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ORIGINAL PAPER

Alterations in Brain Extracellular Dopamine and Glycine Levels Following Combined Administration of the Glycine Transporter Type-1 Inhibitor Org-24461 and Risperidone

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Abstract The most dominant hypotheses for the pathogenesis of schizophrenia have focused primarily upon hyperfunctional dopaminergic and hypofunctional glutamatergic neurotransmission in the central nervous system. The therapeutic efficacy of all atypical antipsychotics is explained in part by antagonism of the dopaminergic neurotransmission, mainly by blockade of D_2 dopamine receptors. *N*-methyl-D-aspartate (NMDA) receptor hypofunction in schizophrenia can be reversed by glycine transporter type-1 (GlyT-1) inhibitors, which regulate glycine concentrations at the vicinity of NMDA receptors. Combined drug administration with D_2 dopamine receptor blockade and activation of hypofunctional NMDA receptors may be needed for a more effective treatment of positive and negative symptoms and the accompanied cognitive deficit in schizophrenia. To investigate this type of combined drug administration, rats were treated with the atypical antipsychotic risperidone together with the GlyT-1 inhibitor Org-24461. Brain microdialysis was applied in the striatum of conscious rats and determinations of extracellular dopamine, DOPAC, HVA, glycine, glutamate, and serine concentrations were carried out using HPLC/electrochemistry. Risperidone increased extracellular concentrations of dopamine but failed to influence those of glycine or glutamate measured in microdialysis samples. Org-24461 injection reduced extracellular dopamine concentrations and elevated extracellular glycine levels but the concentrations of serine and glutamate were not changed. When risperidone and Org-24461 were added in combination, a decrease in extracellular dopamine concentrations was accompanied with sustained elevation of extracellular glycine levels. Interestingly, the extracellular concentrations of glutamate were also enhanced. Our data indicate that coadministration of an antipsychotic with a GlyT-1 inhibitor may normalize hypofunctional NMDA receptor-mediated glutamatergic neurotransmission with reduced dopaminergic side effects characteristic for antipsychotic medication.

Keywords Antipsychotic agents · Glycine transporter type-1 inhibitors · Extracellular glycine and dopamine · Microdialysis · Schizophrenia

Special issue article in honor of Dr. Abel Lajtha.

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Introduction

Deficit of *N*-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmission is widely considered

to be responsible for development of psychotic symptoms as well as for cognitive deficit of schizophrenia [1, 2]. It has been shown that inhibition of glycine transporter type-1 (GlyT-1) leads to enhance synaptic and extrasynaptic glycine brain concentrations [3] and this increase in glycine levels may positively modulate hypofunctional NMDA glutamate receptors [4]. Glycine, which is a mandatory coagonist of NMDA-type receptors in the CNS [5], exerts its positive allosteric modulatory effects by acting on glycine$_B$ binding site at NR1 subunit of NMDA receptors [6]. As glycine$_B$ binding site is not saturated in physiological conditions, elevation of glycine concentrations around NMDA receptors by inhibition of its reuptake may positively influence reduced receptor functions [7, 8]. This may have importance in the treatment of various types of endogenous psychosis characterized by hypofunctional NMDA receptors [9]. Similarly to antipsychotics, GlyT-1 inhibitors exert antipsychotic effects in a number of animal models of schizophrenia [10–12] and they also improve memory impairment in animal tests [13, 14].

Great number of compounds with diverse chemical structures has been reported to inhibit GlyT-1 activity [15, 16]. A major effort has been made to find selective and potent inhibitors of GlyT-1 with pharmacological profile suitable for clinical drug development. Of the GlyT-1 inhibitors, Org-24461 [17] is one of the most wildly used reference compounds (Fig. 1). This compound as well as NFPS (*N*-(3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl)sarcosine, another sarcosine-based GlyT-1 inhibitor [18], decreased glycine reuptake in synaptosomes prepared from human brain tissue, area Brodman 8 [19], further supporting potential clinical efficacy of GlyT-1 inhibitors tested in human phase I clinical trials. At present, SSR504734, JNJ-17305600, and Org-25935 reached clinical trials although detailed reports of their actions in human have not been available [20]. Currently, it is an open question whether GlyT-1 inhibitors administered alone are capable of influencing psychotic symptoms [21] or their combined administration with known antipsychotics is required to manage symptoms of this mental disorder. It has been shown that administration of the GlyT-1 inhibitor sarcosine to schizophrenic patients treated with risperidone improved both negative and positive symptoms as well as cognitive impairment in stable chronic schizophrenia [22] as well as in acute exacerbation [23]. Using brain microdialysis technique in conscious rat striatum, we report here that combined administration of a GlyT-1 inhibitor and an atypical antipsychotic results in a sustained elevation of extracellular glycine concentrations accompanied by a decrease in extracellular dopamine release. A preliminary report of these findings was presented at the thirtieth Annual Meeting of the Society for Neuroscience [24].

Experimental Procedure

All experimental procedures were approved by local Ethical Committees and were in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. Male rats were used in the experiments. Animals were housed five to a cage in a temperature-and humidity-controlled animal facility on a 12 h light/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.

