High dose glycine nutrition affects glial cell morphology in rat hippocampus and cerebellum

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Abstract
Enhancement of N-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission by glycine (Gly) administration may represent a novel pharmacological strategy in schizophrenia. Given the involvement of NMDA receptors in plasticity and excitatory processes, the present study explores effects of Gly on brain cell morphology. Adult rats were randomized to receive, for 2 wk, no dietary supplementation or supplementation with 0–8 or 3–2 g/kg per day Gly. Glial cell morphology was examined using antibodies to glial fibrillary acidic protein (GFAP) and to microglial complement receptor 3 (CR3). Cresyl violet was used for general cellular staining. No evidence of neuronal or microglial pathology was found. Although astrocyte proliferation was not evident in Gly-treated rats, GFAP-like immunoreactivity was dose-dependently increased in the hippocampus (p < 0.01), whereas in cerebellum, a dose dependent decrease in density of astrocytic fibres was demonstrated (p < 0.01). These findings demonstrate for the first time that in vivo administration of high dose Gly may induce brain morphology changes.

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Introduction
Phencyclidine and other dissociative anaesthetics (e.g. ketamine) induce psychotomimetic effects that closely resemble schizophrenia. These agents induce their effects by blocking N-methyl-D-aspartate (NMDA)-type glutamate receptors, indicating that agents which augment NMDA receptor activation may be clinically beneficial in schizophrenia (reviewed in Javitt and Zukin, 1991; Malhorta et al., 1997; Tamminga et al., 1995). Gly, a non-essential amino acid, stimulates NMDA receptors via a strychnine-insensitive Gly-binding site, leading to its proposed use in schizophrenia (Javitt and Zukin, 1991). Despite its poor penetration through the blood–brain barrier, at doses of 0.8 g/kg per day, Gly is effective in reversing PCP-induced behaviours in rodents (Toth and Lajtha, 1986). Recently, controlled studies have shown that addition of high Gly doses (i.e. 0.4 and 0.8 g/kg per day) to the antipsychotic regimens received by treatment-resistant schizophrenic patients can induce, dose-dependently, a significant reduction in negative symptoms (Heresco-Levy et al., 1996). These findings suggest that Gly treatment may represent a novel pharmacotherapeutic approach for schizophrenia.

A cardinal question, given the pivotal role of NMDA receptors in both neuronal plasticity and neurotoxicity processes (for refs. see Javitt and Zukin, 1991) is whether and in what way brain cells may be affected by Gly treatment. Gly constitutes 1–5% of normal nutrition protein and has low permeability across the blood–brain barrier (Oldendorph, 1971). At present it is not known whether in vivo Gly administration can elicit morphological changes in the mammalian brain. In vitro conditions and high Gly concentrations (10 mM) were recently reported to induce neurotoxic neuronal damage in rat hippocampal slice cultures (Newell et al., 1997).

The present study is, to our knowledge, the first to examine effects of in vivo Gly treatment on rat brain cell morphology. The examined Gly regimens were equal to (0.8 g/kg per day) and four times higher (3.2 g/kg per day) than the highest Gly dose employed to date in clinical trials. Markers of glial cell changes were employed since the morphology of these cells has been demonstrated to be particularly sensitive to nutritional and pharmacological interventions (Nakajima and Koisaka, 1993; Shao and McCarthy, 1994).
Method

