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ARTICLE

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Long-lasting recovery of psychotic-like symptoms in isolation-reared rats after chronic but not acute treatment with the cannabinoid antagonist AM251

Erica Zamberletti[1], Daniela Viganò[1,2], Cinzia Guidali[1], Tiziana Rubino[1] and Daniela Parolaro[1,2]

[1] *DBSF and Neuroscience Center, University of Insubria, Busto Arsizio (VA), Italy*
[2] *Zardi-Gori Foundation, Milan, Italy*

Abstract

In this work we investigated the ability of AM251 to reverse schizophrenia-like symptoms produced by a neurodevelopmental animal model based on a social isolation procedure. First, we assessed the validity of our isolation-rearing protocol and, as expected, isolation-reared rats showed hyperlocomotion in a novel environment, cognitive impairment in the novel object recognition (NOR) test and a significant increase in the number of aggressive behaviours in the social interaction test compared to group-housed controls. This behavioural picture was associated with a reduction in CB_1 receptor/G protein coupling in specific brain areas as well as reduced c-Fos immunoreactivity in the prefrontal cortex and caudate putamen. In this model, chronic but not acute treatment with the CB_1 receptor antagonist AM251 counteracted isolation-induced cognitive impairment in the NOR test and aggressive behaviours in the social interaction test. This behavioural recovery was accompanied by the rescue of CB_1 receptor functionality and c-Fos levels in all brain regions altered in isolation-reared rats. Moreover, chronic AM251 also increased c-Fos immunoreactivity in the nucleus accumbens, as previously demonstrated for antipsychotic drugs. Interestingly, the behavioural recovery due to chronic AM251 administration persisted until 10 d after discontinuing the treatment, indicating a long-lasting effect of the cannabinoid antagonist on psychotic-like symptoms.

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Key words: Aggressive behaviour, cannabinoid antagonist, CB_1 receptor, isolation rearing, novel object recognition.

Introduction

Different theories have attempted to clarify the aetiology of schizophrenia but the exact causes of this complex and multifactorial mental disorder remain unknown.

The aetiology of schizophrenia has been largely demonstrated as an involvement of the dopaminergic and glutamatergic systems (Coyle, 2006; Howes & Kapur, 2009) but recent experimental evidence strongly suggest that alterations in the endocannabinoid system may also contribute to the pathogenesis of the disease (De Marchi *et al*. 2003; Giuffrida *et al*. 2004; Leweke *et al*. 1999; Lewis *et al*. 2005; Newell *et al*. 2006; Sundram *et al*. 2005; Ujike *et al*. 2002). Accordingly, a 'cannabinoid hypothesis' of schizophrenia has been suggested (Muller-Vahl & Emrich, 2008).

Based on this hypothesis, the pharmacological manipulation of the endocannabinoid system may represent a promising tool for improving symptoms of the disease; however, the experimental findings concerning the effects of CB_1 receptor (CB_1R) agonists and antagonists on schizophrenia-like symptoms are still controversial, often with different effects depending on the drug, the dose, the species and the model used

Address for correspondence: Professor D. Parolaro, DBSF and Neuroscience Center, University of Insubria, Via A. da Giussano 10, 21052 Busto Arsizio (VA), Italy.
Tel.: +390331339417 *Fax*: +390331339459
Email: daniela.parolaro@uninsubria.it

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for simulating positive or negative symptoms (for review see Parolaro *et al*. 2010). In general, acute administrations of CB_1R agonists reduce the positive symptoms induced by dopaminergic and glutamatergic agents (Gorriti *et al*. 1999; Marcellino *et al*. 2008; Przegalinski *et al*. 2005), whereas the ability of CB_1R antagonists to reverse schizophrenia-like positive symptoms is still under debate (for review see Parolaro *et al*. 2010; Roser *et al*. 2008).

With regard to the negative symptoms of schizophrenia, most studies have investigated the effects of a manipulation of the endocannabinoid system on the prepulse inhibition (PPI) of the acoustic startle reflex. CB_1R agonists cause disruption of the PPI (Martin *et al*. 2003; Nagai *et al*. 2006; Schneider & Koch, 2002) reversed by CB_1R antagonists, in contrast CB_1 antagonists show no effect or even an improvement in NMDA antagonist- or D_2 agonist-induced disruption of PPI (Ballmaier *et al*. 2007; Malone *et al*. 2004; Martin *et al*. 2003). Finally, genetic CB_1 disruption counteracted the social deficit induced by PCP in mice (Haller *et al*. 2005).

We recently demonstrated that the CB_1R antagonist, AM251, restored the cognitive impairment in the novel object recognition (NOR) test and reduced avolition in the forced swim test, a behavioural test commonly also used to assess depression, induced by chronic intermittent PCP treatment, a pharmacological model reproducing some schizophrenia-like symptoms (Guidali *et al*. 2010). This behavioural recovery was correlated with the restoration of CB_1R function in all brain areas altered by PCP administration. Moreover, chronic AM251 co-treatment antagonized the PCP-induced increase in c-Fos expression in the prefrontal cortex (PFC), a key region for the integration of cognitive and negative signs of schizophrenia. In the same brain region, chronic AM251 treatment counteracted the increase in 2-arachidonoylglycerol observed in PCP-treated rats and enhanced anandamide levels in the same cerebral area (Guidali *et al*. 2010). Recently, Seillier's group, using a subchronic PCP model of aspects of schizophrenia in rats, reported that acute AM251 reversed the PCP-induced working-memory deficit but had no effect in the social interaction test both in PCP- and saline-treated rats. Moreover, they found no changes in CB_1R expression, although PCP-treated rats showed an increase in receptor-stimulated [^{35}S]GTPγS binding in the anterior cingulate cortex and nucleus accumbens (NAc), accompanied by a reduction in the CA2/3 fields of the hippocampus (Seillier *et al*. 2010). These findings partially disagree with those previously reported by our group (Viganò *et al*. 2009) and these discrepancies might be explained by differences in drug regimen and the age of the animals used in the studies.