Receptor Binding Assays

Competition binding studies for the GlyT-1 inhibitor Org-24461 and the atypical antipsychotic agent risperidone were performed at multiple serotonin and dopamine D2 receptors. The protocols employed for examination of drug affinities at specific receptor types are summarized in Table 1.

[^{3}H]Glycine Uptake in Rat Brain Synaptosomes

Synaptosomal P_2 fraction was prepared as described previously [25]. Male rats (160–180 g) were decapitated, the brain was removed from the skull and the cerebral cortex or the striatum was dissected on ice. The tissue was

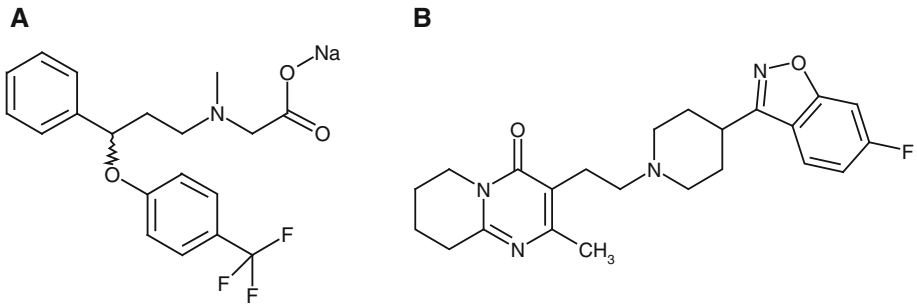

Fig. 1 The chemical structures of **a** Org-24461 (*R*,*S*-(±)*N*-methyl-*N*-[(4-trifluoromethyl) phenoxy]-3-phenyl-propylglycineNa) and **b** risperidone (3-[2-[4-(6-fluoro-1,2-benzisoxyazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one)

Table 1 Inhibition of multiple receptors by Org-24461 and risperidone

Receptors	Org-24461		Risperidone	
	Per cent inhibition			
	10^{-7} M	10^{-5} M	10^{-7} M	10^{-5} M
r5-HT_{1A}	2	2	7	83
h5-HT_{1B}	0	0	16	86
h5-HT_{1D}	0	8	–	–
r5-HT_{2A}	3	0	100	100
p5-HT_{2C}	15	19	67	100
h5-HT_7	12	0	78	100
rD_2	0	1	77	100

Displacement of receptor ligands was expressed as per cent inhibition at 10^{-7} M and 10^{-5} M concentrations for Org-24461, and risperidone. Data are means of at least two determinations performed in triplicate. Receptor binding assays were carried as follows: r5-HT_{1A} [58], r5-HT_{2A} [59], p5-HT_{2C} receptors [60], rD_2 receptors [61]. h5-HT_{1B}, h5-HT_{1D}, and h5-HT_7 receptor competition binding studies were performed according to information sheets obtained from Bio-Xtal (Mundolsheim, France) and Perkin Elmer Life and Analytical Sciences (Shelton, CA, USA). Some of the data were taken from Harsing et al. [10] with permission

homogenized in ice cold 0.32 M sucrose (1:10 g/ml) using a Teflon glass homogenizer (Wheaton, USA). The homogenate was centrifuged at 1,000*g* for 10 min to remove nuclei and debris. The supernatant was then further centrifuged at 12,000*g* for 20 min. The P_2 pellet containing the synaptosomes was resuspended in 0.32 M sucrose (1:15 g/ml) and after slow stirring for 3 min it was used for uptake studies.

Incubation was carried out at pH 7.4 in Krebs solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM D-glucose, 25 mM $NaHCO_3$, 0.3 mM ascorbic acid and 0.01 mM pargyline. Aliquots of synaptosomal preparation (approx. 0.4 mg protein/tube) were preincubated with drugs for 5 min at 37°C in a final volume of 1 ml Krebs solution. The uptake was initiated by adding [^{3}H]glycine (specific activity: 14 Ci/mmole) and the incubation was continued for another 10 min. To achieve 0.3 µM glycine concentration unlabelled glycine was added to an aliquot of [^{3}H]glycine. The uptake was terminated with 3 ml ice cold Krebs solution and rapid vacuum filtration on GF/B Whatman filters soaked in 0.1% polyethylenimine. Filters were washed three times with ice cold saline. The radioactivity trapped on filters was measured by liquid scintillation counting using Packard Ultima Gold cocktail. The non-specific uptake was determined at 0°C incubation. Specific uptake was calculated by the subtraction of non-specific uptake from the total uptake value. Drugs were dissolved and further diluted with DMSO. Each tubes (the control also) contained the same amount of DMSO. In control experiments, DMSO in concentration we used did not affect glycine uptake. Each drug concentration was tested in three parallels of samples. Protein content of the preparation was determined by the method of Lowry et al. [26] using CuEDTA. The value of IC_{50} was calculated by PRISM 3 software using non-linear regression.

