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Chlorpromazine, clozapine and olanzapine inhibit anionic amino acid transport in cultured human fibroblasts

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Summary. We report here that chlorpromazine, a first generation antipsychotic drug, inhibits anionic amino acid transport mediated by system X^-_{AG} (EAAT transporters) in cultured human fibroblasts. With 30 μM chlorpromazine, transport inhibition is detectable after 3 h of treatment, maximal after 48 h (>60%), and referable to a decrease in V_{max}. Chlorpromazine effect is not dependent upon changes of membrane potential and is selective for system X^-_{AG} since transport systems A and y^+ are not affected. Among antipsychotic drugs, the inhibitory effect of chlorpromazine is shared by two dibenzodiazepines, clozapine and olanzapine, while other compounds, such as risperidon, zuclopentixol, sertindol and haloperidol, are not effective. Transport inhibition by clozapine and olanzapine, but not by chlorpromazine, is reversible, suggesting that the mechanisms involved are distinct. These results indicate that a subset of antipsychotic drugs inhibits EAAT transporters in non-nervous tissues and prompt further investigation on possible alterations of glutamate transport in peripheral tissues of schizophrenic patients.

Keywords: Transport – Glutamate – Chlorpromazine – Glutamine – Olanzapine – Clozapine

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's Balanced Salt Solution; FBS, fetal bovine serum; MeAIB, methylaminoisobutyric acid; TPP^+, tetraphenylphosphonium

Introduction

The high affinity, sodium dependent transporters for aspartate and glutamate have been grouped in a family (EAAT, Excitatory Amino Acid Transporters) whose members are endowed with peculiar kinetic and operational features (Kanai and Hediger, 2004). Although some of these transporters are predominantly expressed in the brain, others are expressed also in non-nervous cells. In particular, human fibroblasts are endowed with a system for high affinity glutamate transport, named years ago system X^-_{AG} (Dall'Asta et al., 1983; Gazzola et al., 1981), which is characterized by the operational features of EAAT transporters, such as strict sodium- and potential-dependence (Bussolati et al., 1986) and reactivity towards D-aspartate but not D-glutamate (Gazzola et al., 1981). The presence of EAAT1, EAAT2 and EAAT3, indeed, has been documented in cultured human fibroblasts (Cooper et al., 1998; Zoia et al., 2005). These three transporters present overlapping substrate specificities and similar operational features, although EAAT2, a transporter predominantly expressed in glial models, is peculiarly sensitive to the inhibition by dihydrokainate and kainate (Kanai and Hediger, 2004).

Although the physiologic role played by high affinity anionic amino acid transporters in mesenchymal cells is not yet clear, their regulative features appear similar to those described in nervous models. For example, PKC-dependent regulation of EAAT activity, firstly described in cultured human fibroblasts (Franchi-Gazzola et al., 1990; Franchi-Gazzola et al., 1996), has been thoroughly characterized in glial cell models (Casado et al., 1993; Casado et al., 1991; Dowd and Robinson, 1996). Recently, the interest for these membrane functions in non-nervous cells has been renewed since their alterations seem correlated to pathological states of central nervous system (Tremolizzo et al., 2004).

Since EAAT transporters perform an anionic amino acid transport coupled to fluxes of Na^+, K^+, and H^+ (Zerangue and Kavanaugh, 1996), they build up very steep transmembrane gradients of substrates. As a result, in cultured human fibroblasts as well as in other mesenchymal cells, aspartate and glutamate reach very high intracellular concentrations (Dall'Asta et al., 1994). Providing high intracellular

levels of glutamate, the activity of system X^-_{AG} may be required for the intracellular synthesis of glutamine, at least when cells are starved of the neutral amino acid (McDermott and Butler, 1993; Uggeri et al., 1995).

The increasing evidence for derangements in glutamatergic transmission in the pathogenesis of schizophrenia (see Goff and Coyle, 2001; Tsai and Coyle, 2002 for review) has prompted several investigations on the relationships between this condition and alterations of EAAT expression and/or activity. In particular, the expression of the glial EAAT2 transporter has been found down regulated by the atypical antipsychotic drug clozapine (Melone et al., 2001, 2003). More recently, further studies from the same group have demonstrated that clozapine down regulates EAAT2 expression in astrocytic cultures (Vallejo-Illarramendi et al., 2005) and that the expression of the transporter is increased in the prefrontal cortex of schizophrenics (Matute et al., 2005). In contrast, no information is yet available about changes in anionic amino acid transport caused by antipsychotic drugs in non-nervous cell models. In this report we investigated the effects of antipsychotic drugs on EAAT activity in cultured human fibroblasts and found that three compounds, chlorpromazine, clozapine, and olanzapine, inhibit high affinity anionic amino acid transport in these cells.