In this work we investigated the ability of AM251 to reverse schizophrenia-like symptoms produced by a neurodevelopmental animal model based on the social isolation procedure. Rearing rats in social isolation from weaning produces persistent behavioural and neurochemical alterations compared to group-housed controls (Fone & Porkess, 2008; Lapiz *et al*. 2003). Behavioural changes observed in isolation-reared rats may have translation relevance to several core symptoms of schizophrenia such as locomotor hyperactivity in a novel environment, impaired sensorimotor gating, aggressive behaviour and cognitive impairment (Fone & Porkess, 2008). Interestingly, recent papers showed alterations in several components of the endocannabinoid system in different brain regions of isolation-reared rats, including important areas implicated in the pathophysiology of schizophrenia (Robinson *et al*. 2010; Sciolino *et al*. 2010).

On these bases, in this study we evaluated the effects of a pharmacological manipulation of the endocannabinoid system by acute and chronic AM251 treatment on isolation rearing-induced cognitive impairment and aggressive behaviour. Moreover, we analysed at different time-points the effects of isolation rearing and chronic AM251 treatment on the endocannabinoid system in terms of CB_1R density and functionality.

Materials and methods

Animals

At weaning (PND 21), male Lister Hooded rats (Harlan, Italy) were randomly housed in groups of four (grouped) or alone (isolated). All animals were housed in the same room and had visual, auditory and olfactory contact with animals caged nearby, on a 12-h light/dark cycle (lights on 08:00 hours) and in a temperature- (24 ± 2 °C) and humidity-controlled environment (50 ± 10%) with food and water available *ad libitum*. The isolated animals were left undisturbed in their cages and received the minimal handling associated with husbandry (cage and bedding changed weekly).

All experiments took place during the light phase and were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92) and (D.L. 111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

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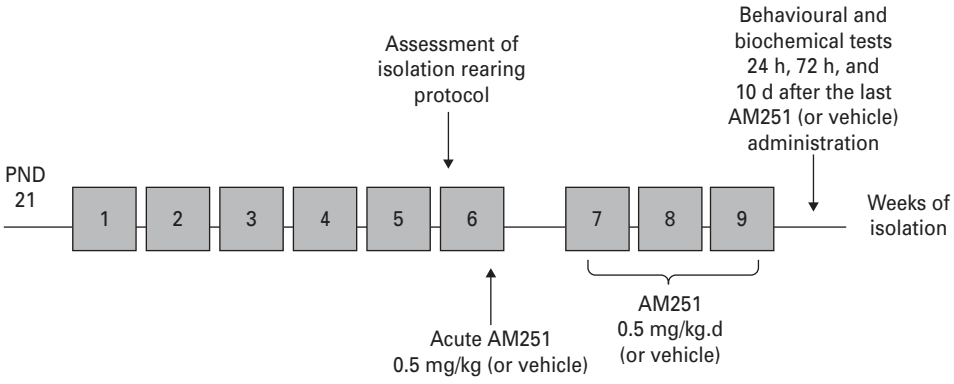

Fig. 1. Treatment schedule.

Drug administration

AM251 (Tocris, Italy) was dissolved in DMSO, Tween-80 and saline (1:1:8). The drug was acutely or chronically administered at 0.5 mg/kg (with the injection volume of 5 ml/kg) i.p.

For acute treatment each animal received a single injection 80 min before the test session, whereas for chronic administration AM251 was given daily for 3 wk and animals underwent a series of behavioural tests 24 h, 72 h and 10 d after the last AM251 (or vehicle) administration. The different behavioural tests were performed on separate groups of animals (Fig. 1).

Behavioural tests

Spontaneous locomotor activity

Rats were placed in a computer-controlled infrared activity monitoring arena. The arena consisted of a clear acrylic box, $43 \times 43 \times 32$ cm (Ugo Basile, Italy) placed in a sound-attenuating room. The cage was fitted with two parallel infrared beams, located 2 cm and 6 cm from the floor and cumulative horizontal and vertical movement counts were recorded for 1 h.

NOR test

The experimental apparatus used for the object recognition test was an open-field box ($60 \times 60 \times 60$ cm) made of Plexiglas, placed in a dimly illuminated room. Animals performed each test individually. A 10-min habituation session preceded the experimental trials. The experiment was performed and analysed as previously described in Viganò *et al*. (2009). Briefly, after habituation each animal was placed in the arena and allowed to explore two identical previously unseen objects for 10 min (familiarization phase). After an inter-trial interval of 1 h one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 3-min test phase. During the test phase the time spent exploring the familiar object (E_f) and the new object (E_n) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows:

$$[(E_n - E_f)/(E_n + E_f)] \times 100.$$

Social interaction test

The test was carried out in a room illuminated with a dim overhead light. On the day of testing, each animal was habituated for 10 min in the test arena ($60 \times 60 \times 60$ cm), an open-field box made of Plexiglas. During the test session, each animal was allowed to freely explore an unfamiliar congener in the arena for 10 min. The arena was cleaned with 0.1% acetic acid and dried after each trial. Social behaviours were defined as sniffing, following, grooming, mounting and nosing. Aggressive behaviours were defined as attacking, biting, tail rattling and aggressive grooming. The whole testing phase was videotaped, analysed by two observers blind to the treatment groups and we recorded the time spent in social behaviours and the number of aggressive behaviours.