Microdialysis of Rat Striatum

On the day of surgery, male rats (280–310 g) were anesthetized with pentobarbital (60 mg/kg ip). After adequate anesthesia had been reached (using toe and tail pinch procedures), rats were placed into a stereotaxic frame (David Kopf Instruments, USA). The skull region was wiped with 2% Betadine solution and a rostrocaudal incision was made to expose the surface of the skull. A CMA/12 guide cannula (CMA/Microdialysis, Sweden) was implanted into the striatal region: AP: −0.4, ML: +3.5, DV: −4.0 mm from the bregma [27]. The guide cannula was secured using two skull screws and dental cement (Duracyl Plus and Liquid). During the implantation, body temperature was maintained using a CMA/150 heating pad (CMA/Microdialysis, Sweden). The CMA/12 Elite microdialysis probe (CMA/Microdialysis, Sweden) was washed first in 70% ethanol, then in distilled water and finally in modified Ringer solution (composition in mM: NaCl 147, KCl 4.0, $CaCl_2$ 1.2, and $MgCl_2$ 1.0), each washing period lasted 5–10 min. Following washing of the probe, it was inserted into a standard solution of neurotransmitters for 1 h. The first 30 min was the equilibration period; the second was for collecting the recovery sample. After this procedure, the probe was washed again in bidistilled water 15–20 min long. 1 day following surgery, rats were weighed and the dialysis probe (2 mm length) was inserted into the brain. The perfusion fluid was modified Ringer solution delivered with a flow rate of 2 µl/min using a CMA/100 microdialysis pump (CMA/Microdialysis, Sweden). Ten fractions were collected after a 2 h long equilibration period. Each fraction was collected 30 min long in 20 µl 0.5 M perchloric acid solution to prevent neurotransmitter degradation. The animals were injected ip with Org-24461 (10 mg/kg) or risperidone (1 mg/kg) or both after the 4th fraction. At the end of the experiments, rats were decapitated; the brain was removed from the skull and fixed in 10% formaldehyde. Later, the brains were sliced coronal to determine the exact position of the probe. Changes in extracellular analytes were normalized to the percent change from the average of the four baseline values i.e. the data obtained before drug administration.

Determination of Extracellular Concentrations of Dopamine, 3,4-Dihydroxyphenylacetic Acid and Homovanillic Acid

Dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured immediately after collecting samples by isocratic reversed-phase high performance liquid chromatography (HPLC)-electrochemistry technique. A Perkin Elmer 200 HPLC system, a degasser with helium and a Thermo Aquasil C18 (3 μm) column with precolumn was used coupled with an ESA Coulochem II detector, analytical cell (ESA Model 5011A) and an analog/digital converter. The mobile phase consisted of 90% buffer (75 mM NaH_2PO_4, 2.8 mM OSA.Na, 50 μM Na_2EDTA) filtered and pH set to 2.6 with H_3PO_4) and 10% acetonitrile was added. The flow rate was 0.6 ml/min and the electrode potentials were C1: −250 mV and C2: +340 mV. Contents of dopamine and its metabolites were quantified by comparing the peak areas obtained with those produced by known concentrations of standards and expressed as pmole/μl [28].

Determination of Extracellular Concentrations of Amino Acids

Microdialysis samples were analyzed by gradient reversed-phase HPLC-electrochemistry using precolumn derivatization by o-phthaldialdehyde/sodium sulphite derivatizing reagent by the method of Rowley et al. [29]. The derivatizing stock solution was prepared by dissolving 5.4 mg *o*-phthaldialdehyde in 100 μl ethanol to which was added 100 μl 1 M sodium sulphite and 1.8 ml 0.1 M sodium tetraborate (pH = 10.4). The reagent was stored in aliquots at −20°C and remained stable for up to 2 weeks. The derivatizing working solution was prepared fresh daily by diluting the stock solution with sodium tetraborate buffer. Derivatization was performed by the autosampler by mixing 12 μl sample with 18 μl working derivatization reagent and allowed to react for 5 min before injection to the analytical system.

An Agilent 1100 HPLC was used. The HPLC system consisted of an autosampler, a degasser, a gradient pump, a reversed phase column (Agilent Zorbax SB-C18, 250 × 4.6 mm, 5 μm) with a precolumn (BST Rutin10 C-18 BD, 20 × 4 mm, 10 μm), an analytical cell (ESA Model 5011A), an electrochemical detector (ESA Coulochem III) and an analog/digital converter. The mobile phase consisted of 0.1 M Na_2HPO_4, filtered, pH was adjusted to 4.5, and methanol was used for gradient elution. The flow rate was 1 ml/min and the working electrode potential was set to +650 mV. Levels of amino acids (glycine, glutamate, and serine) were quantified by known concentrations of standards and expressed as pmole/μl sample.

Statistical Analyses

One-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test and the Student *t*-statistics for two-means were used for statistical analysis of the data. The mean ± S.E.M. was calculated and the number of independent determinations (n) is indicated. A level of probability (P) less than 5% was considered significant.