Three groups of young adult (2–2.5 months old) male albino Sprague–Dawley rats, weighing 230–240 g, were housed in plastic cages and had ad lib. access to food and water. All procedures were conducted in accordance with local and international laws for the care and use of laboratory animals. Each group (n = 4) was assigned to one of three nutritional regimens: no Gly supplementation; 0.8 g/kg per day, and 3.2 g/kg per day Gly supplementation. Gly was mixed into finely ground rodent food pellets based on an estimated daily total food intake of 20 g. After 2 wk, rats were deeply anaesthetized by intraperitoneal injection with pentobarbital sodium. Blood samples were drawn for assessment of Gly, serine (racemate), glutamic acid and glutamine serum levels using HPLC with UV detection (Harharam et al., 1993). Brains were fixed by transcardial perfusion of ice-cold solutions: 50 ml of 0.02 m phosphate-buffered saline (PBS), pH 7.4, followed by 200 ml of 4% paraformaldehyde in 0.1 m PBS, pH 7.4, and containing 4% sucrose. After perfusion, brain blocks were immersed in the same fixative for another hour at 4 °C and then immersed in 10% sucrose in 0.1 m PBS, pH 7.4 at 4 °C until cryostat sectioning. Sections for general cellular staining with cresyl violet were 15 µm thick and were collected and thaw-mounting onto gelatinized slides. Sections for immunohistochemical staining were 30 µm thick and were collected in 0.1 m PBS, transferred to a cryopreservation buffer, and kept at −20 °C until immunohistochemical processing. The cryopreservation buffer contained 40% ethylene glycol and 1% polyvinylpyrrolidone in 0.1 m potassium acetate, pH 6.5. Astrocytes were identified using a monoclonal antibody directed against glial fibrillary acidic protein (GFAP) clone GA5, Sigma, Israel) at a dilution of 1:500 and a secondary goat-anti-mouse antibody conjugated with horseradish peroxidase (Sigma, Israel) diluted 1:100. Microglia were identified using a monoclonal antibody (clone OX42 Cedarlane, Canada) directed against rat complement receptor 3 (CR3) at a dilution of 1:500 and a secondary goat-anti-mouse antibody conjugated with biotin (Sigma, Israel) diluted 1:200, followed by incubation with extravidin-peroxidase (Sigma, Israel) diluted 1:100. Colour reaction was produced by incubation of sections in a solution containing diaminobenzidine (Sigma, USA) at a concentration of 0.0125%, nickel ammonium sulphate at a concentration of 0.05% and hydrogen peroxide at a concentration of 0.0015% for 2 min. To perform densitometric measurements, sections from all treatment groups were processed in parallel.

Analysis consisted of two stages. First, qualitative morphological changes were assessed in frontal cortex, striatum hippocampus and cerebellum, brain regions that have been studied in relation to schizophrenia. From each brain, five parallel tissue sections per region were examined by a careful histological standard examination with the aid of cresyl violet staining and GFAP and CR3 immunohistochemical detection. Second, in regions that appeared to show across-treatment group differences, quantitative analyses were performed on three parallel tissue sections per region from each rat brain. Two types of measurements were performed: optical density of GFAP-like immunoreactivity (GFAP-LI) and counting of astrocytes having GFAP-LI stained soma (cell bodies). The optical density of GFAP-LI was measured over a whole region (dentate gyrus or dorsal lobe of cerebellum), expressed as the absolute value of log 10 of the ratio between transmitted light and incident light through a stained brain section. The number of astrocytes having GFAP-LI stained soma and their percentage of the total number of astrocytes was examined in a series of 10 consecutive 150 × 100 µm fields per section. A total of 300–400 astrocytes per section were sampled from each rat brain.

Comparisons between groups were performed using the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney U test.

Results

Following 2 wk of experimental nutrition, rats of all treatment groups gained weight at similar rates. The Gly serum levels achieved in the 0.8 and 3.2 g/kg per day Gly groups were approx. 2- and 4-fold higher, respectively, than the levels in the group which had not received Gly supplementation (p < 0.01). The levels of other assessed amino acids were not significantly different across groups (Table 1).

In all treatment groups, cresyl violet staining did not reveal any adverse changes in any of the brain regions that were examined. No indication of excitotoxicity such as neuronal swelling or shrinkage or neuronal death, such as pycnotic nuclei or alteration of normal cellular architecture was registered. Similarly, no effects or across-groups differences in microglial morphology were registered following microglia identification by CR3 staining. However, qualitative alterations in GFAP-LI were observed in two brain regions, hilus of hippocampal dentate gyrus and dorsal lobe of cerebellum, which were, therefore, subjected to further quantitative analyses.

In the hilus of the hippocampal dentate gyrus of Gly-treated rats there was a significant (p < 0.01), dose-dependent 3-fold increase in the percentage of astrocytes having GFAP-LI stained cell bodies (Table 2 and Figure
**Table 1.** Body weight and serum amino acids levels after 2 wk of glycine (Gly) nutritional supplementation (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Glycine dose (g/kg per day)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>312.0 ± 9.2</td>
</tr>
<tr>
<td>Serum levels (nmol/ml)</td>
<td>108.2 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>71.8 ± 11.6</td>
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<td></td>
<td>257.8 ± 33.5</td>
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*p = 0.01; p < 0.01.

