cultures. Nonetheless, the results presented here show that antipsychotic drugs can affect NAA and NAAG metabolism in this model system, and therefore it is possible that some of the mechanisms involved have counterparts in human neurochemistry. Based on the current and previous results, it is apparent that NAA and NAAG metabolism is regulated to some extent in differentiated SH-SY5Y cells, with both upregulation and downregulation being reported under different culturing conditions (Arun *et al.* 2004, 2006). Specifically, chronic

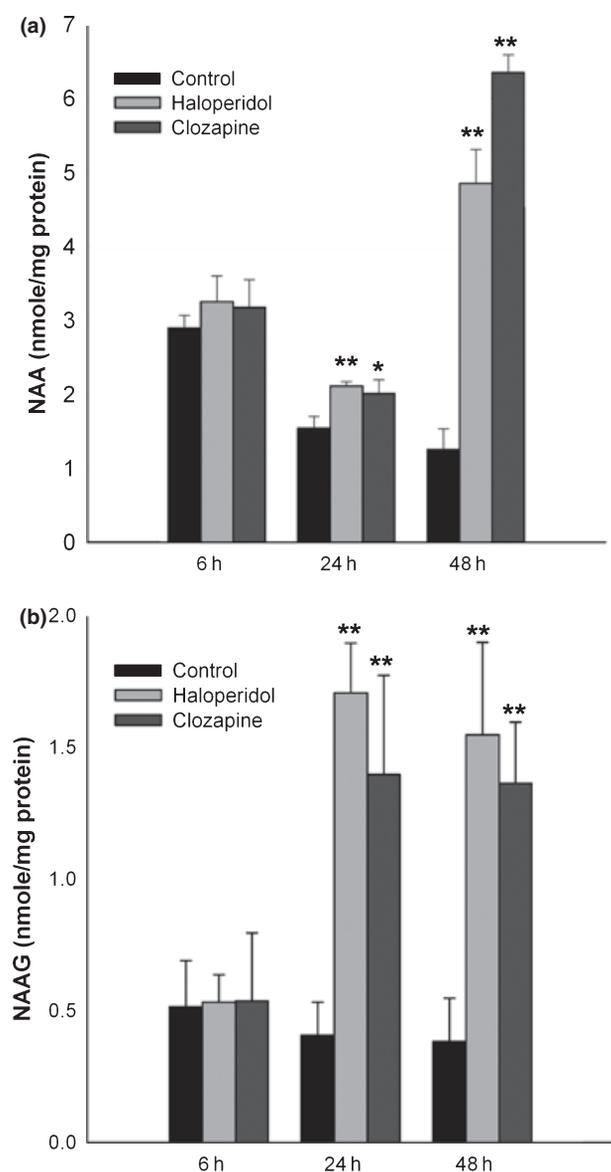


**Fig. 5** Representative HPLC chromatogram showing the effect of haloperidol and clozapine treatment on the peak area/height of NAA and NAAG in SH-SY5Y cells. (a) control (vehicle, DMSO treated) cells, (b) cells treated with 50  $\mu$ M haloperidol and (c) cells treated with 50  $\mu$ M clozapine. Peak heights expressed in milli-absorption units (mAU).

treatment with dibutyryl cyclic AMP increased NAA and NAAG biosynthesis, whereas chronic treatment with phorbol-12-myristate-13-acetate decreased the synthesis of NAA and NAAG in SH-SY5Y cells.

The majority of published studies using MRS to identify metabolite changes in schizophrenia patients report small, regional reductions in NAA or the NAA-creatine ratio (Bertolino *et al.* 1996; Callicott *et al.* 2000a; Deicken *et al.* 2000; Abbott and Bustillo 2006). Researchers typically study metabolite differences between schizophrenia patients and control subjects in relatively few brain locations, rather than using whole-brain measures, and the locations examined often differ from one study to the next. As such, neither the total extent of the NAA loss, or its regional distribution, have been mapped out in any detail (Deicken *et al.* 2000). A recent meta-analysis of MRS studies on NAA levels in schizophrenia found consistent gray matter reductions of approximately 5% to 10% in the frontal lobes (Steen *et al.* 2005). A number of studies have found that NAA levels and NAA-creatine ratios in the medial temporal lobe and the prefrontal cortex are reduced in schizophrenia patients