Materials

(+)Butaclamol HCl, clozapine, cyproheptadine HCl, dopamine HCl, 3,4-dihydroxy-phenylacetic acid (DOPAC), homovanillic acid (HVA), L-glutamate HCl, glycine, L-serine mianserin HCl, serotonin creatinine sulfate, and yohimbine HCl were purchased from Sigma Chemicals Co (St. Louis, MO, USA). GR127935 was obtained from Tocris Cookson Ltd (Bristol, UK). Pentobarbital sodium was obtained from Ceva-Phylaxia, Budapest, Hungary. [^{3}H]GR125743 and [^{3}H]ketanserin were purchased from New England Nuclear Life Science Products (Boston, MA, USA), [^{3}H]glycine, [^{3}H]8-OH-DPAT, [^{3}H]mesulergine, and [^{3}H]spiperone were obtained from Amersham Life Sciences (Buckinghamshire, UK), and [^{3}H]5-carboxamidotryptamine was a product of Perkin Elmer Life and Analytical Sciences (Shelton, CT, USA). Some of the listed chemicals were used for receptor binding assays. Org-24461 (*R*,*S*-(±)*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycineNa) was synthesized by Dr. P. Matyus, Department of Organic Chemistry, Semmelweis University, Budapest, Hungary. Risperidone (3-[2-[4-(6-fluoro-1,2-benzisoxyazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido- [1,2-a]pyrimidin-4-one) was synthesized by Dr. J. Barkoczy at Division of Chemical Research, EGIS Pharmaceuticals Plc, Budapest, Hungary. All other chemicals were of analytical grade.

Results

Receptor Binding Assays

Using receptor binding assays, we measured the displacement of different receptor ligands by Org-24461 in comparison with that of risperidone (Table 1). Each drug was used in 10^{-7} M and 10^{-5} M concentrations. Org-24461 did not exhibit significant affinity to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_7 receptors or D_2 receptors. On the contrary, risperidone showed major affinity to a number of receptors tested, thus, it exhibited preferential affinity to 5-HT_{2A}, 5-HT_{2C}, 5-HT_7 receptors and also to D_2 receptors (Table 1).

Effects on [^{3}H]glycine Uptake in Rat Brain Synaptosomal (P_2) Preparation

The high-affinity [^{3}H]glycine uptake in rat cerebral cortex P_2 synaptosomal preparation was characterized with K_m values of 178 μM and maximal velocity (V_{max}) of 4.9 nmol/mg protein/10 min, respectively. These data correspond to those published in the literature [17]. As shown in Table 2, Org-24461 inhibited the high-affinity [^{3}H]glycine uptake in rat cerebral cortex synaptosomal preparation. The IC_{50} value ($1.3 \pm 0.1 \times 10^{-7}$ M) was in the range of inhibition determined in recombinant GlyT-1b expressed in CHO cells [18]. The IC_{50} values measured for Org-24461 was found identical in cerebral cortex and in striatum. In contrast to the GlyT-1 inhibitor, risperidone exhibited no major inhibitory potency on [^{3}H]glycine uptake determined in rat cerebral cortex or striatum synaptosomes (Table 2).

Effects of Risperidone on Extracellular Dopamine and Glycine Concentrations

Basal extracellular levels of dopamine and those of its metabolites, DOPAC and HVA were found to be stable during a 300 min microdialysis procedure and they did not deviate from the baseline levels [$F(6,21) = 0.654$, $P = 0.654$ for dopamine, $F(6,21) = 0.659$, $P = 0.682$ for DOPAC, and $F(6,21) = 0.538$, $P = 0.773$ for HVA]. Figure 2a shows that 1 mg/kg risperidone (ip injection) increased extracellular dopamine concentrations in the striatum of conscious rats with a peak effect about 60 min after drug administration [$F(6,28) = 2.492$, $P < 0.05$]. The risperidone-induced increase in dopamine levels was associated with elevation of DOPAC and HVA concentrations in the striatal extracellular space [$F(6,28) = 6.716$, $P < 0.001$ and $F(6,28) = 6.652$, $P < 0.001$ for DOPAC and HVA, respectively].

Basal extracellular levels of the amino acids glycine, serine, and glutamate were also found to be stable during microdialysis and they were not different from baseline levels [$F(6,21) = 1.922$, $P = 0.133$ for glycine, $F(6,21) = 0.682$, $P = 0.665$ for serine, and $F(6,21) = 1.305$, $P = 0.297$ for glutamate]. Risperidone, in 1 mg/kg ip dose, did not influence extracellular concentrations of glycine and glutamate in the striatum [$F(6,42) = 1.740$, $P = 0.135$ for glycine and $F(6,42) = 1.352$, $P = 0.256$ for glutamate] (Fig. 2b). The extracellular striatal serine levels, however, were reduced after risperidone administration [$F(6,42) = 4.064$, $P < 0.01$].