Materials and methods

Human foreskin fibroblasts, derived from a 15-year-old donor, were seeded into 2-cm^2 wells of disposable 24-well multidish trays (Falcon) and grown for 3 d in DMEM, supplemented with 10% FBS. Cells were then incubated for 7 days at 1% FBS to reduce the interference of cell proliferation on the activity of system X^-_{AG} (Bussolati et al., 1993). For the experiments, culture medium was replaced with fresh medium (at 1% FBS) with or without the drugs, added from 100 × stock solutions in ethanol. Ethanol concentration was the same in treated and control cells. Glutamate and aspartate, absent in DMEM, are present in serum and in 1%-FBS-supplemented medium their concentrations are, respectively, 10 and 0.5 μM (V. Dall'Asta, unpublished observation).

The transport activity of system X^-_{AG} was evaluated by measuring the influx of the specific substrate L-aspartate (Dall'Asta et al., 1983). Unpublished work from our laboratory ascertained that the operational features of aspartate transport are similar in several dermal fibroblast strains from distinct donors. Unless otherwise stated, cells were incubated for 1 min at 37 °C in EBSS supplemented with L-Asp (10 μM, 3 μCi/ml). At the end of the assay period cells were rapidly washed three times with ice-cold urea (300 mM in water) and extracted in ethanol. The extracts were then added to scintillation fluid and counted for radioactivity with a Wallac Trilux spectrometer (Perkin-Elmer, Wellesley, MA, USA) while cell proteins were measured in the multiwell tray with a modified Lowry procedure (Dall'Asta et al., 1994).

For the kinetic analysis of system X^-_{AG} activity, L-Asp influx data, obtained at different concentrations of the amino acid, were fit to the equation

$$v = \frac{V_{max} \cdot [S]}{Km + [S]} + K_D \cdot [S] \quad (1)$$

Activity assays of amino acid transport systems y^+ and A were performed by using L-arginine and MeAIB as, respectively, preferential or specific substrates (Bussolati et al., 1993).

The intracellular content of the single amino acid species was determined by HPLC analysis with ion exchange chromatography. Cell monolayers were extracted in a 5%-solution of acetic acid in ethanol. The chromatographic analysis of the extracts was carried on with a Biochrom 20 Amino Acid Analyzer (Amersham Pharmacia Biotech) employing a High Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Amersham Biosciences, Little Chanfont, UK) for elution. Cell contents of amino acids are expressed as nmol/mg of protein.

Membrane potential was qualitatively assessed through the initial influx of the lipophilic cation tetraphenylphosphonium (Kimmich et al., 1985).

Serum and culture medium have been obtained from Celbio. [1-^{14}C]-2-Methylaminoisobutyric acid (MeAIB) and L-[2,3-^{3}H]arginine were purchased from Perkin-Elmer. L-[2,3-^{3}H]Aspartate and tetra[^{3}H]phenylphosphonium (TPP^+) bromide were obtained from Amersham Biosciences. Olanzapine was obtained from Eli Lilly (Indianapolis, IN, USA), clozapine from Novartis Farma SpA (Varese, Italy), risperidone and haloperidol from Janssen-Cilag SpA (Milan, Italy), chlorpromazine from Sanofi-Aventis (Milan, Italy), zuclopentixol and sertindol from H. Lundbeck A/S (Copenhagen, Denmark). The source of all the other chemicals was Sigma.

Results

Under the conditions adopted, L-aspartate influx was linear for 90 s in cultured human fibroblasts (Fig. 1A). Preincubation with chlorpromazine severely inhibited the transport of the anionic amino acid. In the representative experiment shown in Fig. 1B, aspartate influx was measured at different times after the addition of chlorpromazine to the culture medium. The uptake of the anionic amino acid decreased progressively in the presence of the drug. After a 3 h incubation transport was inhibited by 30% and, after 48 h of continuous exposure to chlorpromazine, the percentage inhibition of aspartate influx was more than 60%. No inhibition was observed if chlorpromazine was added to the incubation medium only during the assay period, thus excluding a direct interaction of the drug with the transporter (not shown). Dose-dependence analysis of chlorpromazine inhibition on system X^-_{AG} activity pointed to an ID_{50} between 5 and 10 μM (not shown).

