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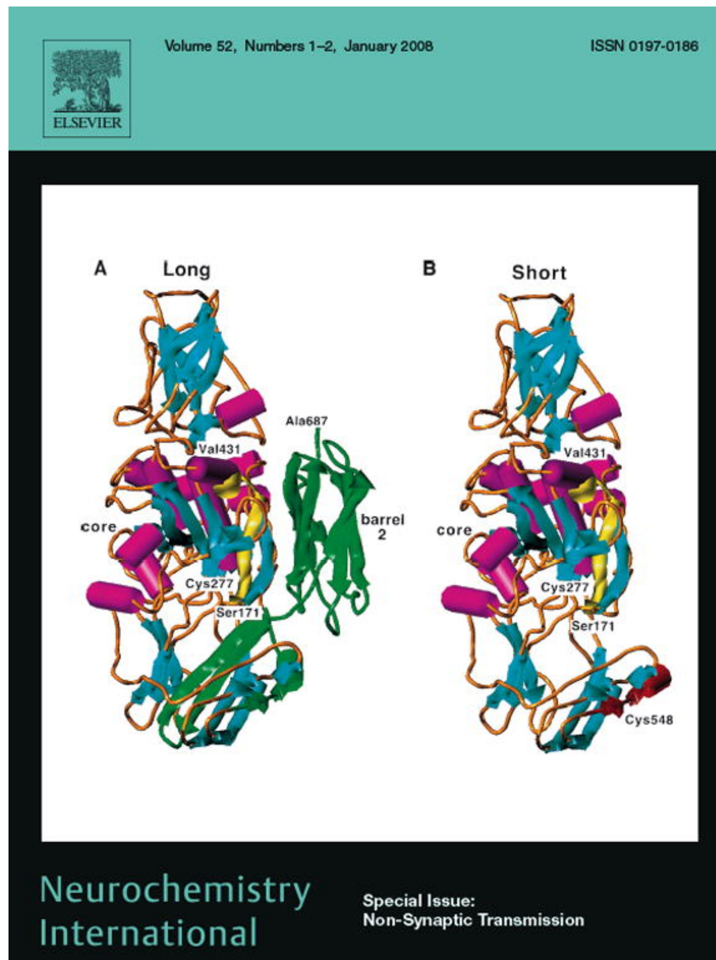

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The synaptic and nonsynaptic glycine transporter type-1 inhibitors Org-24461 and NFPS alter single neuron firing rate in the rat dorsal raphe nucleus Further evidence for a glutamatergic–serotonergic interaction and its role in antipsychotic action

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Abstract

Single neuron firing rate was recorded from dorsal raphe nucleus of anesthetized rats. The firing rate of raphe neurons varied from 4 to 8 discharge per second before drug administration and this neuronal activity was decreased by L-701,324 (2 mg/kg i.v. injection), a competitive antagonist of glycine$_B$ binding site of *N*-methyl-D-aspartate (NMDA) receptors. The glycine transporter type-1 (GlyT1) antagonists Org-24461 (10 mg/kg i.v.) and NFPS (3 mg/kg i.v.) reversed the inhibitory effect of L-701,324 on single neuron activity recorded from dorsal raphe nucleus of the rat. Org-24461 and NFPS both tended to increase the raphe neuronal firing rate also when given alone but their effect was not significant. This finding serves further evidence that glutamate released from axon terminals of the cortico-striatal projection neurons stimulates serotonergic neurons in the raphe nuclei and this effect is mediated at least in part by postsynaptic NMDA receptors. Thus, GlyT1 inhibitors are able to reverse the hypofunctional state of NMDA receptors, suggesting that these drugs may have beneficial therapeutic effects in neurological and psychiatric disorders characterized with impaired NMDA receptor-mediated transmission.
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Keywords: Glycine transporter inhibitors; Serotonergic neurons; Raphe nuclei; NMDA receptors; Org-24461; NFPS