Biochemical assays

For assessment of long-term effects of isolation rearing and AM251 treatment on CB_1R function, biochemical analyses were performed on separate groups of animals not tested for behaviour. Rats were decapitated and brains were rapidly removed, frozen in liquid nitrogen and stored at $-80\ ^\circ$C until processing.

Autoradiographic-binding assays

Coronal sections (20-μm-thick) were cut on a cryostat and mounted on gelatin-coated slides. The sections were stored at $-80\ ^\circ$C until processing.

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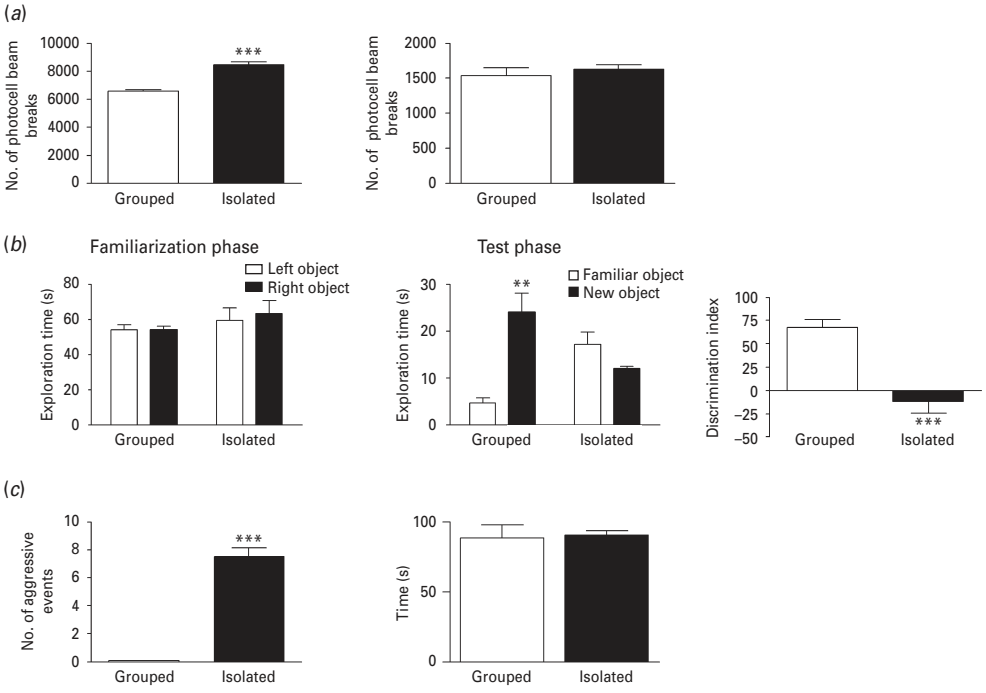

Fig. 2. Behavioural phenotype after 5 wk of isolation rearing. (*a*) Horizontal (*left*) and vertical (*right*) activity assessed in the activity cage. (*b*) Exploration time of identical objects during the familiarization phase (*left*), exploration time of the familiar *vs*. novel object (*centre*) and the discrimination index (*right*) during the test phase in the novel object recognition test. (*c*) Number of aggressive behaviours (*left*) and time spent in social behaviours (*right*) during the social interaction test. Results are expressed as mean ± S.E.M. ** $p < 0.01$ *vs*. familiar object (*t* test), *** $p < 0.001$ *vs*. grouped (*t* test).

[^{3}H]CP-55,940 receptor autoradiographic binding

[^{3}H]CP-55,940 receptor autoradiographic binding was performed as described previously (Rubino *et al*. 2000; Viganò *et al*. 2009).

CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography

This was determined as described previously by our group (Rubino *et al*. 2000; Viganò *et al*. 2009).

Image analysis

The intensity of the autoradiographic films was assessed by measuring the grey levels with an image analysis system consisting of a scanner connected to a PC running Microsoft Windows. The images were analysed using Image-Pro Plus 5.0 (MediaCybernetics, USA) as described previously (Viganò *et al*. 2005).

c-Fos immunohistochemistry

c-Fos expression was assessed as described previously (Guidali *et al*. 2010). Briefly, sections were incubated with a primary antibody to c-Fos (Abcam, UK) diluted 1/50 for 48 h at 4 °C and with biotylinated secondary antibody diluted 1/100 for 1 h at room temperature and finally incubated with avidin–biotin–peroxidase complex (Vector ABC kit, Vector Laboratories, USA) for 1 h at room temperature. Slides were then incubated in chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 5 min. Some control sections were stained without the primary antibody. Positive neurons were counted as described previously (Guidali *et al*. 2010).

Statistical analysis

All analysis were performed using GraphPad Prism 3.0 software and data are reported as mean ± S.E.M. Results were analysed using unpaired Student's *t* test or two-way ANOVA followed up by Bonferroni's *post-hoc* test to examine group differences. The level of statistical significance was set at $p < 0.05$.