(Abbott and Bustillo 2006), and that these reductions parallel alterations in cerebral blood flow as measured with positron emission tomography and functional magnetic resonance imaging (Callicott *et al.* 2000b; Marenco *et al.* 2006). In a recent MRS study comparing NAA concentrations in 14 schizophrenics with 13 control subjects it was found that NAA was decreased significantly in the frontal lobe of affected patients (average, 7.94 mM, compared with healthy subjects average of 8.45 mM,  $p < 0.05$ ) (Tanaka *et al.* 2006). The reduced NAA levels correlated with the severity of negative symptoms and poor performance in the Wisconsin Card Sorting Test. Reductions in NAA levels in the dorsolateral prefrontal cortex of schizophrenic patients have been correlated with poorer performance in the Auditory Verbal Learning Task, indicating connections between cognitive performance and NAA levels (Ohrmann *et al.* 2006). Considering that antipsychotic medications act in part by blocking dopamine  $D_2$  receptors, it is of interest that NAA reductions in prefrontal cortex have been associated with a dysregulation of dopamine release in the striatum of schizophrenia patients (Bertolino *et al.* 2000).



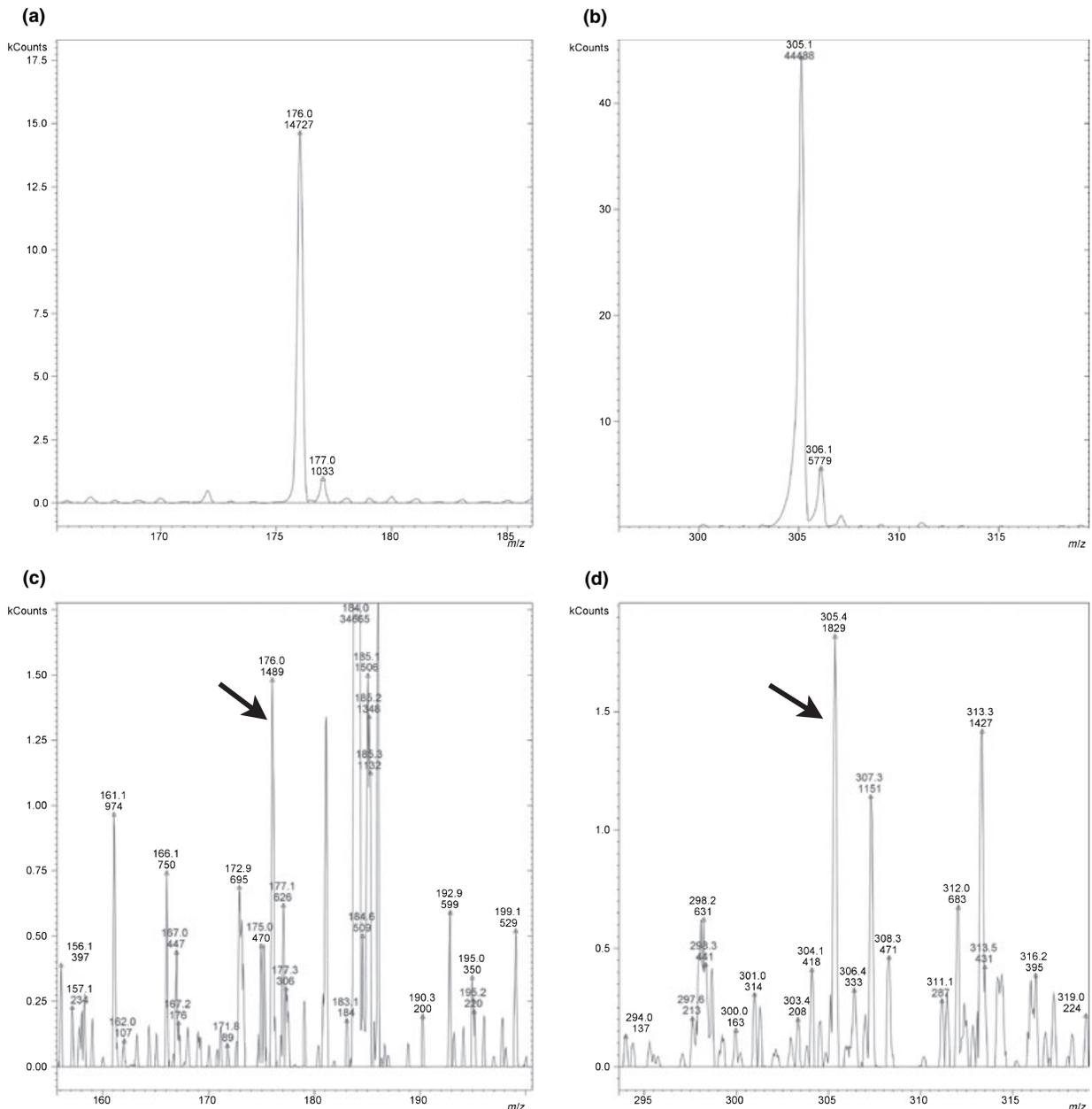
**Fig. 6** Time course of haloperidol and clozapine on NAA (a) and NAAG (b) levels in SH-SY5Y cells. 50  $\mu$ M each of haloperidol or clozapine were added to the cells and incubated for 6, 24 and 48 h. The same volume of DMSO served as the control group. Values of the treated groups at each time point were compared to those of control. Statistical analysis was carried out using Student's *t* test. ( $n = 3$ , \* $p < 0.01$ , \*\* $p < 0.001$ ).