1. Introduction

The dorsal raphe nucleus (DRN) in the brainstem contains at least 50% of all serotonergic neurons of the central nervous system (Wiklund and Bjorklund, 1980). These neurons are medium sized cells with spiny dendritic arborization, which establish local neuronal network with their dendrodendritic connections and recurrent axon collaterals. Beside serotonergic projection neurons, DRN also contains GABAergic interneurons as well as numerous noradrenergic and glutamatergic axon terminals. Morphological and functional observations confirmed the existence of a glutamatergic pathway arising from the median prefrontal cortex (Behzadi et al., 1990; Hajos et al., 1998) whereas serotonergic neurons of DRN form extended projection to the cerebral cortex (Kidd et al., 1991; Sesack et al., 1989). Thus, the raphe-cortical serotonergic and the cortico-raphe glutamatergic projections establish a long-axon neuronal loop between these two brain structures. Interaction between glutamatergic axon terminals and serotonergic neurons forms an excitatory-inhibitory connection by which incoming excitatory signals are converted into inhibitory output projecting back to cerebral cortex where stimulation was generated from.

The primary target of the cortical glutamatergic neurons within the raphe nuclei is the serotonergic projection neurons

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(Tao et al., 1996; Harsing et al., 2004). The excitatory glutamatergic influence in DRN may be mediated by ionotropic glutamate receptors expressed in postsynaptic membrane of serotonergic neurons. In isolated raphe nuclei slices, activation of *N*-methyl-D-aspartate (NMDA) receptors evokes serotonin release (Harsing et al., 2004). The NMDA receptor requires glycine as cotransmitter for its activation (Johnson and Ascher, 1987). At synaptic level, glutamate is released from glutamatergic axon terminals and glycine is released from astroglial cells. Being released into the synaptic cleft, they diffuse to the vicinity of postsynaptic NMDA receptors (Zafra et al., 1997). Operation of glycine transporter type-1 (GlyT1) expressed in astocytes assures to set glycine concentrations at NMDA receptor below saturating levels (Bergeron et al., 1998). Inhibition of GlyT1 enforces NMDA receptor-mediated functions, particularly in conditions when glycine$_B$ binding site at NR1 subunit is blocked by selective inhibitors (Danysz and Parson, 1998). The aim of the present study was to investigate whether the GlyT1 inhibitors Org-24461 and NFPS (Fig. 1) are able to facilitate glutamate-mediated excitation of serotonergic projection neurons in rat DRN.

2. Experimental procedure

2.1. Animals and drugs

Male Wistar rats weighing 250–300 g were obtained from Charles River Hungary. The animals were housed up to five to a cage in a temperature- and humidity-controlled animal facility on a 12-h light:12-h dark cycle (6.00 a.m. on; 6.00 p.m. off) with food and water available ad libitum. The animals were allowed at least 1 week of habituation to their housing prior to experimentation.

NFPS, *N*[3-(4′-fluorophenyl)-3-(4′phenylphenoxy)-propyl]sarcosine (Herdon et al., 2001) and Org-24461, *R,S*-(±)*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycine (Brown et al., 2001) were synthesized by Dr. Peter Matyus, Department of Organic Chemistry, Semmelweis University, Budapest, Hungary. Org-24461 (50 mg/ml) and NFPS (10 mg/ml) were dissolved in dimethyl sulfoxide (DMSO), briefly sonicated and kept in a water bath at 37 °C for ca. 10 min. Immediately before administration; they were diluted in a double volume of saline. L-701,324 was purchased from Tocris Bioscience (Bristol, UK) and dissolved in 25% polyethylene glycol (PEG)-300 and 75% saline. Intravenous administration of drug vehicle alone showed no change in single-unit activity. Urethane was purchased from Reanal (Budapest, Hungary). All other chemicals were of analytical grade.