Results

Behavioural assessment of isolation-rearing protocol

Figure 2 shows the behavioural scene after 5 wk of isolation rearing.

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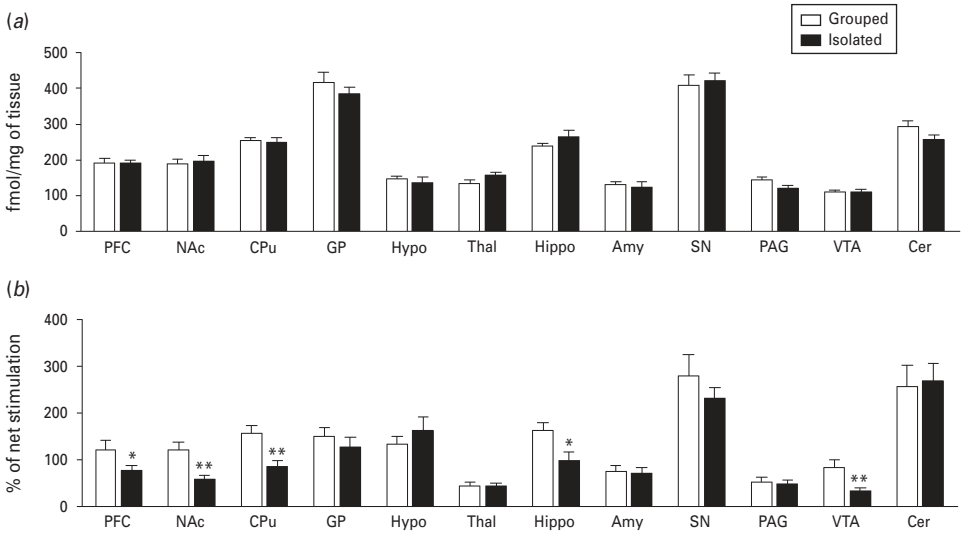

Fig. 3. Effect of 5 wk of isolation rearing on CB_1 receptor density and functionality. (*a*) [^{3}H]CP-55,940 receptor autoradiographic binding. (*b*) CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography. Prefrontal cortex (PFC), nucleus accumbens (NAc), caudate putamen (CPu), globus pallidus (GP), hypothalamus (Hypo), thalamus (Thal), hippocampus (Hippo), amygdala (Amy), substantia nigra (SN), periaqueductal grey (PAG), ventral tegmental area (VTA), cerebellum (Cer). Results are expressed as mean $\pm$ S.E.M. * $p<0.05$, ** $p<0.01$ *vs.* grouped (*t* test).

Isolation-reared rats were significantly more active in the novel environment than group-housed rats ($t=8.820$, $p<0.0001$) without any alteration in rearing activity between the two groups ($t=1.876$, $p=0.0832$) (Fig. 2*a*).

Moreover, the isolation-rearing protocol caused an impairment of cognitive functions as demonstrated by a significant reduction in the discrimination index during the test phase of the NOR test ($t=10.25$, $p<0.0001$) compared to group-reared controls. In both groups there was no difference in the time spent exploring the two identical objects during the familiarization phase, but isolated animals failed to discriminate between the new and familiar object in the test phase (Fig. 2*b*). The locomotor activity was not altered both in grouped and isolated rats (data not shown).

In the social interaction test, no differences were found in the time spent in active behaviours between isolation- and group-reared animals ($t=0.1969$, $p=0.8504$) but isolation rearing caused a significant increase in aggressive behaviours compared to group-reared controls ($t=32.75$, $p<0.0001$) (Fig. 2*c*).

Effects of the isolation-rearing protocol on CB_1R functionality

After 5 wk of isolation rearing, we investigated the effects of housing condition on CB_1R density and functionality.

Figure 3*a* shows the effects of isolation rearing on CB_1R density. Isolation rearing had no effect on CB_1R density in all the cerebral regions analysed.

We found significant changes in CB_1R functionality as shown by the GTPγS binding assay (Fig. 3*b*). Particularly, the isolation-rearing protocol induced a significant reduction in CB_1R functionality in the PFC ($t=2.093$, $p=0.0481$), NAc ($t=3.572$, $p=0.0017$), caudate putamen (CPu) ($t=3.507$, $p=0.0020$), hippocampus (Hippo) ($t=2.546$, $p=0.0216$) and ventral tegmental area (VTA) ($t=3.438$, $p=0.0044$).

Effects of acute AM251 administration on cognitive impairment and aggressive behaviour induced by isolation rearing protocol

In the NOR test two-way ANOVA found acute AM251 treatment did not alter the exploration time in the familiarization phase both in grouped and isolated rats and failed to improve the recognition memory disrupted by social isolation rearing (housing: $F_{1,12}=75.24$, $p<0.0001$; drug: $F_{1,12}=0.0001214$, $p=0.9914$; no interaction) (Fig. 4). Moreover, acute AM251 alone did not affect the discrimination index in socially reared rats and there were no differences in the locomotor activity between all the groups considered (data not shown).