Evidence from other studies (Okada et al., 1987) indicates that chlorpromazine may change membrane potential of cultured fibroblasts. Therefore, we decided to perform an experiment to exclude that inhibition of aspartate transport by chlorpromazine was due to changes in membrane potential induced by the drug. To this purpose, membrane potential was clamped through an incubation at high extracellular potassium (25 mM) in the presence of valinomycin (Bussolati et al., 1989). Under these conditions, membrane potential was not changed by chlorpromazine, as demonstrated by the comparable influx values of the potentiometric probe TPP^+ (Kimmich et al., 1985) obtained in

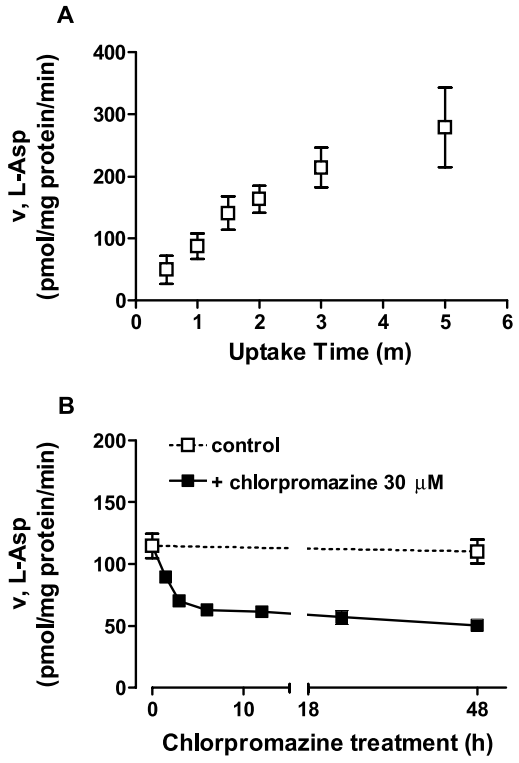

Fig. 1. A Time-course of L-aspartate influx in cultured human fibroblasts. Cultured human fibroblasts were incubated for 7 d in DMEM supplemented with 1% FBS. After this period, influx of 10 μM L-aspartate was determined for the indicated uptake times. Data are means of four independent determinations with S.D. indicated. **B** Effect of chlorpromazine on L-aspartate influx in cultured human fibroblasts. Cultured human fibroblasts were incubated for 7 d in DMEM supplemented with 1% FBS. After this period medium was substituted with fresh medium supplemented with 30 μM chlorpromazine in ethanol or with ethanol alone (control). Influx of L-aspartate was determined at the indicated times of treatment. Data are means of six independent determinations with S.D. indicated when greater of the size of the point

the absence or in the presence of the drug (Table 1). Nevertheless, chlorpromazine still significantly inhibited aspartate transport, indicating that the inhibitory effect was independent from changes in membrane potential.

Table 1. Effect of chlorpromazine on L-aspartate and TPP^+ influx

	$pmol \cdot mg^{-1} \cdot min^{-1}$	
	TPP^+ influx	L-Asp influx
Control	332 ± 29.1	145 ± 11.6
Chlorpromazine 3 h	352 ± 38.7	92 ± 9.5
Chlorpromazine 48 h	339 ± 36.3	61 ± 12.9

Influx of TPP^+ (20 μM, 4 μCi/ml) and of L-Asp (10 μM, 4 μCi/ml) were determined in parallel cultures during, respectively, 20 s and 1 min of incubation in a modified EBSS containing 25 mM K^+ and 100 μM valinomycin. The ionophore had been added 30 min before the assay. Data are means of four independent determinations shown ± S.D.