Table 2 Inhibition of [^{3}H]glycine uptake in rat cerebral cortex and striatum synaptosomal (P_2) preparation by Org-24461 and risperidone

Drugs	Cortex IC_{50} (M)	Striatum IC_{50} (M)
Org-24461	$1.3 \pm 0.1 \times 10^{-7}$	1.8×10^{-7}
Risperidone	$>5.0 \times 10^{-5}$	1.7×10^{-4}

For methods see "Experimental Procedure". Data are mean ± S.E.M., $n = 2$–4

Effects of Org-24461 on Extracellular Dopamine and Glycine Concentrations

Figure 3a shows that 10 mg/kg ip administration of Org-24461 resulted in a decrease in extracellular dopamine levels [F (6, 42) = 5.135, $P < 0.001$]. ANOVA analysis indicated significant changes in DOPAC concentrations in the striatum after GlyT-1 inhibition [$F(6,42) = 3.455$, $P < 0.01$], however, further analysis with Dunnett's post-hoc test showed no significant differences between the control and the treated samples. Extracellular HVA concentrations remained unaltered after ip injection of Org-24461 [$F(6,42) = 1.164$, $P = 0.343$].

As far as the amino acid levels are concerned, Org-24461 selectively increased striatal glycine concentrations [$F(6,35) = 6.706$, $P < 0.001$] (Fig. 3b). A 10 mg/kg ip dose of Org-24461 caused a 2.5-fold increase in extracellular glycine concentrations that reached statistically significance between 180 and 300 min. The elevation in extracellular glycine concentrations after drug administration persisted for a period of 90 min then it declined towards the baseline levels. After Org-24461, striatal concentrations of glutamate and serine were not changed [$F(6,35) = 0.198$, $P = 0.975$ for glutamate and $F(6,35) = 1.211$, $P = 0.324$ for serine].

Combined Administration of Risperidone and Org-24461 on Extracellular Dopamine and Glycine Concentrations

When risperidone (1 mg/kg) and Org-24461 (10 mg/kg) were injected ip in combination, striatal extracellular dopamine concentrations remained unchanged [$F(6,35) = 0.528$, $P = 0.782$] (Fig. 4a). ANOVA analysis indicated significant increases of DOPAC [$F(6,35) = 2.640$, $P < 0.05$] and HVA concentrations [$F(6,35) = 2.652$, $P < 0.05$] in the striatum after combined drug administration.

Combined administration of risperidone and Org-24461 resulted in increases of both striatal glycine and glutamate extracellular concentrations, [$F(6,35) = 5.624$, $P < 0.001$ for glycine and $F(6,34) = 4.336$, $P < 0.01$ for glutamate], those of serine, however, did not change in response to combined drug treatment [$F(6,35) = 1.929$, $P = 0.103$ for serine] (Fig. 4b).

The elevations of dopamine and glycine concentrations over a 180 min microdialysis period were also calculated as cumulative percent changes above baseline efflux in

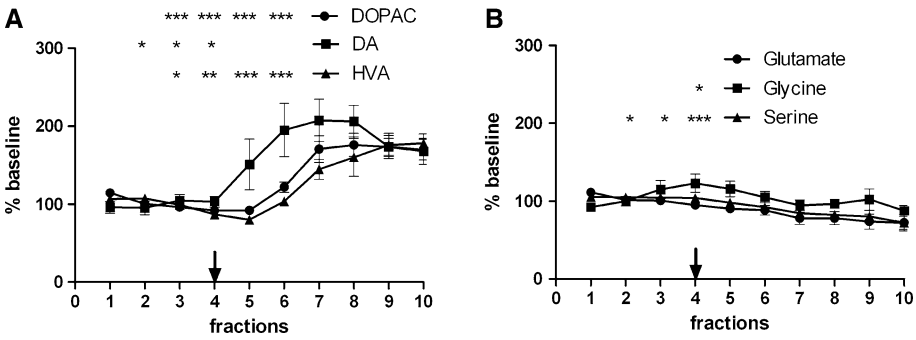

Fig. 2 **a** Effect of risperidone (1 mg/kg injected ip) on the extracellular concentrations of dopamine and its metabolites, DOPAC and HVA in striatum of conscious rat. Drug administration is indicated by the *arrow*. The ordinate shows the concentrations of dopamine or its metabolites expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 2.90 ± 0.32 fmole/µl for dopamine and 0.77 ± 0.04 and 0.45 ± 0.04 pmol/µl for DOPAC and HVA, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 5$. **b** Effect of risperidone (1 mg/kg injected ip) on the extracellular concentrations of glycine, serine, and glutamate in striatum of conscious rat. Drug administration is indicated by the *arrow*. The ordinate shows the concentrations of the amino acids expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 1.61 ± 0.14, 5.01 ± 0.33, and 1.17 ± 0.14 pmol/µl for glycine, serine, and glutamate, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 7$