With regard to the effects of antipsychotic medications on brain NAA levels, Okumura and colleagues (Okumura *et al.* 1959) reported that 30–60 days of intraperitoneal administration of chlorpromazine in rats had no effect on NAA concentrations measured using chromatographic determination in whole brain extracts. In a more recent study, seven days of intraperitoneal administration of haloperidol or clozapine did not result in detectable changes in subcortical NAA levels in rats, while the same treatment regimen with

Olanzapine caused small reductions of NAA in the brain as measured by MRS (Lindquist *et al.* 2000). Using a within-subject design study conducted in 23 schizophrenic patients, Bertolino and colleagues (Bertolino *et al.* 2001) concluded that antipsychotic drugs increase NAA levels selectively in the dorsolateral prefrontal cortex, suggesting that these drugs modify the function of populations of cortical neurons in a regionally specific manner. Heimberg *et al.* also reported higher NAA levels in the frontal lobes of patients treated with atypical antipsychotic drugs as compared to those treated with typical antipsychotics (Heimberg *et al.* 1998). On the other hand, a longitudinal follow up study conducted in 10 schizophrenic patients (Bustillo *et al.* 2002) showed that after 1 year of antipsychotic drug treatment NAA levels were decreased in the frontal lobes, and the authors concluded that the well-described reduced frontal NAA in schizophrenia may not be a trait of the disease but may represent medication effects, or progression of the disease.

High performance liquid chromatography analysis of homogenates of different rat brain regions including cortex, striatum, thalamus, hippocampus and cerebellum after six weeks of antipsychotic drug treatment found no detectable changes in NAA levels (Bustillo *et al.* 2004). In another study, chronic administration of haloperidol in rats resulted in a significant increase in NAA concentrations in the striatum, but failed to show any significant changes in the other regions such as frontal cortex, temporal cortex, thalamus, hippocampus, amygdala or the nucleus accumbens (Harte *et al.* 2005). But a recent MRS study conducted by Bustillo and coworkers (Bustillo *et al.* 2006) involving chronic administration of haloperidol for six months reported that antipsychotic drugs did not result in changes in NAA levels in various brain regions (medial frontal and cingulate cortex, striatum, nucleus accumbens, dorsal and ventral hippocampus, amygdala and temporal cortex). In a subsequent study, the same investigators found that global NAA levels as measured by MRS were positively correlated with cognitive performance, but that the levels did not respond to antipsychotic treatment (Bustillo *et al.* 2007).

If the present results with antipsychotic drug treatment are indicative of the response of select (dopamine  $D_2$  receptor-bearing) neuronal populations *in vivo* then it is possible that earlier conflicting results concerning the effects of antipsychotic drugs on NAA levels in the brain could be due to opposing factors. For example, if schizophrenia is associated with reduced NAA levels in certain brain regions, and antipsychotic drugs increase NAA levels *in vivo*, then the two factors may tend to counteract one another, leading to conflicting results in different studies. Further, if only select neuronal populations respond to antipsychotic treatment by increasing levels of NAA and NAAG, then additional interpretive complications arise due to the fact that various MRS studies often examine different brain regions for changing metabolite levels.



**Fig. 7** Standard mass spectrograms for NAA (a) and NAAG (b) showing molecular masses of 176 *m/z* and 305 *m/z*, respectively. Mass spectrograms of SH-SY5Y cell extract samples after removal of

protein and passage through a cation exchange column, showing corresponding mass peaks of NAA (arrow in c) and NAAG (arrow in d).