2.2. Recording single neuron activity from rat DRN

Rats were anesthetized with urethane (1 g/kg i.p.). A polyethylene cannula was inserted into the left femoral vein. Then the head of the animals was fixed, the skull was opened on a 6 mm × 6 mm square around the lambda point using a dental drill and the dura was cut. The opening was sealed with bone wax, and the animal was put aside for at least 30 min. For recording, the rat was mounted in a stereotaxic frame (Narishige SR-6N) and a wolfram microelectrode (World Precision Instruments, WPI TM33B20) was advanced into the area of DRN (AP: 8; L: 0; and V: 5.8–6.5 mm, Paxinos and Watson, 1998) by a single axis micromanipulator (Narishige SM15). Extracellular single-unit activity was recorded and amplified, amplified gain: 1000×; low-pass filter at 0.1 kHz, high-pass filter 5 kHz. The amplified biological signals were registered in a Tektronix oscilloscope and sampled by a PC-based computer using Neurosys 1.1.0.357 software (Experimetria, Hungary). As soon as the firing in DRN reached a stabile rate, control activity was recorded for 10 min. Then, with uninterrupted recording, L-701,324 was injected i.v. in a dose of 2 mg/kg and activity was recorded for another 10-min period. GlyT1 inhibitors were added i.v. (Org-24461 10 mg/kg, NFPS 3 mg/kg) and neuronal single-unit activity was further recorded for a 10-min period.

To verify the position of the microelectrode, dc current (1 mA for 15 s) was delivered through the recording electrode when all recording was completed. The animals received then an overdose of urethane and were perfused transcardially with saline followed by buffered formalin. Brain sections (70 μm) containing the raphe nuclei were cut using a freezing microtome and the sections were washed, dehydrated with alcohol and Nissl staining was used for evaluation. A further proof that activity of neurons from a correct location was recorded was provided by changes in neuronal activity after L-701,324 administration (see below).

2.3. Evaluation of the records and statistical analysis

For evaluation, the records were played back and a threshold level was set so that all extracellular spikes, but no noise, crossed it. Crossings were counted as events, from which neuronal firing rate was calculated by the software. For data analysis, firing rates were collected for 250 s before injection of L-701,324 (control period) and for 250 and 500 s periods before and after the administration of the GlyT1 inhibitors. All group data were presented as mean ± S.D. and one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) tests were used to compare differences between group mean data with control group mean. Differences between control and experimental responses with $P < 0.05$ were considered significant, *n* indicate the number of experiments.

3. Results

Our preliminary experiments indicated that single neuron firing registered from rat DRN was due to activation of

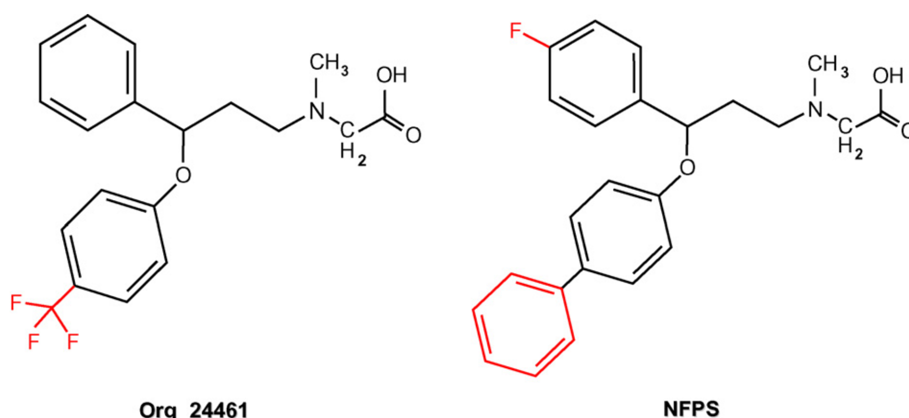

Fig. 1. The chemical structures of the glycine transporter type-1 inhibitors Org-24461 and NFPS.

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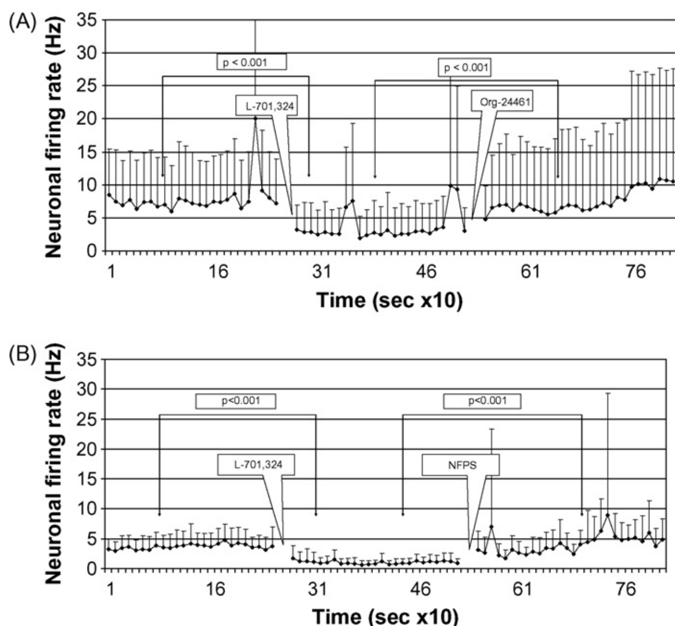

