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ORIGINAL PAPER

Effects of atypical (risperidone) and typical (haloperidol) antipsychotic agents on astroglial functions

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Abstract Although classical and atypical antipsychotics may have different neurotoxic effects, their underlying mechanisms remain to be elucidated, especially regarding neuroglial function. In the present study, we compared the atypical antipsychotic risperidone (0.01–10 μM) with the typical antipsychotic haloperidol (0.01–10 μM) regarding different aspects such as glutamate uptake, glutamine synthetase (GS) activity, glutathione (GSH) content, and intracellular reactive oxygen species (ROS) production in C6 astroglial cells. Risperidone significantly increased glutamate uptake (up to 27%), GS activity (14%), and GSH content (up to 17%). In contrast, haloperidol was not able to change any of these glial functions. However, at concentration of 10 μM, haloperidol increased (12%) ROS production. Our data contribute to the clarification of different hypothesis concerning the putative neural responses after stimulus with different antipsychotics, and may establish important insights about how brain rewiring could be enhanced.

Keywords Astrocyte · Antipsychotics · Autism · C6 astroglial cells · Haloperidol · Risperidone

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Introduction

Schizophrenia is still one of the most mysterious and costliest mental disorders in terms of human suffering and societal expenditure. Once the diagnosis is made, antipsychotic drugs which block dopamine D2 receptors are the main treatment. However, during the last 10 years, a reevaluation of therapeutically strategies in schizophrenia has been emerging with the new introduction of atypical antipsychotics [6, 40]. In general, one could speculate that typical antipsychotic agents, such as haloperidol, may seem to be more effective in reducing positive symptoms but not particularly useful against negative ones and/or or cognitive deficits. On the other hand, atypical antipsychotic agents have gained popularity over typical antipsychotics due to their theoretical efficacy in controlling both positive and negative symptoms. However, this could vary within the drug of study. For instance, amisulpride has indeed shown a great versatility as an antipsychotic drug on both positive and negative symptoms [27].

Both antipsychotics risperidone and haloperidol act on multiple neurotransmitter receptors, although each drug could be characterized by its specific receptor-binding profile. Risperidone is an atypical second-generation antipsychotic available world-wide since early 1990s [16]. The main pharmacological activities of risperidone (9-hydroxyrisperidone, as the principal active metabolite) include serotonin 5-HT2 receptor blockade and dopamine D2 antagonism [16, 24]. Besides, haloperidol acts primarily through dopamine D2 receptors with lower activity at D1, D3, D4, 5-HT2A, and α1 adrenergic receptors [17].

Even though such individual characteristics may help us to understand some of the different therapeutic and side-effect profiles of both typical and atypical antipsychotics exerted on patients, there is still much to learn about

additional neural cells effects as well as the ones concerning neuroglial functions. Astrocytes, a subtype of glial cells in the central nervous system (CNS), are dynamic signaling elements that integrate neuronal inputs and exhibit calcium excitability. In addition, they are able to modulate neighboring neurons, neuronal survival, and neurite formation. They also take part in the blood brain barrier (BBB) and participate in the maintenance of glutathione levels in the brain [1, 37]. Emerging evidences indicate that signaling between perisynaptic astrocytes and neurons at the tripartite synapse plays an important role during the critical period when neural circuits are formed and refined [1, 37, 41]. An imbalance in glioneurotransmission in tripartite synapse was proposed as a model of the pathophysiology of schizophrenia [25].

Astrocytes express a wide variety of functional receptors for many different neurotransmitter such as glutamate, GABA, as well as adrenergic, purinergic, dopaminergic, and serotonergic receptors [26, 29]. Glutamate is the major excitatory transmitter in transferring information between neurons, but is now also known to activate astrocytes through different transporters and receptors. Glutamate released from presynaptic terminals is primarily transported to astrocytes, where it is converted into glutamine via the enzyme GS (EC 6.3.1.2) pathway. The glutamine is released back to the neurons, where glutamate is regenerated via phosphate-dependent glutaminase (EC:3.5.1.2), a mitochondrial enzyme [9], whereas, glutamate has another important fate in astrocytes, particularly in the synthesis of the tripeptide L-γ-glutamyl-L-cysteinyl-glycine or glutathione (GSH), [11]. Among neural cells, astrocytes are more resistant to oxidative stress and provide a protective role for neurons, mainly due to their higher GSH content. Hence, the cell's ability to reduce or synthesize GSH is an important key factor that may determine how the cell could manage oxidative stress-induced neurotoxicity [23]. Moreover, it has been described a reduced glial/neuronal ratio in patients with mood disorders [3]. However, how astrocytes contribute to brain function and behaviors associated with depression, schizophrenia, or anxiety is currently poorly understood.