In the social interaction test acute AM251 did not significantly reduce the number of aggressive events

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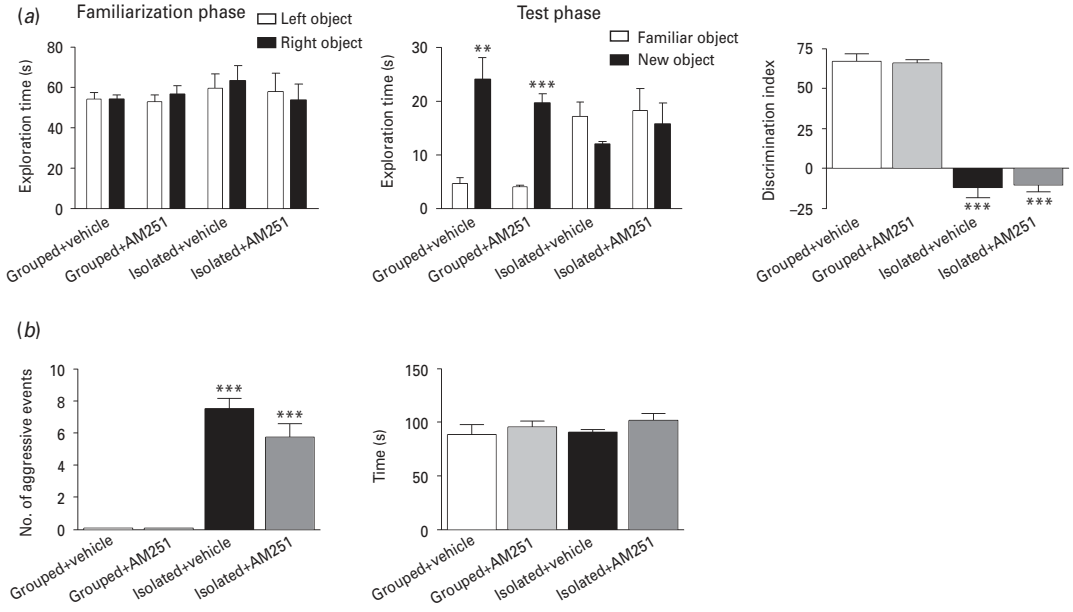

Fig. 4. (*a*) Effect of acute AM251 treatment (0.5 mg/kg) on isolation-induced cognitive deficit in the novel object recognition test. The test was performed 80 min after AM251 administration. *Left*: The exploration time of identical objects during the familiarization phase; *centre*: the exploration time of the familiar *vs.* novel object; *right*: the discrimination index during the test phase. (*b*) Effect of acute AM251 treatment (0.5 mg/kg) in the social interaction test. The test was performed 80 min after AM251 administration. *Left*: The number of aggressive behaviours during the test session; *right*: the time spent in active social behaviours. Data are expressed as mean ± S.E.M. ** $p<0.01$, *** $p<0.001$ *vs.* familiar object (*t* test); *** $p<0.001$ *vs.* grouped + vehicle (Bonferroni's *post-hoc* test).

in isolation-reared rats (housing: $F_{1,12}=148.6$, $p=0.9914$; drug: $F_{1,12}=2.673$, $p=0.1280$; no interaction). Neither housing conditions or AM251 treatment affected the time spent in social behaviours during the social interaction test (housing: $F_{1,12}=0.3768$, $p=0.5508$; drug: $F_{1,12}=1.844$, $p=0.1995$; no interactions) (Fig. 4*b*).

Effects of chronic AM251 treatment on isolation rearing-induced behavioural alterations

Figure 5 shows the effects of chronic AM251 administration on the cognitive impairment induced by isolation rearing revealed by two-way ANOVA. The discrimination index impaired in isolation-reared rats was restored after 3 wk of chronic AM251 treatment (housing: $F_{1,12}=6.834$, $p=0.0226$; drug: $F_{1,12}=4.755$, $p=0.0498$; drug × housing interaction: $F_{1,12}=19.43$, $p=0.0023$) (Fig. 5*a*) and the recovery of this parameter was still evident at 72 h (housing: $F_{1,12}=5.482$, $p=0.0373$; drug: $F_{1,12}=12.27$, $p=0.0044$; no interaction) (Fig. 5*b*) and 10 d after the last AM251 administration (housing: $F_{1,12}=10.48$, $p=0.0071$; drug: $F_{1,12}=6.403$, $p=0.0264$; drug × housing interaction: $F_{1,12}=10.39$, $p=0.0073$) (Fig. 5*c*). Neither housing conditions nor AM251 treatment altered the time spent exploring the two identical objects during the familiarization phase and AM251 alone did not affect the recognition memory in socially reared rats. The locomotor activity was not altered in any of the groups analysed (data not shown).

Figure 6 shows the effects of chronic AM251 treatment on the aggressive behaviours in the social interaction test (Fig. 6*a*). We observed a reduction in the number of aggressive behaviours in isolation-reared rats 72 h and 10 d after the last AM251 administration compared to what was observed in the social interaction test performed 24 h after the last injection. However, the aggressive behaviours in isolation-reared rats were still significantly increased compared to group-reared controls at both time-points. Isolation-reared rats chronically administered with AM251 showed a significant reduction in the number of aggressive events in the social interaction test performed 24 h after discontinuing treatment (drug: $F_{1,12}=13.71$, $p=0.0030$; drug × housing interaction: $F_{1,12}=13.71$, $p=0.0030$) and this recovery was still evident at 72 h (drug: $F_{1,12}=11.37$, $p=0.0056$; drug × housing interaction: $F_{1,12}=11.37$, $p=0.0056$) and 10 d (drug: $F_{1,12}=6.716$, $p=0.0236$; drug × housing interaction: $F_{1,12}=6.716$, $p=0.0236$) after the last AM251 administration.