The activity of other transport systems for amino acids was also measured in chlorpromazine-treated fibroblasts (Fig. 2). The sodium independent influx of L-arginine, a

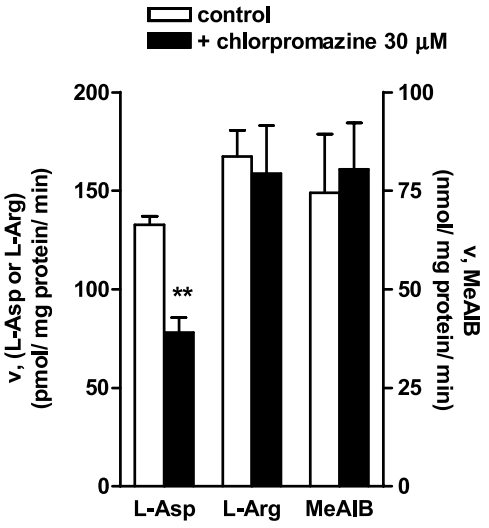

Fig. 2. Effect of chlorpromazine on the influx of aspartate, arginine, and MeAIB in cultured human fibroblasts. Cultured human fibroblasts were incubated for 7 d in DMEM supplemented with 1% FBS. After this period medium was substituted with fresh medium supplemented with 30 μM chlorpromazine in ethanol or with ethanol alone (control). Uptakes of aspartate (10 μM, 3 μCi/ml, 1 min), arginine (20 μM, 2 μCi/ml, 30 s), and MeAIB (100 μM, 1 μCi/ml, 1 min) were determined as described under Materials and methods. Data are means of four independent determinations with S.D. indicated. $^{**}p < 0.01$ vs. control, untreated cells

Table 2. Effect of chlorpromazine on the intracellular amino acid pool

Amino acid	Cell content (nmol/mg of protein)		Change (%)
	Control	+ Chlorpromazine 30 μM	
Asp	74	39	−41
Thr	73	67	−8
Ser	22	22	0
Glu	188	135	−22
Gln	219	191	−13
Pro	35	17	−51
Gly	97	100	+3
Val	12	22	+83
Ile	11	21	+91
Leu	15	23	+53
Tyr	9	15	+67
Phe	9	14	+56
Lys	6	9	+50
His	4	8	+100
Arg	14	18	+29

Cells were incubated for 48 h in the absence or in the presence of 30 μM chlorpromazine. At the end of the incubation, the cell content of amino acids was measured through HPLC as described in Materials and methods. The contents of other amino acids, not indicated here, were below 2 nmol/mg of protein. The experiment was repeated twice with comparable results

preferential substrate of the membrane potential sensitive, sodium independent transport system y^+ for cationic amino acids (Dall'Asta et al., 2000), was substantially unchanged by the drug. Also the influx of MeAIB, the characterizing transport substrate of the membrane potential sensitive, sodium-dependent system A, was not significantly affected by the treatment.

The analysis of the intracellular amino acid pool in control and chlorpromazine-treated cells is shown in Table 2. The major components of the pool were glutamine, glycine, and glutamate. Under the conditions of maximal transport inhibition (incubation in the presence of 30 μM chlorpromazine for 48 h), the intracellular pools of glutamate and aspartate were markedly decreased. The cell contents of glutamine and proline were also lowered, while other neutral and cationic amino acids were not affected by the treatment or even higher in chlorpromazine-treated cells.

Several experiments were performed to test the effects of other compounds used as antipsychotic drugs (Fig. 3). No change in aspartate transport was detected after 48 h-incubations in the presence of risperidon, zuclopentixol, sertindol, or haloperidol (employed at a concentration of 100 μM). On the contrary, two dibenzodiazepines, clozapine and olanzapine, significantly inhibited L-aspartate influx at a concentration of 50 or 100 μM, respectively. Neither clozapine nor olanzapine significantly affected L-aspartate influx if added only during the transport assay (not shown).

The kinetic analysis of aspartate influx demonstrated that, in both control and drug-treated cells, the transport of the anionic amino acid occurred through a single, saturable component and a non saturable pathway, formally undistinguishable from diffusion in the range of concentrations employed. Table 3 reports the kinetic parameters of aspartate influx obtained in cells treated with chlorpromazine, clozapine, and olanzapine. While chlorpromazine and olanzapine only produced a significant decrease of transport V_{max}, cells treated with clozapine exhibited also a significant decrease of K_m, as well as a lowered diffusion constant K_D.