Fig. 2. The effects of the GlyT1 inhibitor Org-24461 (A) and NFPS (B) on single neuron firing rate in DRN of anesthetized rats. Org-24461 and NFPS were injected i.v. in doses of 10 and 3 mg/kg, respectively, after inhibition of neuronal firing by the glycine$_B$ binding site antagonist L-701,324. L-701,324 was administered to rats in an i.v. dose of 2 mg/kg. ANOVA followed by the least significant difference test (LSD), mean ± S.D., $n = 6$ in Org-24461-treated group and $n = 11$ in NFPS-treated group.

serotonergic neurons. This was evidenced by firing rate characteristics (Blier et al., 1992) and by the fact that i.v. administration of 8-OH-DPAT, and 5-HT$_{1A}$ receptor agonist, markedly reduced neuronal firing rate in DRN of the rat.

During the control period the rate of neuronal single-unit firing, recorded from DRN of anesthetized rats varied between 3 and 8 Hz with no excessive variations in time (Fig. 2A). Then, in the first series of experiments, L-701,324 was injected i.v. in a dose of 2 mg/kg, which reduced firing rate significantly by approximately 50% (Table 1). This effect increased the detection sensitivity of the putative effect of the agent tested, indicated that the recorded activity was of DRN neurons having glutamate receptors. Org-24461, injected in a dose of 10 mg/kg i.v. 3–5 min after L-701,324 administration, raised the firing rate of DRN approximately to the control level (Fig. 2A and Table 1). The increase started within 1 min after injection, peaked at 4–5 min and then ceased gradually.

In the second series of experiments, 2 mg/kg L-701,324 was injected as before and it decreased neuronal firing rate in DRN by ca. 70% (Table 1). On i.p. administration of 3 mg/kg NFPS, this decrease was reversed (Fig. 2B and Table 1), so that the firing rate was higher than that in the control. The time course of the rate change was similar to that seen after Org-24461.

Table 1
Effect of Org-24461 and NFPS on single neuron firing rate recorded from dorsal raphe nucleus of anesthetized rats

	Control	L-701,324 (2 mg/kg)	Org-24461 (10 mg/kg)
Frequency (Hz)	7.92 ± 8.97	3.64 ± 6.55***	7.64 ± 11.35$^{###}$
	Control	L-701,324 (2 mg/kg)	NFPS (3 mg/kg)
Frequency (Hz)	3.69 ± 0.42	1.05 ± 0.27***	4.95 ± 1.96$^{###}$

Single-unit activity was recorded from DRN of anesthetized rats. L-701,324, a glycine$_B$ binding site competitive antagonist, and Org-24461 and NFPS, inhibitors of GlyT1, were administered i.v. in doses indicated. Mean ± S.D., $n = 6$ in Org-24461-treated group and $n = 11$ in NFPS-treated group. ANOVA followed by the least significant difference (LSD) test.

*** $P < 0.001$ vs. control.

$^{###}$ $P < 0.001$ vs. L-701,324.

4. Discussion

It has been suggested that hypofunction of NMDA receptors may be involved in the etiology of schizophrenia (Coyle et al., 2003). The glutamatergic theory of schizophrenia is based upon the cognitive and behavioral effects of the NMDA receptor antagonist phencyclidine (PCP) and ketamine in animals and human. In fact, subanesthetic doses of dissociative anaesthetics exacerbate psychotic symptoms, reverse remission in schizophrenic patients and evoke psychotic state in normal subjects (Millan, 2002). The similarities of PCP-precipitated behavior with appearance of symptoms of schizophrenia have prompted the use of PCP and ketamine in pharmacological models of schizophrenia in both preclinical and clinical studies (Mathé, 1998).