The possible relationship between glial cells and antipsychotic drugs has been extensively speculated and studied [2, 13]. However, it still remained unclear which and to what extent was the specific effect exerted on glial cells upon neuroleptic treatment. In such direction, our laboratory has already reported how risperidone is able to modulate important glial functions [30, 31]. In the present comparative study, we further investigated the effects of two different antipsychotics, risperidone (atypical) and haloperidol (typical), concerning different aspects such as glutamate uptake, GS activity, and GSH levels in the C6 astroglial cell model. Moreover, we investigated the different effects of both antipsychotics on the formation of reactive oxygen species (ROS).

Experimental procedures

Materials

Risperidone (Risperdal®) and Haloperidol (Haldol®) were a gift from Janssen-Cilag (São Paulo, SP, Brazil) a Brazilian division of Johnson & Johnson. Standard GSH, *o*-phthaldialdehyde, γ-glutamylhydroxamate, DCF-DA, and cell culture materials were purchased from Sigma (St. Louis, MO, USA), except for Dulbecco's modified Eagle's medium (DMEM) which was purchased from Gibco BRL (Carlbad, CA, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil) and L-[^{3}H]Glutamate was purchased from Amersham International (UK). All other chemicals were purchased from local commercial suppliers.

Cell culture and risperidone treatment

The C6 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was cultured as previously described [10]. The C6 lineage cells are an astrocyte-like cell line that is widely used to study cellular functions such as glutamate uptake, glutamine synthetase (GS) activity, S100B secretion, and oxidative stress [7, 10, 30, 31].

The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% FBS, 2.5 mg/mL Fungizone® and 100 U/L gentamicin. Cells were kept at a temperature of 37°C in an atmosphere of 5% CO_2/95% air. Exponentially growing cells were detached from the culture flasks by using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and then seeded (5×10^3 cells/cm^2) in 24- or 6-well plates. When cells reached confluence, the culture medium was removed by suction, and the cells were incubated for 6 h at 37°C in an atmosphere of 5% CO_2/95% air in DMEM (pH 7.4) without serum in the absence (control) or presence of risperidone (0.01, 0.1, 1 and 10 μM) or haloperidol (0.01, 0.1, 1 and 10 μM). Control conditions were performed in the presence of an equivalent volume of risperidone/haloperidol vehicle (containing 0.2% benzoic acid and 0.75% tartaric acid). It is important to mention that, in all parameters analyzed, the results obtained with vehicle were not different from those obtained under basal conditions. Membrane integrity loss was determined by fluorescent image analysis of propidium iodide (PI) uptake [10]. Cells were incubated with 7.5 μM PI, concomitantly with treatments ($N = 3$, performed in triplicate). Images were acquired by digital camera (Sound Vision Inc., Wayland, MA, USA) at the end

of incubations, using a TE-FM Epi-Fluorescence accessory. Optical density was determined with Optiquant version 02.00 software (Packard Instrument Company). Density values obtained were expressed as density light units (DLU).

Glutamate uptake assay

Glutamate uptake was performed as previously described [12] with some modifications. Briefly, C6 glioma cells were incubated at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4{\cdot}7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.4. The assay was started by addition of 0.1 mM L-glutamate and 0.33 μCi/ml L-[2,3-^{3}H] glutamate. Incubation was stopped after 10 min by removing the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M of NaOH. Radioactivity was measured in a scintillation counter. Sodium independent uptake was determined by using *N*-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the total uptake in order to obtain the specific uptake. Values are expressed as nmol/mg protein/min.

Glutamine synthetase (GS) activity

The enzymatic assay was performed as previously described [10]. Briefly, homogenate (0.1 mL) was added to 0.1 mL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 10 ATP and incubated for 15 min at 37°C. The reaction was stopped by adding 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent. Values are expressed as nmoles of γ-glutamylhydroxamate/mg protein/min.

Glutathione (GSH) content assay

Glutathione levels (nmol/mg protein) were measured as previously described [4]. Cell homogenates were diluted in ten volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured by using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM).

Evaluation of intracellular ROS production

Intracellular ROS production was detected by using the nonfluorescent cell permeating compound, 2′–7′-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound, 2′–7′-dichlorofluorescein (DCFH). C6 cells were treated with DCF-DA (10 μM) for 30 min at 37°C. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max Gemini XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [31, 32]. Values were obtained as fluorescence units/mg protein and expressed as % of control.

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard [22].

Statistical analysis

Data represent means ± SE and were analyzed statistically by one-way ANOVA, followed by the Tukey test. Values of $P < 0.05$ were considered to be significant.