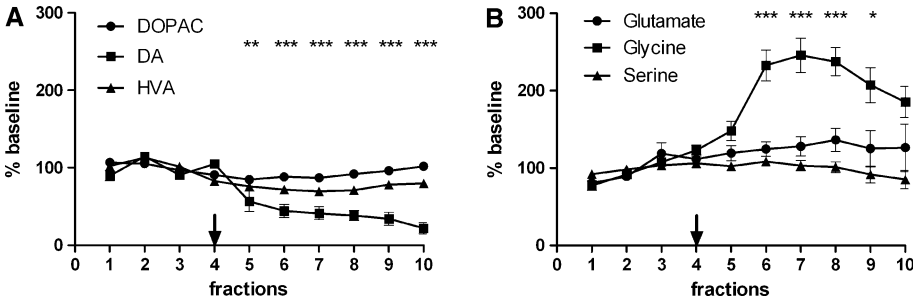

Fig. 3 **a** Effect of Org-24461 (10 mg/kg injected ip) on the extracellular concentrations of dopamine and its metabolites, DOPAC and HVA in striatum of conscious rat. Drug administration is indicated by the *arrow*. The ordinate shows the concentrations of dopamine or its metabolites expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 1.08 ± 0.22 fmole/µl for dopamine and 0.48 ± 0.02, and 0.40 ± 0.02 pmol/µl for DOPAC and HVA, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 7$. **b** Effect of Org-24461 (10 mg/kg injected ip) on the extracellular concentrations of glycine, serine, and glutamate in striatum of conscious rat. Drug administration is indicated by the *arrow*. The ordinate shows the concentrations of the amino acids expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 1.99 ± 0.12, 4.46 ± 0.43, and 2.41 ± 0.31 pmol/µl for glycine, serine, and glutamate, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 6$

response to combined administration of risperidone and Org-24461. As shown in Fig. 5, Org-24461 reduced the stimulatory effect of risperdone on dopamine efflux. The cumulative efflux of glycine evoked by the GlyT-1 inhibitor was tended to decline after combined administration of Org-24461 and risperidone but these changes were not, however, statistically significant.

Discussion

The major question addressed in our study was whether combined administration of GlyT-1 inhibitors and atypical antipsychotic drugs possesses any advantageous effects on dopaminergic and NMDA receptor-mediated glutamatergic neurotransmissions compared to single drug administration. To answer this question, Org-24461, a GlyT-1 inhibitor with antipsychotic profile [10, 17], was injected with the atypical antipsychotic risperidone. Using microdialysis, we found that combined administration of Org-24461 with risperidone reduced extracellular dopamine concentrations with sustained high levels of extracellular glycine in rat striatum.

Several authors demonstrated that both sarcosine-containing GlyT-1 inhibitors and those without sarcosine moiety increase extracellular glycine levels in various brain

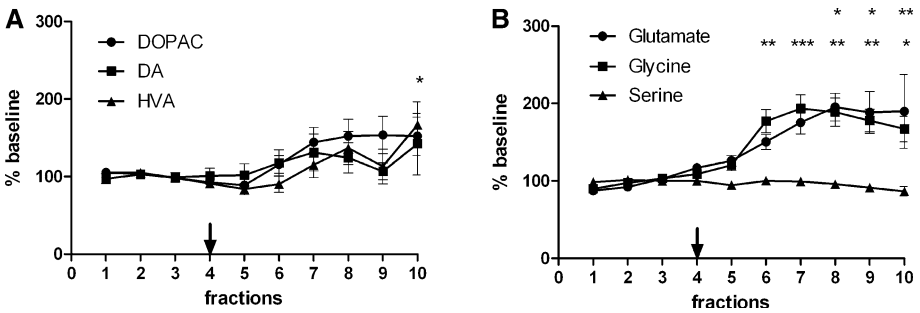

Fig. 4 **a** Effect of Org-24461 and risperidone (10 and 1 mg/kg injected ip) on the extracellular concentrations of dopamine and its metabolites, DOPAC and HVA in striatum of conscious rat. Drug co-administration is indicated by the *arrow*. The ordinate shows the concentrations of dopamine or its metabolites expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 0.91 ± 0.14 fmole/µl for dopamine and 0.49 ± 0.06, and 0.43 ± 0.05 pmol/µl for DOPAC and HVA, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 6$. **b** Effect of Org-24461 and risperidone (10 and 1 mg/kg injected ip) on the extracellular concentrations of glycine, serine, and glutamate in striatum of conscious rat. Drug co-administration is indicated by the *arrow*. The ordinate shows the concentrations of the amino acids expressed as the percentage of the baseline concentration, which is the mean of values measured in fractions 1 to 4. 100% was considered 1.85 ± 0.27, 4.64 ± 0.24, and 1.71 ± 0.25 pmol/µl for glycine, serine, and glutamate, respectively. One-way ANOVA followed by the Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control value measured immediately before drug administration. Each point is the mean ± S.E.M., $n = 6$