A reduced NMDA receptor function has been established in our study by using L-701,324, an antagonist on glycine$_B$ binding site of NMDA receptor that readily crosses the blood–brain barrier. We found that blockade of glycine$_B$ binding sites in DRN reduces single neuron firing rate and this inhibition can be suspended by subsequent administration of GlyT1 inhibitors. Similarly to our finding, it has been shown that GlyT1 inhibition potentiates NMDA-mediated neuronal cell firing responses in rat prefrontal cortical neurons (Chen et al., 2003). We have shown previously that stimulation of NMDA receptors leads to increase of serotonin release from rat raphe nuclei slices (Harsing et al., 2004). The present experiments, showing enhanced neuronal firing rate after indirect activation of NMDA receptors by GlyT1 inhibitors, are in accordance. Thus, both *in vitro* and *in vivo* experiments served direct evidence for the existence of a glutamatergic–serotonergic interaction in the raphe nuclei.

The prefrontal cortex receives serotonergic innervation from DRN (Hajos et al., 1998). The raphe-cortical projection exerts stimulation of GABA interneurons through 5-HT$_{2A}$ receptors and inhibition of glutamatergic pyramidal cells via 5-HT$_{1A}$ receptors in the prefrontal cortex (Carlsson et al., 1997; Aghajanian and Marek, 2000). This dual action of serotonin results in an increased GABAergic inhibition and a reduced glutamatergic stimulation in cortical neuronal circuitry. The GABA/glutamate balance in the prefrontal cortex may be particularly important in schizophrenia when the impaired thalamic filter leads to increased transmission of sensory information from the periphery to the cerebral cortex (Aghajanian and Marek, 2000). The extreme levels of sensory inputs disrupt integrative cortical functions, which mirrors in

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desynchronized EEG activity and hallucinations (Sebban et al., 2002).

In schizophrenia, NMDA receptors in the central nervous system may be impaired (Tamminga, 1998) and those expressed in the cell membrane of raphe serotonergic neurons might also be altered. Decreased activity of glutamate carboxy peptidase and variants in the gene for neuroregulin I have been implicated to explain NMDA receptor hypofunction in subjects with schizophrenia (Lewis and Gonzalez-Burgos, 2006). Since cortical glutamatergic input activates raphe serotonergic neurons via NMDA receptors (Harsing et al., 2006), their hypofunctional state will lead to reduced serotonergic transmission in their axon terminal fields, including the prefrontal cortex.

Direct agonists of glycine$_B$ binding site may restore NMDA receptor hypofunctionality characteristics for psychotic state (Millan, 2002) and GlyT1 inhibitors may act accordingly (Mork et al., 2005). GlyT1 inhibitors will increase availability of synaptic glycine at NMDA receptors and restoration of glutamatergic influence on raphe serotonergic neurons may normalize GABA/glutamate balance in the cerebral cortex leading to an overall inhibition in prefrontal neuronal circuitry. Thus, effects of GlyT1 inhibitors on raphe nuclei NMDA receptors may be part of their antipsychotic action reported earlier (Javitt, 2002; Harsing et al., 2003, 2005; Thomsen, 2006). The potential site of action of GlyT1 inhibitors in the neuronal circuitry of DRN is shown in Fig. 3.