Results

Risperidone, but not haloperidol, increases glutamate uptake

As shown in Fig. 1, basal glutamate uptake was 0.33 ± 0.006 nmol/mg protein/min. Glutamate uptake was increased by risperidone: 10% at 0.1 μM (0.36 ± 0.007),

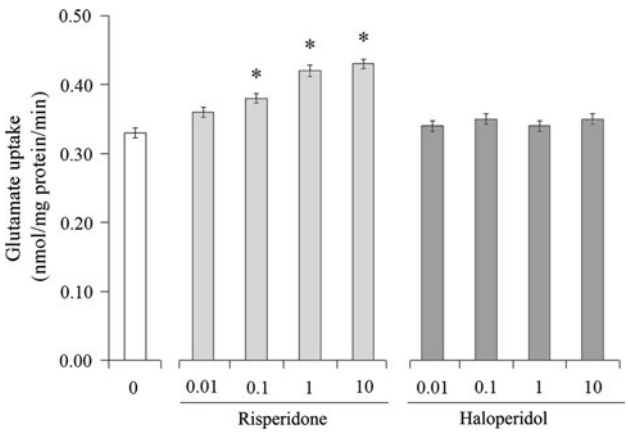

Fig. 1 Glutamate uptake in C6 astroglial cells in the presence or absence of risperidone and/or haloperidol. Cells were treated with risperidone or haloperidol for 6 h in DMEM without serum. Data represent means ± SE from three experimental determinations performed in triplicate. *Significantly different from control, $P < 0.05$

15% at 1 μM (0.38 ± 0.006) and 27% at 10 μM (0.42 ± 0.007), $P < 0.05$. In contrast, glutamate uptake was not affected by haloperidol. The concentration of haloperidol used in this and in other assays was not increased due to the impairment of membrane integrity observed at 100 μM haloperidol, as measured by PI uptake assay. At this concentration, there was a significant increase (18%) in PI uptake, from 0.22 ± 0.033 (control) to 0.26 ± 0.041 Gross DLU, $P < 0.05$. Concentrations of haloperidol up to 10 μM did not affect membrane integrity.

Risperidone, but not haloperidol, increases glutamine synthetase activity

Figure 2 shows the effect of risperidone and haloperidol on GS activity. Basal GS activity was increased by risperidone at 1 and 10 μM (14 and 18%, respectively), $P < 0.05$. However, haloperidol, did not affect the activity of such enzyme.

Risperidone, but not haloperidol, increases GSH content

Next, we examined the effects of risperidone and haloperidol on GSH content (Fig. 3). Risperidone increased the amount of GSH at concentrations of 0.1 (12%), 1 (19%) and 10 (58%) μM (from 14.4 ± 0.3 to 16.1 ± 0.32; 17.2 ± 0.42 to 22.7 ± 0.5 nmol/mg protein, respectively), $P < 0.05$. Haloperidol did not affect GSH content.

Haloperidol, but not risperidone, increases ROS production

Figure 4 shows the effect of risperidone and haloperidol on ROS production. As observed, 10 μM haloperidol increased DCFH oxidation (about 12%), $P < 0.05$. This parameter was not affected by risperidone.

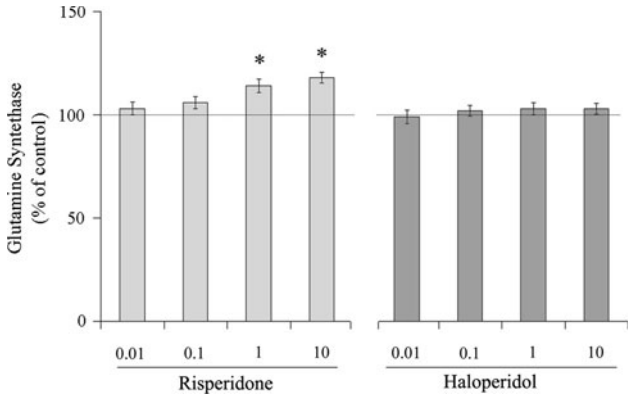

Fig. 2 Glutamine synthetase activity in C6 astroglial cells in the presence or absence of risperidone and/or haloperidol. Cells were treated with risperidone or haloperidol for 6 h in DMEM without serum. Data represent means ± SE from three experimental determinations performed in triplicate. *Significantly different from control, $P < 0.05$