Table 3. Kinetic constants of L-aspartate influx. Effect of selected antipsychotic drugs

	V_{max} ($pmol \cdot mg^{-1} \cdot min^{-1}$)	K_m (μM)	K_D (min^{-1})
Control	265 ± 17.2	20.2 ± 2.64	0.329 ± 0.056
Chlorpromazine (30 μM)	140 ± 12.2	17.8 ± 3.27	0.327 ± 0.043
Clozapine (50 μM)	150 ± 9.0	10.4 ± 1.63	0.139 ± 0.034
Olanzapine (100 μM)	229 ± 11.6	22.9 ± 2.19	0.252 ± 0.040

Cells were incubated for 48 h in the absence (control) or in the presence of the indicated compounds. The parameters were obtained by fitting experimental data to Eq. (1) (see Materials and methods) and are shown ± S.E. In all cases R^2 was >0.950

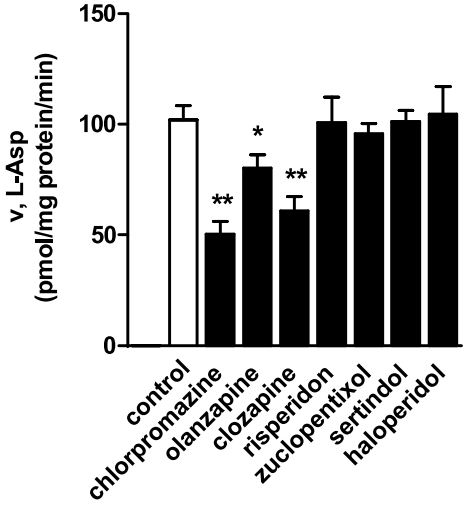

Fig. 3. Effect of antipsychotic drugs on the influx of aspartate in cultured human fibroblasts. Cultured human fibroblasts were incubated for 7 d in DMEM supplemented with 1% FBS. After this period medium was substituted with fresh medium supplemented with the indicated drugs at a concentration of 30 μM (chlorpromazine), 50 μM (clozapine) or 100 μM (all other compounds). The influx of aspartate was determined as described in Materials and methods. Data are means of four independent determinations with S.D. indicated. $^*p < 0.05$, $^{**}p < 0.01$ vs. control, untreated cells

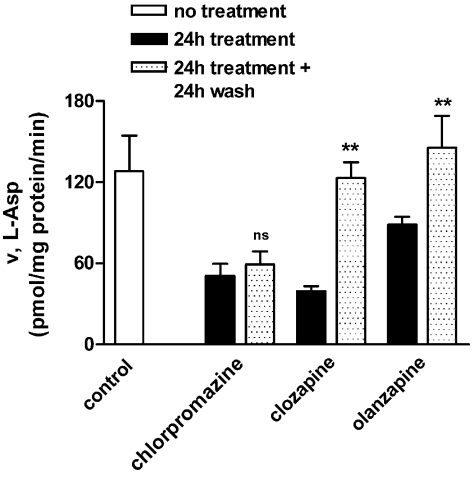

Fig. 4. Reversibility of the inhibition of system X^-_{AG} activity by antipsychotic drugs. After 24 h of incubation in the presence of 30 μM chlorpromazine, 50 μM clozapine, or 100 μM olanzapine, cells were washed twice in plain DMEM and then incubated in fresh medium (+1% fetal bovine serum) in the absence of drugs. Data are means of six independent determinations with S.D. indicated. $^{**}p < 0.01$ vs. cells treated with the indicated drug for 24 h

To assess the reversibility of the effect of the three compounds on the activity of system X^-_{AG}, cells were treated for 24 h with the inhibitors and then rescued for 24 h in their absence. The results (Fig. 4) show that, while chlorpromazine-treated cells exhibited a low aspartate influx even after the drug-free incubation, transport activities measured in cells pretreated with olanzapine or clozapine were not significantly different from control, untreated cells.

Discussion

Our results demonstrate that in cultured human fibroblasts anionic amino acid transport is substantially hindered after an incubation with chlorpromazine, a phenotiazine used as a first generation antipsychotic agent. The inhibitory effect of chlorpromazine is shared by clozapine and olanzapine, two dibenzodiazepines also used to treat schizophrenia, but not by other antipsychotic drugs. Several EAAT transporters, such as EAAT1, EAAT2. and EAAT3, are expressed in cultured human fibroblasts (Cooper et al., 1998; Zoia et al., 2005) and may, therefore, account for anionic amino acid transport in these cells. Preliminary results obtained in our laboratory indicate that, under the conditions employed, dihydrokainate inhibits aspartate transport by 30–40%, pointing to a significant contribution of EAAT2 (M. G. Bianchi, unpublished observation). However, further investigations will be needed to identify which EAAT transporter(s) is (are) inhibited by the antipsychotic agents in human fibroblasts.

