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ORIGINAL ARTICLE

Effects of Isolation Rearing and Early Antipsychotic Intervention on Oxidative Stress-induced Apoptosis and Brain-derived Neurotrophic Factor in Hippocampus in a Rat Model of Schizophrenia

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Background: Oxidative stress-induced neuronal dysfunction has been considered an essential factor for the development of schizophrenia. However, a longitudinal and causal relation between the impacts of developmental stress and oxidative stress remains unsolved. The present study aimed to examine whether the oxidative stress-relevant dysfunctions of the apoptotic index can be induced in rats of isolation rearing (IR, a rodent model of schizophrenia) and to see if the intervention of antipsychotics can reverse these dysfunctions. **Materials and Methods:** Pharmacological manipulation (risperidone [RIS] [1 mg/kg/day], olanzapine [OLA] [2.5 mg/kg/day], or saline [SAL] vehicle) was introduced 4 weeks (adolescence) or 8 weeks (young adulthood) after IR (i.e., rats were 7- or 11-week-old). The regime of RIS, OLA, or SAL was continued for 9 weeks. Locomotor activity was employed to validate the IR effect. Rats' hippocampus immediately after sacrifice was removed to measure messenger RNA expression of Bax, Bcl-2, brain-derived neurotrophic factor (BDNF) and the plasma level of nitric oxide (NO). **Results:** The results showed: (i) IR rats were more hyperactive. (ii) RIS may exert anti-apoptotic effects on IR rats, particularly at their adolescent age (as indexed by increased Bcl-2 and decreased Bax/Bcl-2 ratio). (iii) The therapeutic potential of RIS can be also observed in the change of BDNF in an age-independent manner, in which RIS effectively increased the BDNF level in IR but not social (SOC) rats. (iv) Plasma NO was not altered. **Conclusion:** The study results support the utility of the IR paradigm in exploring mental disorders with neurodevelopmental origin in which early pharmacological intervention may provide a therapeutic benefit in the overloaded oxidative stress and the dysfunction of BDNF.

Key words: Brain-derived neurotrophic factor, early antipsychotic intervention, isolation rearing, oxidative stress, schizophrenia

INTRODUCTION

Schizophrenia is a multidimensional mental disorder in which patients suffer from progressively psychosocial impairment and cognitive decline, including distortions of thought and perception (positive symptoms) and lack of motivation or spirit (negative symptoms) across time in a deteriorating manner.[1] Although the pathoetiology of this disorder remains unclear, oxidative stress-induced neuronal dysfunction has been considered a key factor.[2,3]

Oxidative stress indicates an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the antioxidant defenses (i.e., an ability of the biological system to repair the damage caused by ROS).[4] Excessive oxidative stress may finally lead to cellular apoptosis which is regulated by, for example, the Bcl-2 family in which Bcl-2 is anti-apoptotic but Bax is pro-apoptotic.[5] Recently, increasing evidence suggests a relevance of oxidative stress and mental illness with long-term environmental or social distress impact.[6] This is important because it exemplifies the possibility that life stress can exert its effects on the events at the cellular level.

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On the other hand, brain-derived neurotrophic factor (BDNF) is a neurotrophic factor abundant in brain, responsible for regulating the events of neurotransmission, such as differentiation, growth, and plasticity.[7] Its role in the pathogenesis of schizophrenia has been addressed recently in terms of the interplay with oxidative stress,[8] however, elaboration on this hypothesis by performing studies with large sample sized in humans appears difficult due to ethical reasons, for example, it is difficult to separate these effects from the influence of antipsychotics because patients are seldom drug naive. A longitudinal and causal relation between the impacts of developmental stress and antipsychotics thus need to be clarified by using an appropriate animal model.