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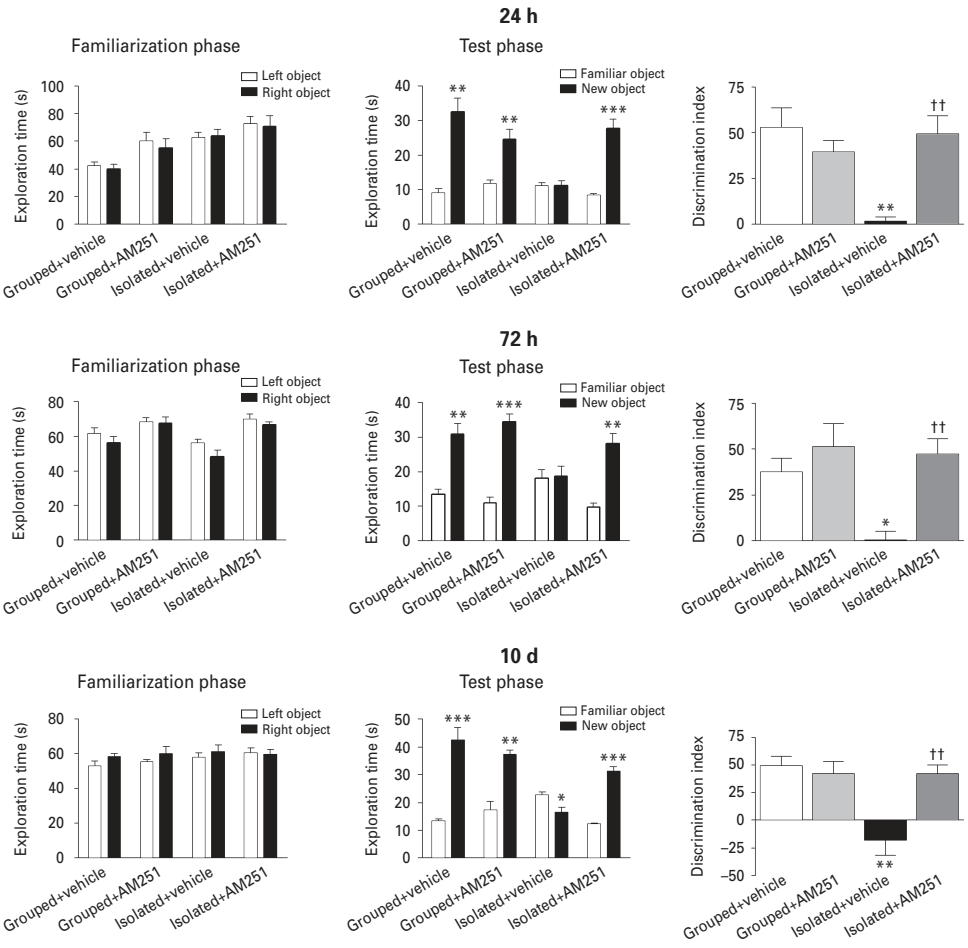

Fig. 5. Effect of chronic AM251 treatment (0.5 mg/kg) on isolation-induced cognitive deficit in the novel object recognition test performed 24 h, 72 h and 10 d after the last AM251 administration. *Left*: The exploration time of identical objects during the familiarization phase; *centre*: the exploration time of the familiar *vs.* novel object; *right*: the discrimination index during the test phase. Data are expressed as mean ± S.E.M. ** $p<0.01$, *** $p<0.001$ *vs.* familiar object (t test), ** $p<0.01$, * $p<0.05$ *vs.* grouped + vehicle; †† $p<0.01$ *vs.* isolated + vehicle (Bonferroni's *post-hoc* test).

Chronic AM251 did not alter the time spent in social behaviours.

Effects of AM251 chronic treatment on CB_1R functionality

After 3 wk of chronic AM251 (or vehicle) treatment we found no alterations in CB_1R density in group-housed or isolation-reared rats, in all the brain regions analysed by two-way ANOVA (Fig. 7*a*).

Fig. 7*b* represents the results of CP-55,940-stimulated GTPγS autoradiographic-binding assay performed 24 h after the last chronic AM251 (or vehicle) administration.

AM251 had no effect on CB_1R functionality in group-housed controls but counteracted the alterations observed in rats reared in isolation in the PFC, NAc, Hippo, and VTA. After 3 wk of chronic vehicle treatment, isolation-reared rats still showed a significant reduction in the CB_1R functionality in the PFC, NAc, Hippo, and VTA compared to group-housed controls (PFC, housing: $F_{1,28}=6.610$, $p=0.0157$; NAc: housing: $F_{1,28}=7.083$, $p=0.0127$; Hippo, housing: $F_{1,28}=8.549$, $p=0.0068$; VTA, housing: $F_{1,28}=8.549$, $p=0.0068$), indicating that these alterations were not influenced by daily handling. The reduction reported in isolation-reared rats were counteracted by AM251 chronic administration (PFC, drug × housing interaction: $F_{1,28}=8.881$, $p=0.0059$; NAc, drug × housing interaction: $F_{1,28}=5.848$, $p=0.0223$; Hippo, drug × housing interaction: $F_{1,28}=8.410$, $p=0.0072$).