**Table 2.** Glial fibrillary acidic protein-like immunoreactivity (GFAP-LI) parameters in rat hilus of hippocampal dentate gyrus and dorsal lobe of cerebellum after 2 wk of glycine (Gly) nutritional supplementation (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Parameter</th>
<th>Glycine dose (g/kg per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Hilus of hippocampal dentate</td>
<td>% astrocytes with GFAP-LI stained cell</td>
<td>21.0 ± 2.9</td>
</tr>
<tr>
<td>gyrus</td>
<td>body</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optical density</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Number of astrocytes per section</td>
<td>171.2 ± 6.2</td>
</tr>
<tr>
<td>Cerebellum, dorsal lobe</td>
<td>% astrocytes with GFAP-LI stained cell</td>
<td>47.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>body</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optical density</td>
<td>0.24 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>Number of astrocytes per section</td>
<td>133.0 ± 3.0</td>
</tr>
</tbody>
</table>

*†H = 9.3; p < 0.01.  
†The 3.2 g/kg per day dose had a greater effect than the 0.8 g/kg per day dose (Mann–Whitney U = 1.0; p < 0.04).

IA vs. B). This increase occurred although the total number of astrocytes and overall optical density were unaffected. In contrast, in the dorsal lobe of cerebellum, the percentage of astrocytes showing GFAP-LI did not differ across treatment groups (Table 2), although the overall GFAP-LI staining optical density decreased significantly (*p < 0.01) in the Gly-treated rats. This decrease appeared to be due to a diminished size of the astrocytic processes which normally fill the spaces between astrocyte cell bodies (Figure 1C vs. D). As in the other brain regions examined, the total number of astrocytes was unaffected.

**Discussion**

Due to the small sample examined, the results of the present study should be considered preliminary. Nevertheless, they represent, to our knowledge, the first demonstration of induction of brain cell morphological changes by in vivo Gly administration. The main findings of the present study are that following 2 wk of Gly nutritional supplementation in rat, with doses equal to and higher than those presently used in schizophrenia clinical trials: (1) GFAP-LI was dose-dependently increased in the hippocampal dentate gyrus and decreased in the cerebellar dorsal lobe and (2) no pathological changes were observed in any of the brain regions examined. These observations were paralleled by significant dose-dependent increases in Gly serum levels.

The increase in GFAP-LI in the hippocampal dentate gyrus was in the percentage of astrocytes with GFAP-LI staining of the soma. In the ‘resting’ state, astrocytic processes are GFAP-LI whereas the soma appears pale or altogether absent. From this state, astrocytes may be ‘aroused’ to varying degrees of activation. In paradigms involving neurotoxicity, astrocytes proliferate, the dendritic tree is enlarged and GFAP-LI appears in the soma.
Figure 1. Glial fibrillary acidic protein-like immunoreactivity (GFAP-LI) in control rats (A and C) and in rats given 0.8 g/kg per day glycine (Gly) as nutritional supplementation for 2 wk (B and D). Some astrocytes in the hippocampal dentate gyrus (arrows point to examples in A), had GFAP-LI staining in the cell body. In Gly-treated rats (B), there was an increase in the percentage of astrocytes having GFAP-LI stained cell bodies. In addition, in Gly-treated (D), in the dorsal lobe of the cerebellum, the density of GFAP-LI staining decreased (compared to controls, demonstrated in C), associated with shrinkage of astrocytic processes. Bar, 50 µm.

(McDonald and Johnston, 1990). These changes give the astrocyte a ‘hypertrophied’ appearance. In paradigms involving synaptic reorganization without neurotoxicity, the dendritic tree is enlarged and astrocytic soma also becomes GFAP-LI but there is no proliferation of astrocytes (Sirevaag et al., 1991; Jones et al., 1996). It is not clear what the functional significance of GFAP-LI in astrocytic soma staining may be. The GFAP mRNA is distributed in both soma and astrocytic processes (Landry et al., 1994) and the relationship between astrocytic functional state and the mobilization of GFAP mRNA and protein is still under investigation. In the present study, in Gly-treated rats, GFAP-LI in the soma increased in a significant percentage of astrocytes but there was no detectable change in the dendritic tree. This probably reflects a moderate level of astrocytic activation, not typical of pathological reactions. It should be noted that astrocytes contain metabolic pathways utilizing Gly (Sato et al., 1991) and have been reported to take up Gly in vitro (Fedele and Foster, 1992) so that increases in Gly availability may be handled by astrocytes within the normal range of their physiology.