Rat of isolation rearing (IR) is a commonly used method in which weanling rats are increased in social separation. Generally, they are allowed to see, smell, and hear other rats but not to have social contact with them. The IR rats exhibit profound psychological, behavioral, and neurochemical changes when they grow up,[9] similar to the full-blown symptoms of schizophrenia in patients' adulthood. IR rats are thus suitable for modeling mental disorders with a pathoetiology based on developmental hypothesis,[10] such as schizophrenia. The hallmark features of IR-induced behavioral changes are locomotor hyperactivity and a defect in sensorimotor gating function and are is particularly useful in examining the pathogenic/therapeutic mechanism of schizophrenia.[11,12] Here, if the neurodevelopmental process has a crucial impact on the oxidative stress-related abnormalities in schizophrenia, it is expected that these abnormalities may be revealed in rats reared in social isolation (i.e., IR rats), and pharmacological interventions at an early stage of IR might reverse the dysfunctions. For the pharmacological interventions, the treatment of schizophrenia has been greatly improved with the extensive use of second-generation antipsychotics (SGAs) in recent years. It helps schizophrenic patients by relieving both their positive and negative symptoms with a lower risk of extrapyramidal side effects.[13]

In the present study, we examined the IR effects on oxidative stress-relevant dysfunctions of apoptotic index and BDNF, and to see if clinical antipsychotics may reverse these dysfunctions. For these, we employed longitudinal regimes of two commonly used SGAs, risperidone (RIS) and olanzapine (OLA). IR rats in adolescence (7-week-old) or young adulthood (11-week-old) were randomly entered into a drug protocol (RIS [1 mg/kg/day] or OLA [2.5 mg/kg/day] for 9 weeks) to examine the IR and drug effects on the Bcl-2 and Bax and the BDNF in hippocampus. Hippocampal neurogenesis has recently been considered to play an important role in the development of schizophrenia, particularly in terms of receiving a stressful or traumatic impact early in life,[14-16] such as the long-term environmentally stressful situation in rats of IR.[17] Finally, as nitric oxide (NO) may act in a positive feedback loop with BDNF to regulate synaptic plasticity,[18] but in an opposite manner as antioxidants do (schizophrenia is accompanied by increased NO but decreased antioxidant level),[19] the plasma level of NO would be measured in the present study too.

The result of the present study may help clarify the role of oxidative stress in IR-induced abnormalities and is of great relevance to the evidence-based clinical strategy that detecting and intervening in schizophrenia at an early stage may protect neurons from further deterioration and may lead to a better long-term outcome.[20-22]

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (BioLASCO, Taiwan) were used in all experiments. The rats were 21–23-day-old and had been weaned on arrival at the animal center of the National Defense Medical Center (NDMC, Taipei, Taiwan). Rats in the IR group were housed singly. They could see, hear, and smell others but were kept separate and denied physical contact. The control group for IR was a social rearing (SOC) group. These rats were from the same batch as the IR rats but were reared socially (n = 2 for each cage). All rats were housed in a temperature (21°C–25°C) and humidity (40%–60%) controlled holding facility with 12 h light/dark cycles (light on from 700 to 1900) and received food and water *ad libitum*. After weaning (3 weeks postnatal age), rats were randomly and equally assigned to the IR or SOC group. Pharmacological manipulation (RIS [1 mg/kg/day], OLA [2.5 mg/kg/day], or saline [SAL] vehicle) was introduced 4 weeks (adolescence) or 8 weeks (young adulthood) later (i.e., rats were 7- or 11-week-old). The regime of RIS, OLA, or SAL was continued for 9 weeks. Locomotor activity was employed to validate the IR effect. Immediately after completion of the final blood sampling, the rats were sacrificed, and their hippocampus was removed to measure messenger RNA (mRNA) expression of Bax, Bcl-2, and BDNF. All experimental procedures were approved by the Laboratory Animal Center from the NDMC (AAALAC full accreditation).

Locomotor activity

Locomotor activity was measured using a computerized automated activity monitoring system (MED Associates, Inc., St. Albans, VT, USA). The system included four plexiglass chambers (43 cm × 43 cm × 30 cm) equipped with an infrared array of 16 photodetectors and corresponding light sources that emitted photobeams 3 cm apart and 4.5 cm above the chamber floor. Travel distance was recorded at the assigned intervals and analyzed with Med-Associates software.