It is also possible that the inconsistent findings in the above studies may in part reflect methodological differences. Most of the HPLC analyses carried out to date employed UV absorption detection at 200–230 nm, a relatively insensitive technique prone to interference from other endogenous organic compounds. In the present study, we first used cation-exchange chromatography to eliminate positively charged compounds from solubilized cell homogenates. Following derivatization, the resultant phenacyl esters absorb

at 250 nm and hence the interference from other UV absorbing compounds is minimized. Furthermore, the phenacyl ester of NAA is well separated from the potentially interfering ester of NAAG and other related compounds. Based on these facts, we conclude that the present method of NAA and NAAG analysis is more reliable, sensitive and reproducible than using standard HPLC with UV detection at 200 to 230 nm. Regarding proton MRS techniques, whereas the peak at 2.01 ppm in an MRS spectrum of human brain is

predominantly attributed to NAA, this signal includes smaller contributions from other acetylated compounds such as NAAG and N-acetyl neuraminic acid, as well as underlying coupled resonances from glutamate and glutamine (Moffett *et al.* 2007). These facts, as well as other factors related to MRS techniques including voxel size, overlying tissue interference and volume effects, make it difficult to precisely measure NAA levels in the brain by this method.

In light of the fact that NAA is known to be involved in the myelination of axons by oligodendrocytes (Madhavarao *et al.* 2005; Ledeen *et al.* 2006; Moffett *et al.* 2007) it is noteworthy that reductions in white matter volume and oligodendrocyte markers have been reported in schizophrenia (Hakak *et al.* 2001; Flynn *et al.* 2003; Tkachev *et al.* 2003). For example, expression of the gene for aspartoacylase, the enzyme that catabolizes NAA, is reported to be significantly reduced in the prefrontal cortex in schizophrenia patients (Tkachev *et al.* 2007), and corpus callosum size is reduced (Goghari *et al.* 2005). In this regard it may be relevant that chronic antipsychotic treatment has been reported to reduce astrocyte and oligodendrocyte numbers in macaque monkeys, suggesting that the white matter loss observed in medicated schizophrenic patients could have a drug-related component (Konopaske *et al.* 2008). The connections between drug effects, reduced NAA levels, apparent white matter and oligodendrocyte loss, and the etiology of schizophrenia remain to be determined.

The observed increase in NAAG levels in dopaminergic-like human neuroblastoma cells in response to haloperidol and clozapine treatment in the present study may have important implications. We have previously reported that increasing NAA levels exogenously leads to increased NAAG biosynthesis in SH-SY5Y cells (Arun *et al.* 2006), so it is possible that the increase in NAAG levels with antipsychotic drug treatment in the present study could be due to the increased biosynthesis of NAA. However, the large increase in NAAG levels preceded the large increase in NAA levels after antipsychotic drug treatment (Fig. 6). This does not support the idea that increased NAAG synthesis results from elevated NAA concentrations alone. Rather, the temporal sequence whereby NAAG levels rise more rapidly than those of NAA suggests that dopamine D<sub>2</sub> blockade may affect changes in NAAG and NAA expression via distinct mechanisms.

Following its synaptic release, NAAG is reported to activate group II metabotropic glutamate receptors (mGluR2/3) on pre-synaptic sites (Wroblewska *et al.* 1997) leading to the inhibition of neurotransmitter release, including that of glutamate (Xi *et al.* 2002; Zhong *et al.* 2006). As such, NAAG action is thought to reduce glutamate excitotoxicity (Slusher *et al.* 1999). In contrast, activity of the NAAG hydrolyzing enzyme, GCP II, relieves the mGluR2/3 inhibition on synaptic glutamate release, while simultaneously

increasing the level of extracellular glutamate as NAAG is broken down into NAA and glutamate in the extracellular space. Thus, depending on the activity levels of GCP II, NAAG can either reduce or enhance glutamatergic neurotransmission. Low hydrolytic enzyme activity reduces neuronal excitability by allowing NAAG to persist in the synaptic cleft, whereas high levels of GCP II activity may enhance excitability. Additional evidence has been presented suggesting that NAAG also acts as an endogenous antagonist at the NMDA glutamate receptor (Bergeron *et al.* 2005, 2007), which would support the general conclusion that NAAG reduces neuronal excitability. However, contradictory data have been published concerning antagonist actions of NAAG at NMDA receptors, with high concentrations of NAAG (100–200  $\mu$ M) failing to reduce the peak current elicited by 10  $\mu$ M NMDA in cerebellar granule cells in culture (Losi *et al.* 2004).