Still, the question of whether the glial activation observed in the present study may intensify with longer periods of Gly supplementation remains open at this stage, and is currently under investigation. Such intensification may occur if neurotoxicity develops. Typical excitotoxicity features such as neuronal shrinkage as revealed using cresyl violet staining (McDonald and Johnson, 1990) or induction of a macrophage-like functional state of microglia as visualized by CR3 staining (Nakajima and Kohsaka, 1993) were not observed in the present study. Additional sensitive methods such as silver staining are currently being tested to further explore any evidence of toxicity. Nevertheless, in the present study, after a relatively ‘acute’ treatment of 2 wk, astrocyte proliferation, which is typical of neurotoxicity, did not occur. This suggests that if there is enhancement of NMDA receptor-mediated neurotransmission by nutritional Gly then it is moderate and/or develops sufficiently
slowly to allow for adjustments, which preclude toxicity. These may include modulation of Gly transporter function and/or down-regulation of some elements of the response to NMDA such as synthesis of NMDA receptors and/or calcium channels. These questions are currently under investigation.

An intriguing finding in the present study shows an increase in GFAP-LI in the hippocampus but a decrease in the cerebellum. The morphology of both these regions is known to retain a high potential for plasticity in adulthood (Alcantara et al., 1992) and there is evidence that amino acid neurotransmitters including glutamate and taurine regulate plasticity in these brain regions during early development and in adulthood (Magnusson, 1996). However, differences in distribution of NMDA-receptor subtypes in hippocampus vs. cerebellum may contribute to the observed differences in GFAP reactions. Thus, the NR2B subtype of NMDA receptors, has the highest affinity to Gly (Honer et al., 1998). This subunit is more abundant in the hippocampus whereas the NR2C subunit is more abundant in the cerebellum (Monyer et al., 1994). Astrocytes also express NMDA receptors (Conti et al., 1996), so that differential regional variation in the subtype of NMDA receptor expressed in astrocytes may contribute to the differential effects observed in this study.

In addition, the differential effects of Gly in hippocampus vs. cerebellum may depend on how exogenous Gly affects neurotransmission in each brain region. In the hippocampal dentate gyrus, due to high concentration of NMDA receptors (McDonald and Johnston, 1990), exogenous Gly is likely to enhance neurotransmission. Increased neuronal activity may result in fluctuations of extracellular pH to which astrocytes are known to respond (Chesler and Kaila, 1992). In the cerebellum, the inhibitory action of Gly (Zafra et al., 1995) may result in reduced extracellular pH-associated activation of astrocytes. Another mechanism may involve trophic factors. Astrocyte morphology is modulated by neuronal factors (Shao and McCarthy, 1994) and astrocytes generate trophic factors that support synaptic plasticity (Meshul et al., 1987). If the net effect of exogenous Gly is to reduce neuronal activity in the cerebellum, this may be accompanied by reduced synthesis of trophic factors and, as a consequence, shrinkage of astrocytic processes resulting in an overall decrease in the density of astrocytic fibres as observed in the cerebellum in the present study.

Another issue which emerges in the present study is the potential effects of amino acids related to Gly. While the present studies were performed with Gly, and serine levels were not significantly increased, there is evidence that d-serine may also function as an endogenous modulator of NMDA function (Schell et al., 1995). Whether or not similar effects on glial morphology are observed following chronic treatment with d-serine needs to be determined.

The present findings support the concept that Gly, despite its poor permeability, penetrates across the blood–brain barrier and affects brain cell morphology. However, further documentation is necessary on Gly levels in the brain as a function of nutritional dose and duration of treatment, to help elucidate mechanisms underlying the present observations. Thus, it is not clear why doubling of astrocyte staining for GFAP occurred with the low Gly dose as there was only a small further increase with the doubling of plasma Gly levels.

Future studies should aim to further elucidate the nature of brain cell morphological changes induced by in vivo Gly administration. The use of high Gly doses and longer periods of treatment may help achieve this aim. The implications of this line of investigation could be far-reaching, given the present interest in NMDA receptor-related treatments in schizophrenia and other neuropsychiatric syndromes (Heresco-Levy and Javitt, 1998).

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