Messenger RNA expression of hippocampal Bax, Bcl-2, and brain-derived neurotrophic factor

Total RNA of rat hippocampus was extracted by according to manufacturer's protocol of EZ-RNA II total RNA isolation kit (Catalog No. 20-410-100, Biological Industries, USA). The quality and concentration of total RNA were determined by spectrophotometry (NanoDrop ND-1000® Spectrophotometer, Thermo Scientific). Subsequently, one microgram of total RNA was reverse-transcribed into complementary DNA using the RNA-to-Ct™ 1-Step Kit (One-Step Real-Time-Polymerase chain reaction [RT-PCR] Master Mixes, Thermo Scientific). Specific primers for rat Bax (Rn01480160_g1), Bcl-2 (Rn99999125_m1), BDNF (Rn02531967_s1) and GAPDH (Rn99999916_s1) were purchased from Applied Biosystems (USA) and to use for quantitative RT-PCR (7500 Real-Time PCR System, Applied Biosystems, USA). The reaction was incubated at 50°C for 2 min, 95°C for 10 min, followed by forty cycles of denature at 95°C for 15 s and annealing/extension at 60°C for 30 s. All mRNA quantities of target genes were analyzed in triplicates. GAPDH gene expression was used as an internal control standard for normalization and the SAL-treated group served as a calibrator sample for the comparative $2^{\Delta\Delta Ct}$ method.[23,24]

Nitric oxide

NO production was indexed by the amount of nitrite and nitrate. Plasma NO was measured in terms of nitrate/nitrite levels following adopted from methods of Green *et al*.[25] and Kalaivani *et al*.[26] In brief, plasma, SAL and 5-sulfosalicylic acid were added. The precipitate was removed by centrifugation at 10,000 rpm for 10 min, 4°C. Sample (80 µl), enzyme cofactor mixture (10 µl), and nitrate reductase mixture (10 µl) were added by sequence and placed for 3 h at room temperature. Griess reagents (50 µl R1 and 50 µl R2 were added by sequence). The mixture was then allowed to stand for 20 min. The absorption at 540 nm was measured in spectrophotometer (Thermo Scientific Multiskan, Scan Jose, CA, USA).

Drug

RIS and OLA (Sigma-Aldrich Co., LLC) were dissolved in 1 N acetic acid and titrated to a final pH of 7 with 1 N NaOH. Drugs were prepared to produce a total injection volume of 1.0 ml/kg and were injected subcutaneously for 9 weeks (RIS 1 mg/kg/day, OLA 2.5 mg/kg/day and SAL at the same injection volume). The dosages used in this study have previously been shown to recover prepulse inhibition (PPI) in rats.[27,28]

Statistical analyses

Statistical analyses were performed across the groups using a multi-factor analysis of variance using rearing condition, treatment, and timing as the between-subjects factor. Further analyses and *post hoc* multiple comparisons were performed if necessary. For brevity, *F*-values are only provided for significant effects. The value of $P < 0.05$ was considered statistically significant.

RESULTS

Locomotor activity

For locomotor activity at baseline, IR/older rats were more hyperactive. There were main effects of rearing condition ($F_{(1,60)} = 23.12$, $P = 0.000$) and intervention age ($F_{(1,60)} = 18.80$, $P = 0.000$) (adolescent IR: 7145 ± 402; adolescent SOC: 5710 ± 329; young adult IR: 9790 ± 442; young adult SOC: 7237 ± 385). Treatment with RIS or OLA had no effect on locomotor activity.