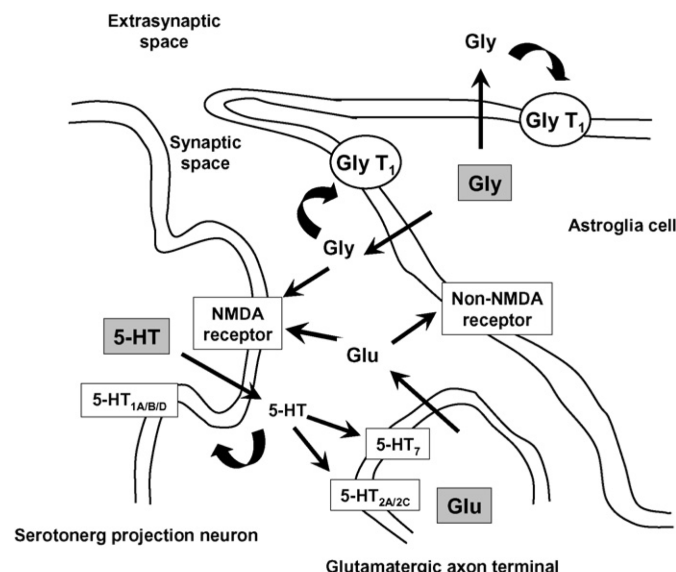

Fig. 3. A possible model for the site of action of GlyT1 inhibitors, Org 24461 and NFPS in DRN of the rat. Serotonin released from dendrites or recurrent axon collaterals of serotonergic projection neurons inhibits its own release through 5-HT$_{1A}$ and 5-HT$_{1B/1D}$ receptors. Glutamate release from glutamatergic axon terminals in the raphe nuclei is under the control of excitatory 5-HT$_{2A/2C}$ receptors and inhibitory 5-HT$_7$ receptors (Bagdy et al., 2000; Harsing et al., 2004). There are NMDA receptors located postsynaptically in the cell membrane of serotonergic projection neurons (Harsing, 2006) and the coagonist glycine required for NMDA receptor activation is released from neighboring astroglial cells. Glycine release from glial cells is regulated by non-NMDA glutamate receptors (Harsing et al., 2006). Glycine concentrations in the synaptic cleft are determined by the activity of GlyT1 expressed in glial cell membrane. However, glycine may also be released from astroglial cells into the extrasynaptic space, its concentrations in the biophase are regulated by nonsynaptic GlyT1. Whether currently known GlyT1 modulators exert equal inhibition on synaptic and nonsynaptic GlyT1 needs further clarifications.

There have been a number of publications indicating the disruption of raphe neurochemical transmission in schizophrenia or animal models of psychosis (Akhondzadeh, 2001; Craven et al., 2005). Thus, raphe serotonergic pathways have been shown to participate in psychotomimetic drug-induced locomotion and prepulse inhibition in rats (Kusljic and van den Buuse, 2004). Furthermore, it has been shown that destruction of raphe serotonergic neurons by the neurotoxin 5,7-dihydroxytryptamine enhances the psychomimetic effects of phencyclidine (Kusljic et al., 2005). The present findings serve additional information to the potential role of midbrain raphe nuclei in the pathology of psychosis and may provide further evidence for the potential antipsychotic role of drugs inhibiting GlyT1.

GlyT1, which may be involved in mediation of schizophrenia, has been detected in different locations in the CNS (Fig. 3). The primarily action of synaptic GlyT1 is regulation of glycine concentrations in glutamatergic synapses (Aragon and Lopez-Corcuera, 2005) whereas those located nonsynaptically establish glycine concentration gradient between the extrasynaptic solution and within the synapse (Vandenberg and Aubrey, 2001). Thus, GlyT1 inhibitors may alter not only glycine concentrations within the synaptic cleft (Javitt, 2007) but also in other brain compartments, such as the extracellular space as revealed by microdialysis studies (Marko et al., 2006). Nonsynaptic GlyT1 may also have important role in the regulation of nonsynaptic chemical neurotransmission, a novel form in neuronal communication first described by Vizi (1984, 2005). Inhibitors of GlyT1 may participate in CNS drug actions including their antipsychotic effects (Harsing et al., 2003). Whether synaptic and nonsynaptic GlyT1s represent the same or different splice variants of the transporter protein remains to be elucidated (Thomsen, 2006).

In conclusion, the potential antipsychotic effects of GlyT1 inhibitors in human have been arisen since the early discovery of this group of compounds (Toth and Lajtha, 1986; Javitt and Frusciante, 1997). There are currently two GlyT1 inhibitors (SSR504734 and JNJ-17305600) in human clinical phase I investigation as antischizophrenic agents (Depoortere et al., 2005; Thomsen, 2006). Our findings, that the GlyT1 inhibitors Org-24461 and NFPS enhance firing rate of raphe serotonergic neurons *in vivo*, may provide additional information about the mode of antipsychotic action of GlyT1 inhibitor compounds.

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