The concentrations of the compounds used in this study are much higher than their therapeutic plasma levels. However, it should be considered that the exposure to these lipophilic drugs may last for months and years. Moreover, after treatment interruption, drug metabolites are excreted for several weeks, indicating a significant drug accumulation in the organism that, however, may not be homogeneous. For instance, phenotiazines, the group that includes chlorpromazine, attain levels significantly higher in brain than in plasma and are much more concentrated in neurons than in glial cells (Daniel et al., 2001). Although these considerations suggest that drug concentrations in some intracellular compartment may be much higher than the therapeutic levels detected in organic fluids, the evaluation of the biological significance of the results presented here will require their extension to other cell models as well as a careful assessment of the experimental conditions adopted.

Chlorpromazine, clozapine, and olanzapine produce a clear-cut decrease in transport V_{max}, whereas only clozapine also alters the other kinetic parameters of anionic amino acid transport. Thus, the mechanisms involved in transport inhibition appear distinct and clozapine may have peculiar effects. Distinct mechanisms of action are also suggested by the observation that, while clozapine and olanzapine effects are fully reversible, inhibition by chlorpromazine persists notwithstanding a drug-free incubation. However, a competition between the three drugs and anionic amino acids for the transporter is excluded for all the three drugs by the results of the kinetic analysis. A direct inhibitory interaction with the transporter should be also excluded because the three drugs do not inhibit aspartate transport if present only during the assay. Moreover, at least for chlorpromazine, the results obtained are also not compatible with an effect due to interference with membrane potential or ion gradients, since the drug does not substantially affect either the sodium dependent and membrane potential sensitive system A or the sodium independent system y^+ (Bussolati et al., 1989; Dall'Asta et al., 1991). Consistently, the effect of chlorpromazine on aspartate transport was still clearly detectable (see Table 1) when membrane potential had been clamped through an incubation with high extracellular potassium and valinomycin, as demonstrated by the comparable influx of the potentiometric probe TPP^+ (Bussolati et al., 1989; Kimmich et al., 1985) detected in the presence and in the absence of the drug.

The antipsychotic effects of chlorpromazine, an inhibitor of calmodulin known since many years (Levin and Weiss, 1976) have been usually referred to the interference with dopamine D2 receptors (see Kapur and Mamo (2003) for an historical review) while no experimental evidence is yet available about possible effects of the drug on EAAT activity and/or expression. However, it is known that the drug deeply affects membrane structure, as a prototype of the so-called cationic amphiphilic drugs, and produces complex effects on various membrane functions, such as the activity of Na^+, K^+, or Ca^{++} channels (Awayda et al., 2004; Kon et al., 1994; McNaughton et al., 2001), and clathrin-dependent endocytosis (Wang et al., 1993), thereby interfering with the trafficking of membrane proteins (Petris et al., 2003). Although there is no information about similar effects of olanzapine and clozapine, interference with transporter trafficking between membrane and intracellular compartments may be a mechanism worthy of further investigation, in light of recent data that imply EAAT recycling as an important control step in the regulation of anionic amino acid transport (Fournier et al., 2004; Guillet et al., 2005).

While this report constitutes the first investigation on the effect of olanzapine on anionic amino acid transport, recent contributions have demonstrated that EAAT2 expression and activity are inhibited by clozapine in rat central nervous system (Melone et al., 2001, 2003; Vallejo-Illarramendi et al., 2005). Inhibition of both EAAT2 and EAAT3 expression in rat brain were also described after chronic treatment with clozapine by another group (Schmitt et al., 2003). Consistently with the results presented here, haloperidol was found to be completely ineffective in that study (Schmitt et al., 2003).

The inhibition of high affinity transport of anionic amino acids by chlorpromazine produces selective alterations of the intracellular pool of amino acids. In particular, lowered cell contents were detected for the anionic amino acids aspartate and glutamate, as well as for the neutral amino acids glutamine and proline, both metabolically related to glutamate. These results confirm the metabolic role of EAAT transporters in the maintenance of the intracellular pool of glutamate and related amino acids in non-nervous cells (McDermott and Butler, 1993; Uggeri et al., 1995). Moreover, since alterations of EAAT activity in cultured human fibroblasts have been recently correlated with derangements of glutamate transport observed in neurological disorders (Tremolizzo et al., 2004; Zoia et al., 2005), these data should prompt further studies about EAAT expression and/or activity in peripheral, non-nervous cell models obtained from schizophrenic patients.

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