Bax, Bcl-2

Several indexes of apoptosis in the hippocampus were investigated with PCR. Our results revealed that for the SAL regime, lower Bax levels were found in adolescent rats (intervention age, $F_{(1,20)} = 5.58$, $P < 0.05$) which were due to a higher Bax level in young adult SOC rats. A higher Bcl-2 level was observed in rats treated with RIS (treatment, $F_{(1,40)} = 8.85$, $P < 0.01$), which was due to the higher Bax levels in both adolescent and young adult IR rats. Our results also showed that RIS but not OLA reduced the ratio of Bax/Bcl-2 in IR-adolescent rats (main effect of rearing condition, $F_{(1,20)} = 5.14$, $P < 0.05$, and effect of rearing condition × intervention age, $F_{(1,20)} = 4.76$, $P < 0.05$). *Post hoc* multiple comparisons confirmed that it was due to the difference between RIS/adolescent and SAL/adolescent rats [Figures 1 and 2].

Brain-derived neurotrophic factor

Our results showed that RIS and OLA can increase the BDNF level (treatment, $F_{(1,40)} = 12.6$, $P < 0.01$). RIS and OLA-treated IR rats had an overall higher BDNF level ($t_{(22)} = 3.84$, $P < 0.01$ for RIS and $t_{(22)} = 2.16$, $P < 0.05$ for OLA) [Figure 3].

Nitric oxide

There were no statistically significant effects of rearing condition, intervention age, and treatment in amount of plasma NO [Figure 4].

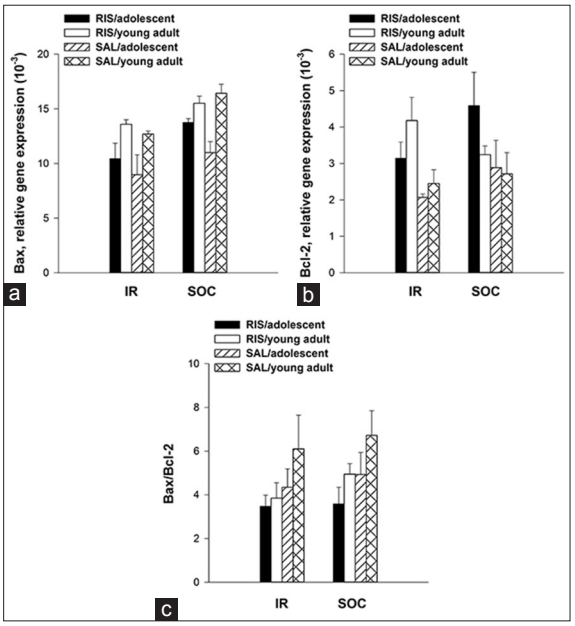

Figure 1: For saline regime, lower Bax levels appear in the adolescent rats which were due to a higher Bax level in young adult SOC rats (a). Risperidone-treated isolation rearing rats (both adolescent and young adult rats) have a higher Bcl-2 level (b). Risperidone also reduces the ratio of Bax/Bcl-2 particularly in adolescent isolation rearing rats (c). For each subgroup, $n = 6$. Data are represented as group average + standard error of mean. (IR: Isolation reared rats; SOC: Social control rats; RIS: Risperidone; OLA: Olanzapine; SAL: Saline)