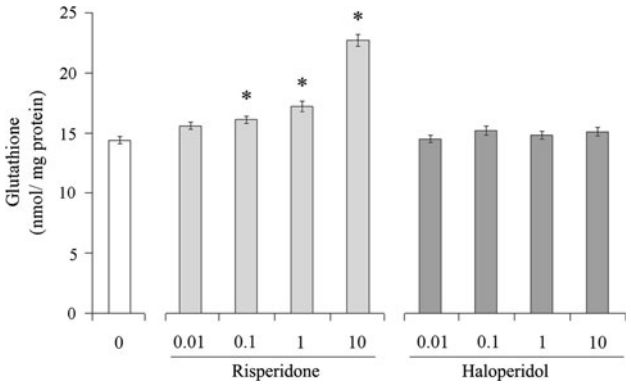

Fig. 3 Glutathione levels in C6 astroglial cells in the presence or absence of risperidone and/or haloperidol. Cells were treated with risperidone or haloperidol for 6 h in DMEM without serum. Data represent means ± SE from three experimental determinations performed in triplicate. *Significantly different from control, $P < 0.05$

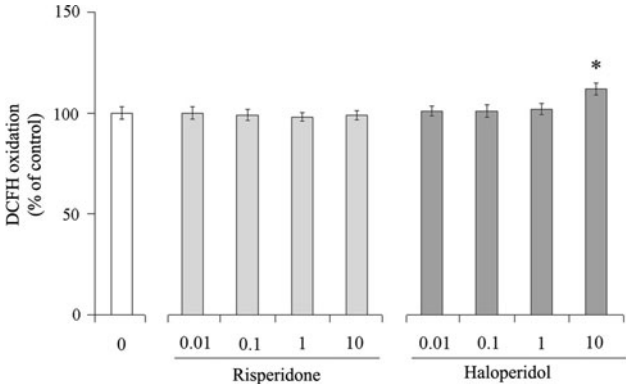

Fig. 4 Intracellular ROS production in C6 astroglial cells in the presence or absence of risperidone and/or haloperidol. Cells were treated with risperidone or haloperidol for 6 h in DMEM without serum. Data represent means ± SE from three experimental determinations performed in triplicate. *Significantly different from control, $P < 0.05$

Discussion

The use of atypical antipsychotics has recently emerged as an effective strategy in treatment-resistant mood and anxiety disorders [18, 28]. Although some of the atypical antipsychotics could be considered as the first-line approach for patients with treatment-resistant bipolar disorder and obsessive–compulsive disorder, there is still paucity in terms of evidence-based studies from a clinical point of view. A recently published meta-analysis comparing first with second generation antipsychotics [19] showed that some, but not all, of the second generation

compounds are superior over the first one, in terms of positive or negative symptoms-related improvement.

Conventional antipsychotic-associated neurotoxicity became one of the principal goals to improve within drug development. In fact, atypical antipsychotics such as risperidone, do not present mentioned sides effects as it was previously described [39]. This study pointed that haloperidol-induced neurotoxicity results in inhibition of survival-associated pathways such Akt and significant activation of caspase-3 apoptotic-mediated signals, in comparison to risperidone, which did not show similar capacity to injure neurons. In this context, our work contributes to the understanding of the putative antipsychotic effects on astroglial cells, especially related to glutamate function, since astrocytes are intimately associated with glutamatergic transmission, synaptic plasticity, and neuroprotection [1, 8, 9].

We previously reported that risperidone was able to modulate glutamate metabolism by astroglial cells, suggesting that risperidone might exert neuroprotective effects against brain disorders, and such effects may also involve modulation of astrocyte functions. In the present study, we compared the effects of risperidone (0.01–10 μM) and haloperidol (0.01–10 μM) regarding to different astroglial parameters to better understand the mechanisms by which antipsychotics may function in astrocytes. Despite the fact that our in vitro data demonstrated a relevant modulation of astroglial functions upon risperidone treatment and therefore, positive neuroprotective effects in oxidative stress and/or excitoxicity-associated disorders, some relevant limitations should be under discussion. First of all, we do not know yet whether those results are cell line-dependent or on the contrary, they could be relay on other neural cell contexts. In addition, mean risperidone and haloperidol concentrations in humans are, respectively, around 0.05 and 0.03 μM in plasma [14] and around 0.01 and 0.5 μM in brain tissue [42]. However, during in vitro experiments, drug concentrations need to be usually higher in order to mimic same long-term physiological cell activities and their functions. In our previous work, on C6 astroglial cell model [30], we investigated the effect of 10 μM risperidone as previous studies performed to determine dopamine-sensitivity in primary astroglial cultures [33]. Indeed, in vitro studies remain a powerful tool for generation of a new hypothesis that could be then studied in vivo and later, generalized to the human scenario. Since animal models are necessary to further investigate our in vitro findings and to study the relationship between biological, biochemical and behavioral variables within environmental factors, it would remain to be done a number of in vivo studies that may confirm our current results.