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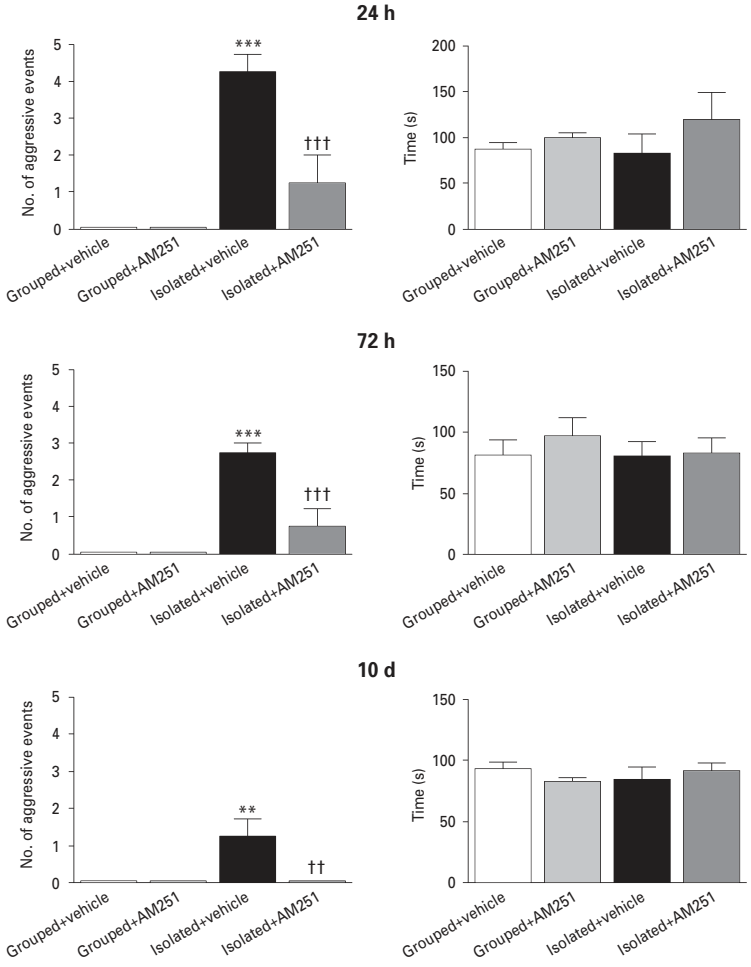

Fig. 6. Effect of chronic AM251 treatment (0.5 mg/kg) during the social interaction test performed 24 h, 72 h and 10 d after the last AM251 administration. *Left*: Number of aggressive events; *right*: time spent in active social behaviour during the test session. Results are expressed as mean ± S.E.M. ** $p<0.01$, *** $p<0.001$ *vs.* grouped + vehicle; †† $p<0.01$, ††† $p<0.01$ *vs.* isolated + vehicle (Bonferroni's *post-hoc* test).

c-Fos immunohistochemistry

Figure 8*a* shows the effects on c-Fos immunoreactivity in rats reared in isolation for 5 wk in the PFC, CPu, and NAc. Isolation rearing significantly reduced c-Fos expression in the PFC ($t=5.346$, $p<0.0001$) and CPu ($t=3.583$, $p=0.0038$) compared to group-housed controls, but had no effect on c-Fos expression in the NAc.

Figure 8*b* represents the effects of isolation rearing and chronic AM251 (or vehicle) administration on c-Fos expression. Two-way ANOVA showed the reduction in c-Fos observed in the PFC was still evident in isolation-reared rats after 3 wk of chronic vehicle treatment (housing: $F_{1,28}=4.927$, $p=0.0357$). Moreover, chronic AM251 administration counteracted the reduction in c-Fos expression observed in the PFC of isolation-reared rats (drug × housing interaction: $F_{1,28}=4.804$, $p=0.0379$). Instead, chronic handling due to chronic vehicle treatment recovered the reduction in c-Fos expression observed in the CPu of rats reared in isolation and AM251 treatment did not show any further effect.

Chronic AM251 administration *per se* significantly increased c-Fos expression in NAc ($F_{1,25}=20.52$, $p=0.0001$) in isolation and group-reared rats.

Discussion

In the present study, using the social isolation paradigm as a model of psychotic-like behaviours, we demonstrated that alterations in CB_1R functionality

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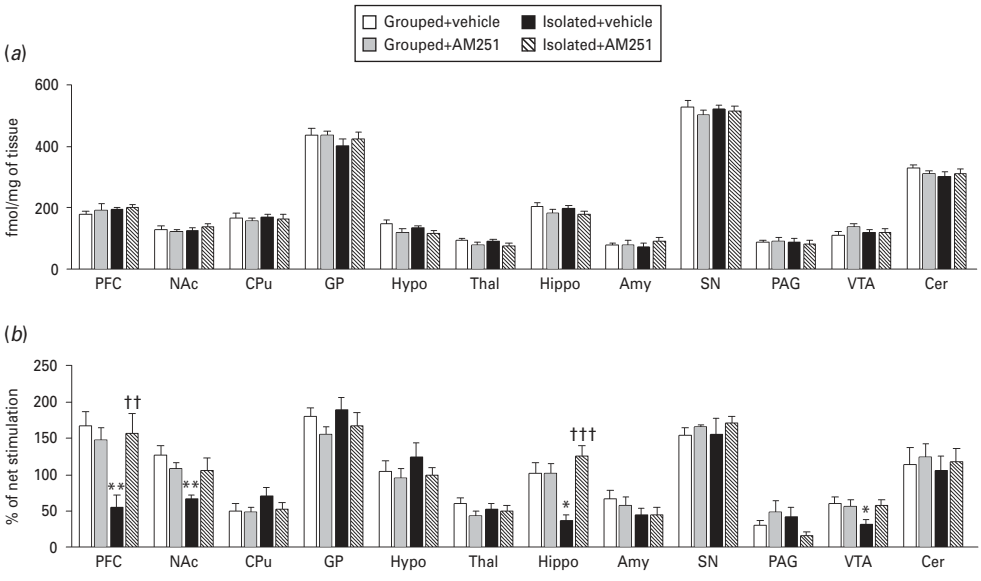