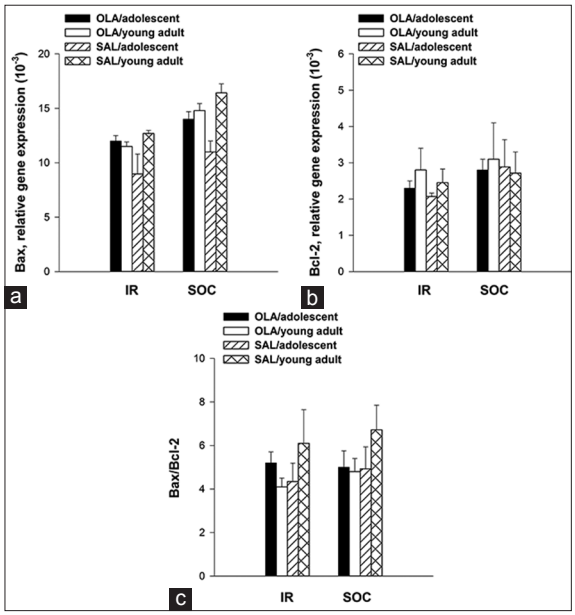

Figure 2: There are no statistically significant effects of rearing condition, intervention age and treatment in Bax level (a), Bcl-2 (b) and the ratio of Bax/Bcl-2 (c). For each subgroup, $n = 6$. Data are represented as group average + standard error of mean. (IR: Isolation reared rats; SOC: Social control rats; RIS: Risperidone; OLA: Olanzapine; SAL: Saline)

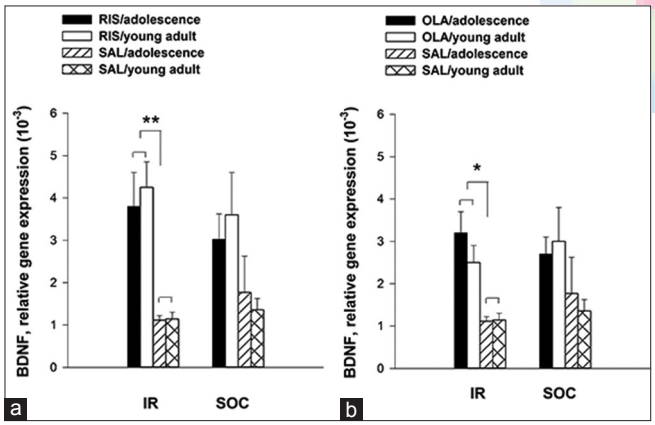

Figure 3: Risperidone (a) and olanzapine (b) can increase the brain-derived neurotrophic factor level. The drug-increased brain-derived neurotrophic factor is more pronounced in isolation rearing rats (pooled groups of different age, for each subgroup, $n = 6$). Data are represented as group average + standard error of mean. *$P < 0.05$ versus saline, **$P < 0.01$ versus saline. (IR: Isolation reared rats; SOC: Social control rats; RIS: Risperidone; OLA: Olanzapine; SAL: Saline)

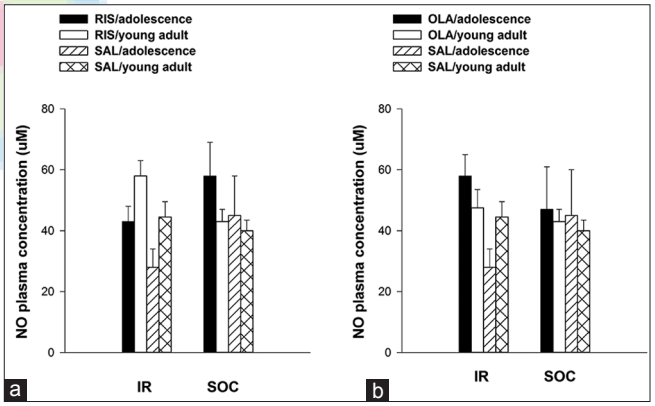

Figure 4: Risperidone (a) and olanzapine (b) have no effects on NO plasma concentration. There are no statistically significant effects of rearing condition, intervention age, and treatment in plasma nitric oxide. For each subgroup, $n = 6$. Data are represented as group average + standard error of mean. (IR: Isolation reared rats; SOC: Social control rats; RIS: Risperidone; OLA: Olanzapine; SAL: Saline)

DISCUSSION

The oxidative stress-induced dysfunctions are crucial in the pathoetiology of schizophrenia, and as the latter is a disorder with the developmental origin, an animal model of developmental impact on brain function appears suitable paradigm to examine the hypothesis that the neurodevelopmental process has a crucial impact on the oxidative stress-related abnormalities. The present study initially confirmed the validity of IR by showing IR-induced locomotor hyperactivity, and then for the first time demonstrated that RIS may exert anti-apoptotic effects on IR rats, particularly at their adolescent age (as indexed by increased Bcl-2 and decreased Bax/Bcl-2 ratio). The therapeutic potential of RIS can be also observed in the change of BDNF in an age-independent manner, in

which RIS effectively increased the BDNF level in IR but not SOC rats. Our results, in general, support the hypothesis that developmental impact may reflect in the oxidative stress level. These findings are further discussed as the following.