Another important aspect to be discussed is the oxidative stress implicated in the pathogenesis of diverse disease states, which may be a common pathogenic mechanism underlying many major psychiatric disorders, as the brain has comparatively greater vulnerability to oxidative damage. This could partly be caused by alterations in dopaminergic and glutamatergic activity that are implicated in these illnesses [35]. Glutamate and dopamine are highly redox reactive molecules and produce ROS during normal neurotransmission. Alterations to these neurotransmitter pathways may therefore increase the oxidative burden in the brain [35].

In addition, oxidative stress associated with typical antipsychotic treatment has been reported as one of the mechanisms involved in the pathogenesis of extrapyramidal side effects [28]. Extensive evidence indicates that an unbalanced production of free radicals is associated with chronic haloperidol use and might contribute to the onset of tardive dyskinesia and cell damage [15, 28]. This effect can be related, at least in part, to a reduction in specific endogenous antioxidant mechanisms, such as a decrease in GSH levels [34, 35]. Therefore, the observed in vitro effect of risperidone on the GSH content could indicate a new possible role for the reported in vivo effect of this antipsychotic. These reports are consistent with the haloperidol-induced ROS production showed in the present study, indicating that such antipsychotic may induce a redox imbalance, followed by ROS production. In addition, both in vitro and in vivo studies have demonstrated that several atypical antipsychotics possess a capacity to protect neuronal cells from a variety of pathological insults [15].

Typical antipsychotics, can partially solve not only the neurotransmitter dysfunction but also aggravate oxidative stress expanding brain damage in schizophrenic patients. Some studies have reported more gray matter loss in patients treated with haloperidol than atypical antipsychotics [21]. In fact, our data shows that risperidone was able to induce a significant increase in glutamate uptake, GS activity, and GSH content. In contrast, haloperidol was not able to change any of these glial functions even at higher doses. Our data contribute to the concept that atypical antipsychotics are additionally neuroprotective compounds. Risperidone improved antioxidant defense (increment of glutathione) and decreased the oxidant activity of extracellular glutamate (increment of glutamate uptake). Therefore, this compound not only appears to improve neurotransmitter balance but also contributes to decrease oxidative stress, both underlying the brain damage of the schizophrenia [28]. Also, it is important to emphasize that this improvement is mediated by astrocytes assuming their key role in glutamate uptake and glutathione synthesis in central nervous system [9].

Our data are in agreement with other studies concerning enhanced effects of risperidone in comparison with haloperidol and their corresponding receptor affinity [20] as

well as regarding previously reported rotenone-induced neurotoxicity in PC12 cells [38]. Further investigations in different models still remain necessary. Immunohistochemical analysis of brain slices and astrocytic/neuronal co-cultures, in order to either confirm or discard the effect of haloperidol on glutamate metabolism, seem to be the logical next steps.

It is commonly accepted that the role of glutamatergic system in the development of schizophrenia and indeed, atypical neuroleptics function through affecting such system, at least to a minor extent. In fact, it was already described relevant changes on glutamine synthetase-like protein (GSLP) upon treatment with atypical neuroleptic olanzapine, in the platelets of chronic schizophrenia patients [5]. Moreover, survival analysis study of olanzapine 28-week-treated patients showed that the higher levels of GSLP measure in platelets before treatment the shorter time under treatment would be required to get a positive drug-induced response from a clinical point of view. Therefore, whether an increase in GS levels upon treatment with atypical neuroleptics could be taken as a positive sign or not might be a bit speculative because additional works showed no change of GS in cortical areas of schizophrenia patients, for instance [36]. However, since our present in vitro data performs a comparative study between both typical and atypical neuroleptics, an important increase in the expression/activity of glutamine synthetase by the tested atypical ones (among other compounds) could have significant importance at least in a circumstantial context.

Our data provide some evidences for delineating the mechanisms of atypical antipsychotics-induced protective actions, such as prevention of glutamate-mediated excitotoxicity by stimulating its astrocytic uptake and increasing the levels of GSH, which is an important non-enzymatic antioxidant for the CNS. These data contribute to the available knowledge regarding to neural responses after antipsychotic-induced stimulus, and it could give rise to important insights about how to promote brain rewiring in different psychiatric diseases. In this context, future studies are necessary not only for an improved comprehension about the direct relationship between astrocytes and antipsychotics-triggered mechanisms of action but also for the future development of novel potential strategies which could ameliorate the effects of disintegrative diseases in the developing brain.

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