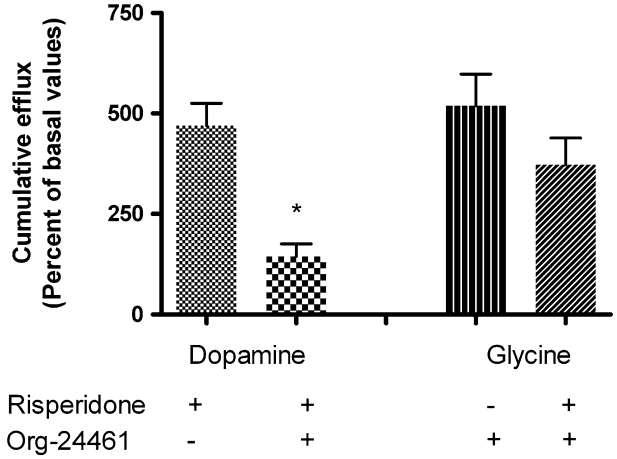

Fig. 5 Cumulative efflux of dopamine and glycine measured by microdialysis from the striatum of conscious rats after risperidone (1 mg/kg ip), Org-24461 (10 mg/kg ip) or their combination. Drugs were injected after collection of fraction 4. Area under the curve for dopamine and glycine efflux were calculated from the amount of dopamine or glycine outflow collected during the 180 min post-treatment periods (from fractions 5 to fraction 10). Basal efflux was determined in fraction 4. Student *t*-statistics for two-means ($t = 5.314$, $df = 9$, *** $P < 0.001$ for dopamine and $t = 1.419$, $df = 10$, $P = 0.1864$ for glycine), mean ± S.E.M

areas [3, 30, 31]. Similarly to these findings, we found that Org-24461 selectively increased extracellular glycine concentrations in conscious rat striatum whereas increases in serine or glutamate concentrations were not seen in the microdialysis samples. Since Org-24461 and other GlyT-1 inhibitors do not exhibit major binding affinity to most neurotransmitter receptors, elevation of glycine concentrations in the vicinity of impaired NMDA receptors may be responsible for their antipsychotic [10, 11, 32] and procognitive [14, 33] effects.

It has been shown that the GlyT-1 inhibitors glycyldodecylamide and ALX5311 reduced NMDA-induced [^{3}H]dopamine release from mouse striatal slice preparations [34, 35]. We found here that Org-24461 also decreased extracellular dopamine concentrations in the striatum when microdialysis was used in conscious rats. This effect of Org-24461 was associated, however, with no major alterations in extracellular levels of the dopamine metabolites, DOPAC and HVA. The site of action of GlyT-1 inhibitors to reduce striatal dopamine release may be the GABAergic neurons, which receive NMDA receptor-mediated glutamatergic innervation from the cerebral cortex, and their recurrent axon collaterals terminate on nigrostriatal dopaminergic nerve endings [36]. It is possible that GlyT-1 inhibitors decrease dopamine release in the striatum indirectly as these drugs were shown to facilitate GABA release, which then in turn inhibits dopamine efflux [35, 37]. The decrease in dopamine release after Org-24461 can be attributed to effects on NMDA receptors as this compound, in contrast to risperidone, does not exhibit significant binding affinity to D_2 dopamine receptors. GlyT-1 inhibitors acting by activation of hypofunctional NMDA receptors may normalize both dopaminergic hyperactivity and GABAergic hypoactivity as was demonstrated by changes in opposite direction of neurotransmitter release [35]. Thus, shifting dopaminergic/GABAergic balance may be a common action of various GlyT-1 inhibitors at least in striatum and this change in neurotransmission interaction may have importance in their antipsychotic effects [38].

It is generally accepted that almost all drugs effective in schizophrenia antagonize D_2 dopamine autoreceptors and thereby elevate extracellular dopamine levels [39]. Risperidone also produced an increase in extracellular dopamine concentrations in our microdialysis studies and this increase was associated with an elevation of extracellular levels of DOPAC and HVA. Risperidone is known to exhibit high affinity to D_2 receptors and our data on receptor binding studies are in line with previous findings. It is thus highly probable that the increased extracellular dopamine levels in the striatum after risperidone administration is due to an inhibition of presynaptic release-mediating D_2 autoreceptors located on nigrostriatal dopaminergic axon terminals. Besides their autoreceptor function, D_2 receptors are also present on postsynaptic membranes of medium size spiny GABAergic projection neurons. Dopamine released from nigrostriatal nerve terminals may directly inhibit GABAergic neuronal activity via postsynaptic D_2 receptors in the striatum [40]. Antipsychotics, which block D_2 receptors on GABAergic neurons, will enhance GABAergic tone in the striatum, an effect believed to be important in the action of antipsychotics [38]. Thus, antipsychotic agents, similarly to GlyT-1 inhibitors, may change disturbed dopaminergic/GABAergic balance, the former blocking D_2 receptors and latter activating NMDA receptor-mediated glutamatergic neurotransmission.

Atypical antipsychotic drugs like risperidone and olanzapine and even clozapine exhibited only a weak inhibition on the activity of GlyT-1a expressed in human placenta choriocarcinoma cells [41]. In accordance, risperidone, which exerted a poor inhibitory effect on GlyT-1 in rat brain synaptosomes, failed to influence extracellular glycine and glutamate levels in striatum of conscious rats. It needs to further elucidate whether lack of inhibitory effects of most currently used antipsychotics on GlyT-1 activity is related to their limited effects on negative symptoms of schizophrenia and the cognitive deficit accompanied.