The present study demonstrated a varied sensitivities and impact of IR and SGAs acting on the oxidative stress-relevant biological variables. Whereas only RIS may be beneficial for younger generation (i.e., adolescent stage) of IR rats in terms of the anti-apoptotic change (as seen in the increased Bcl-2 level and decreased Bax/Bcl-2 ratio), both RIS and OLA were helpful for IR rats in an age-independent manner in terms of the neurogenesis (as seen in the high levels of BDNF in IR but not SOC rats, no matter when was the intervention timing). The underlying mechanism may be complicated. However, it could be interpreted in several aspects.

First, compared to BDNF, Bcl-2 family is a more direct pathway to reflect the imbalance of oxidative stress,[4] the therapeutic effects of RIS appear more pronounced in younger generation of IR rats, suggesting a developmental specificity of IR-induced characteristics. Second, the antipsychotic effects on oxidative stress are controversial, while RIS previously has been reported to augment apoptosis through enhancing Bax in a stroke rodent model[29] and reducing Bcl-2/bax gene expression ratio in an *in vitro* study,[30] Keilhoff *et al*. on the contrary reported that RIS can promote the survival of young neurons in the hippocampus by enhancing the expression of the anti-apoptotic protein Bcl-2.[31] Our hippocampal results apparently support the study of Keilhoff's group and highlight the specific feature of hippocampus, in which it is among the most important brain areas to interpret the pathoetiology of schizophrenia.[32-34]

BDNF, on the other hand, interpret the pathogenesis of schizophrenia from an aspect of neuronal function, including brain development, neuroplasticity, and synaptic connectivity.[35] Cognitive dysfunction and epigenetic alterations of the BDNF gene are induced by social isolation during early adolescence.[36] The involvement of BDNF in the IR is highly possible, as BDNF is involved in the chloride homeostasis-related central expression of KCC2/NKCC1,[37] which can be also adjusted by IR in SPAK knockout mice.[12] In terms of the relationship between oxidative stress and BDNF system, although interactions between them were reported, which is along with the developmental hypothesis,[8] the results of the present study indicate that the change of BDNF appears not the primary outcome following oxidative stress such as the Bcl-2 family. Further study to elucidate the causal relationships between oxidative stress and the BDNF system is necessary. The BDNF data are of particular relevance to research on schizophrenia, as the substance is active during a critical period of neuroplasticity in schizophrenia development.[38,39] However, the neuroprotective profile of antipsychotics varies. For example, BDNF gene expression is increased by olanzapine,[40] but decreased by haloperidol and a high dose of RIS.[41] Our data demonstrated that chronic RIS administration at a relative low dosage (i.e., 1 mg/kg) might benefit BDNF gene expression, possibly through its $5HT_2$ blockade activity.[41]

It is noted that in the present study, RIS seems superior to OLA in adjusting the balance of oxidative stress in younger IR rats, indicating a better therapeutic index of RIS in restoring the abnormalities with developmental origin, which is along with what reported previously that long-term RIS but not OLA to regulate the factors relevant to oxidative stress.[42] Note in terms of medicinal chemistry, RIS belongs to the benzisoxazole class, which is different from OLA, a commonly used SGA belonging to thienobenzodiazepine class. Here, the superiority of RIS than OLA in restoring IR-induced abnormalities is a cross-model phenomenon as it was also reported in a paradigm of repeated treatment with phencyclidine, a substance known to induced PPI deficit in rodents and psychosis in human.[43,44]

The duration of antipsychotic intervention could be one of the factors to determine its effects on oxidative stress. Previous evidence revealed that a shorter antipsychotic regime causes sabotage effects, however a longer one (for example, 180 days, rather than 90 days) turns to be beneficial in terms of the antioxidant enzymes and membrane lipid peroxidation.[42] As the drugged time in our study was 9 weeks, it possible explains the observation that the failure of RIS effects on Bax, as the intervention duration is insufficient to cause a valid effect.