It has been suggested previously that alterations in NAAG metabolism may be associated with the pathophysiology of schizophrenia (Tsai *et al.* 1995). Combined with the fact that NAAG appears to be involved in regulating the release of multiple classical neurotransmitters (Galli *et al.* 1991; Zhao *et al.* 2001; Mateo and Porter 2007), probably by agonist activity at mGluR2/3 receptors, it is plausible that schizophrenia involves improper regulation of the release of multiple neurotransmitters, including glutamate and dopamine. While there is a growing consensus that altered NAAG physiology has some role in the pathophysiology of schizophrenia, there are conflicting data regarding how perturbations in NAAG activity might be related to the development of the disease. Evidence has been presented suggesting that decreased glutamate neurotransmission in corticolimbic areas of the brain is involved (Tsai and Coyle 2002; Javitt 2004; Javitt and Coyle 2004). For example, NAAG levels were found to be increased in the prefrontal cortex and hippocampus of schizophrenia patients, whereas glutamate levels and the activity of the NAAG hydrolyzing enzyme GCP II were reduced (Tsai *et al.* 1995). Under this hypothesis, NAAG acts to antagonize NMDA receptor activity (Bergeron *et al.* 2005, 2007), and excess NAAG in specific brain regions leads to hypo-activity of glutamate neurotransmission.

However, the glutamate hypo-activity hypothesis of schizophrenia can not account for a significant body of data which links mGluR2/3 receptor activation to reductions in psychotomimetic actions of NMDA channel blockers such as phencyclidine (PCP). PCP and other so-called open channel NMDA antagonists have been reported to increase glutamate efflux (Moghaddam and Adams 1998), and reproduce many of the negative symptoms of schizophrenia. Olszewski and colleagues have reported that an inhibitor (ZJ43) of the hydrolytic enzyme of NAAG, GCP II, significantly reduced the overall motor behavior and some of the stereotypic behaviors elicited by PCP in rats (Olszewski *et al.* 2004, 2007). These effects of ZJ43 were blocked by the mGluR2/3

antagonist, LY341495, which supports the conclusion that GPC II inhibition led to increased group II metabotropic glutamate receptor activation by NAAG (Olszewski *et al.* 2004). Under this hypothesis, NAAG acts to reduce glutamate efflux. As such, studies into the effects of psychotomimetic drugs and mGluR2/3 agonists implicate hyperactivity of glutamatergic forebrain systems in the etiology of schizophrenia, and suggest that mGluR2/3-mediated regulation of glutamate release has therapeutic effects in reducing the cognitive defects in schizophrenia patients. If correct, then increasing NAAG synthesis and release, and reducing NAAG hydrolysis, would be effective in reducing the symptoms of schizophrenia.

Recently, a randomized phase 2 clinical trial has been conducted with the mGluR2/3 agonist LY404039, and efficacy compared with placebo and olanzapine (Patil *et al.* 2007). Subjects were given an oral prodrug LY2140023, and evaluated for improvements in both positive and negative symptoms of schizophrenia. The mGluR2/3 agonist was nearly as effective as olanzapine in reducing both positive and negative symptoms, supporting the idea that decreased NAAG synthesis and release may be one etiological mechanism in schizophrenia.

Evidence suggests that both excessive glutamatergic activity and reduced GABAergic inhibition in brain regions such as cingulate and prefrontal cortices may be responsible in part for the development of schizophrenia (reviewed in Benes 2000). A number of alterations in GABA-related transcripts (Hashimoto *et al.* 2008) and protein expression (Akbarian *et al.* 1995; Lewis 2000) have been reported in the prefrontal cortex in schizophrenia patients. It is thought that the alterations in GABAergic neurotransmission lead to dysregulation of glutamatergic activity, which in turn is responsible in part for the pathophysiology of schizophrenia. NAAG is expressed extensively in interneurons of neocortex in rat (Moffett and Nambodiri 1995), and monkey (Moffett and Nambodiri 2006), and is colocalized with the GABA synthesizing enzyme glutamic acid decarboxylase (Moffett 2003). Because NAAG has been shown to reduce glutamate release (Xi *et al.* 2002; Zhong *et al.* 2006), it is plausible that loss of, or reduced inhibitory activity in NAAG and GABA-expressing interneurons in select cortical areas may play some role in the development of schizophrenia. These observations together with our present findings that antipsychotic drugs increase NAAG levels in an *in vitro* model of dopaminergic neurons support the idea that increased NAAG biosynthesis resulting from dopamine D<sub>2</sub> receptor blockade may play some role in the therapeutic actions of these medications.

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