In the next series of experiments, the GlyT-1 inhibitor Org-24461 and the antipsychotic risperidone were combined in order to determine their effects on extracellular glycine and dopamine levels in rat striatum. Org-24461, which decreased striatal dopamine levels by itself, abolished the stimulatory effects of risperidone on extracellular dopamine concentrations. In combined administration, Org-24461 also decreased risperidone-induced elevation of DOPAC and HVA levels but in a much less extent. On the other hand, when added in combination, Org-24461 was still capable of increasing striatal glycine levels in the presence of risperidone assuring elevated glycine concentrations for activation of $glycine_B$ binding sites at NMDA receptors. The indirect stimulation of NMDA receptors by Org-24461 and blockade of D_2 receptors by risperidone may beneficially alter subcortical disturbed balance between dopaminergic and glutamatergic systems characteristic for schizophrenia. This shift in neurotransmitter balance will then produce stimulation of GABAergic outflow either by NMDA receptor-mediated stimulation or by suspension D_2 receptor-mediated inhibition of GABAergic neurons or both.

The most surprising effect of simultaneous administration of Org-24461 and risperidone was an elevation in extracellular glutamate concentrations in striatum of conscious rats. This effect was unexpected as neither Org-24461 nor risperidone altered glutamate concentrations in striatum when they were added separately in the doses we used. Although the mechanism how extracellular glutamate concentrations in striatum are increased after combined drug administration is not clear it is possible to draw some speculations. Besides GlyT-1, which is expressed in astroglial cells [42] and glutamatergic synapses [43, 44], other tranporters like System A or glycine heterotransporter-2 (GlyT-2) are also involved in glycine transport [45, 46]. These transporters may have particular importance in regulation of synaptic glycine concentrations when GlyT-1 is blocked by specific inhibitors. Selective GlyT-1 inhibitors like NFPS exhibit only low affinity interaction with System A-mediated glycine transport and they are almost completely ineffective in inhibition of GlyT-2 activity [45, 47]. The dual involvement of GlyT-1 and GlyT-2 in glycine-induced glutamate efflux was demonstrated by using confocal microscopy experiments in the spinal cord and by direct release measurements in rat cerebral cortex synaptosomes [46, 48]. These findings suggest that GlyT-1/GlyT-2 may also be operative in glycine transport at glutamatergic corticostriatal nerve endings. Following GlyT-1 inhibition, elevated synaptic glycine still can be taken up by transporters capable of carrying glycine into glutamatergic axon terminals inducing glutamate efflux from cytoplasmic pools by reversal of glutamate transporter operation. This was suggested by Raiteri and coworkers [46] who demonstrated that [^{3}H]aspartate release evoked by glycine was reduced in part by the non-transportable glutamate transporter inhibitor D,L-TBAO. Our results also suggest that glycine-induced glutamate release in the presence of a GlyT-1 inhibitor may be strengthened when glutamatergic axon terminals are disinhibited by simultaneous blockade of the inhibitory D_2 receptors by antipsychotics. In striatum, reciprocal innervation between dopaminergic and glutamatergic axon terminals occurs regulating dopamine and glutamate release by NMDA and D_2 dopamine receptors, respectively [49]. Thus, risperidon blocking D_2 dopamine receptors located on glutamatergic axon terminals and the concomitantly occurring GlyT-1 inhibition by Org-24461 may lead to elevation in striatal glutamate release observed after combined drug administration.

In conclusion, we have found here that combined treatment with GlyT-1 inhibitors and antipsychotics may beneficially influence disturbed dopaminergic/glutamatergic balance in schizophrenia by reducing dopamine efflux accompanied with elevated release of glycine and glutamate in the striatum. These findings suggest an efficient modulation of neurotransmitter dysregulation in schizophrenia when two different antipsychotics, the GlyT-1 inhibitors and the atypical antischizophrenic drugs, are added in combination. Clinical findings suggest the importance of this type of drug combination in the therapy as substitutional administration of the GlyT-1 inhibitor sarcosine to patients treated with risperidone led to beneficial alteration of positive and negative symptoms as well as cognitive deficit and general well-being of psychotic patients [22]. Sarcosine-derived GlyT-1 inhibitors, like Org-24461, have been shown, however, to suffer from a range of deleterious side effects including ataxia, hypoactivity, and respiratory depression [50]. Because of these adverse effects [51], several non-sarcosine-based GlyT-1 inhibitors have been synthesized and studied. Of these series of GlyT-1 inhibitors, compound RG1678 has recently been tested successfully in a phase II clinical study [52]. Our results suggest that combined administration of GlyT-1 inhibitors with antipsychotic drugs, which strengthens glutamatergic tone in striatum, may open a novel route to influence psychotic symptoms and cognitive deficit in schizophrenia [53, 54]. It is important to point out when drug combination is used that, unlike treatment with risperidone or other antipsychotics, adjuvant therapy with the GlyT-1 inhibitor sarcosine or the direct glycine_B binding site agonists glycine or cycloserine did not improve psychotic symptoms in patients receiving clozapine treatment [55–57]. It has been suggested that clozapine may directly activate glycine recognition site and/or NMDA-mediated neurotransmission pointing further out the importance of glutamate and glycine interaction in schizophrenia.

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