Note although NO may serve as a signaling molecule involves in the development and treatment of schizophrenia, the alterations of NO metabolism are not unique to schizophrenia, thus NO is not a suitable biological index for this disease.[45] The involvement of NO in schizophrenia is complicated, as both overproduction and underproduction of NO have been suggested.[46,47] This study showed that the plasma NO was not sensitive to both IR procedure and antipsychotic regimes. It is also possible that the peripheral NO fails to reflect the pathology of schizophrenia in central nervous systems and the benefit bringing from the antipsychotics (i.e., enhancement of nitrergic activity).[47]

It is interesting that in the present study, only IR rats appeared effective to the RIS treatment. This is important for clinical implication in terms of the concept of early antipsychotic intervention. In general, our results add to the evidence to support the concept of early antipsychotic intervention.[12] Thus, how early to start antipsychotic treatment is debatable. For those who consider not taking antipsychotics in the prodromal phase of schizophrenia based on the issues of false-positive

diagnosis,[48,49] risking of stigmatizing patients,[50,51] and the long-term side effects of drugs,[52,53] and thus results in an ethical dilemma regarding early antipsychotic intervention.[54] On the other hand, if considering schizophrenia a developmental disorder as hypothesized, the biological method should help impede the progress of the disorder and prevent the patients from receiving too much biochemical disturbance before they are aware of the symptoms, thus the pharmacological interventions at an early stage of IR might be beneficial. The study apparently supports the concept of early antipsychotic intervention, in a more general viewpoint of stress. Thus, the present study demonstrates an example in which a long-term environmental stress may cause effects onto the cellular events of ROS-oxidative stress and the employment of antipsychotic regime in an earlier stage of IR may be beneficial. Further as RIS did not cause any negative effect on Bax/Bcl-2 or BDNF in adolescent rats compared to young adult controls, and this result may demonstrate a safety profile for early treatment in prodromal schizophrenic patients.

CLINICAL IMPLICATION AND CONCLUSION

Although pharmacological intervention with antipsychotics has been proven effective in managing the symptoms of schizophrenic patients, an earlier intervention is an ethical dilemma. False-positive diagnosis (as the symptoms are not full-blown), potential risk of stigmatizing patients, and the long-term side effects appear against the earlier pharmacological intervention. On the other hand, if considering schizophrenia to be a developmental disorder, the biological method should help impede the progress of the disorder thus prevent the patients from receiving too much biochemical disturbance before they are aware of the symptoms.[12] The present study to a degree supports the early intervention of SGAs in terms of the oxidative stress-induced dysfunctions.

The hippocampal data obtained from the present study may support the use of SGA in managing the apoptosis-related abnormalities.[55] In general, developmental stage plays a crucial role in determining the pharmacological efficacy onto the oxidative-stress relevant dysfunctions, thus earlier pharmacological intervention appears more beneficial in reversing the overloaded oxidative stress-induced abnormalities, as RIS may exert anti-apoptotic effects on IR rats at their adolescent age (as indexed by increased Bcl-2 and decreased Bax/Bcl-2 ratio), which is also in line with the sensorimotor gating profile.[56] Our results support the neurodevelopmental hypothesis of schizophrenia and validate the utility of the IR paradigm in exploring mental disorders with neurodevelopmental origin. In terms of translational medicine, our results also demonstrated that the oxidative stress profile can be one of the biological markers reflecting the efficacy of antipsychotics, in which earlier intervention would lead to a better progress.

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Conflicts of interest

There are no conflicts of interest.

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