Fig. 7. Effect of chronic AM251 on CB_1 receptor density and functionality. (*a*) [^{3}H]CP-55,940 receptor autoradiographic binding. (*b*) CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography. Results are expressed as mean $\pm$ S.E.M. * $p<0.05$, ** $p<0.01$ *vs.* grouped + vehicle; †† $p<0.01$, ††† $p<0.001$ *vs.* isolated + vehicle (Bonferroni's *post-hoc* test).

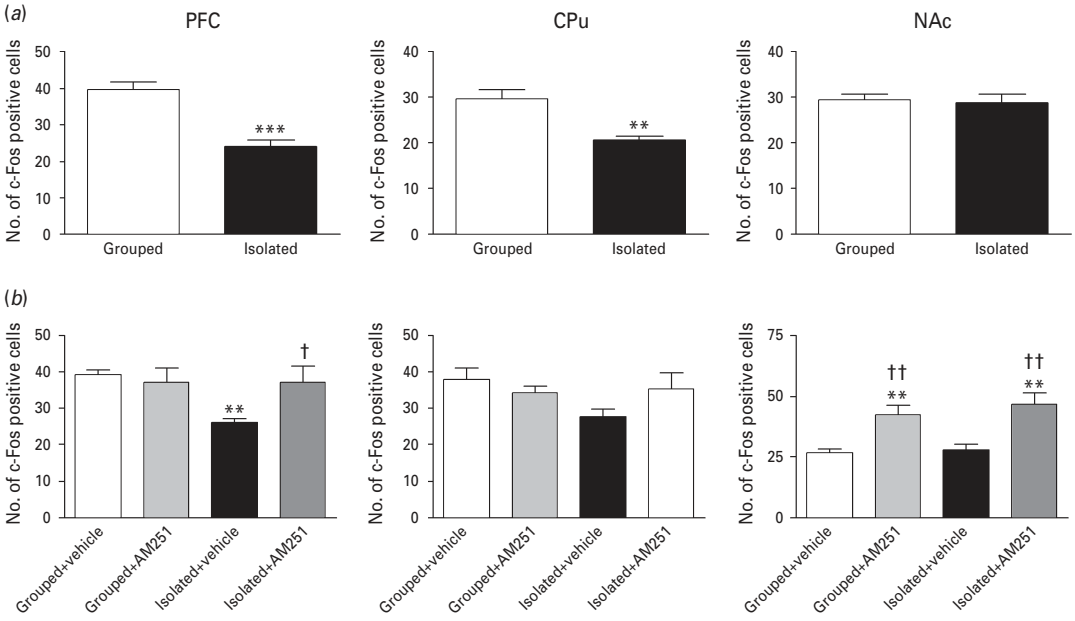

Fig. 8. Effect of (*a*) social isolation protocol and (*b*) chronic AM251 treatment on c-Fos immunoreactivity in the prefrontal cortex (PFC), nucleus accumbens (NAc) and caudate putamen (CPu). (*a*) c-Fos immunoreactivity evaluated after 5 wk of isolation rearing. (*b*) c-Fos immunoreactivity after 3 wk of chronic AM251 administration. Results are expressed as mean $\pm$ S.E.M. ** $p<0.01$, *** $p<0.001$ *vs.* grouped (*t* test); ** $p<0.01$ *vs.* grouped + vehicle; † $p<0.05$, †† $p<0.01$ *vs.* isolated + vehicle (Bonferroni's *post-hoc* test). For abbreviations see Fig. 3 legend.

represent one of the molecular mechanisms contributing to the behavioural phenotype observed in isolation-reared rats. In addition, we clearly highlighted that chronic AM251 exerted an apparent beneficial action in terms of reversing the behavioural phenotype and the alterations in CB_1R functionality as well as the reduction in neuronal activity induced by isolation.

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First, we assessed the validity of the isolation-rearing protocol before performing other behavioural and neurochemical analyses. In fact, animals reared in isolation show a pattern of behavioural alterations such as hyperlocomotion in a novel environment (Bakshi & Geyer, 1999; Einon & Morgan, 1976) cognitive impairment (Bianchi *et al*. 2006; Fone & Porkess, 2008; Hellemans *et al*. 2004; Lapiz *et al*. 2000; Lu *et al*. 2003) and an increase in aggressive behaviours in the social interaction test (Toth *et al*. 2008; Vale & Montgomery, 1997; Wongwitdecha & Marsden, 1996). In our study, after 5 wk of isolation rearing, rats presented a marked increase in total horizontal locomotor activity, a cognitive impairment in the NOR test and a significant increase in the number of aggressive behaviours.

Recent studies have demonstrated a close association between the behavioural phenotype induced by isolation rearing and the presence of alterations in the endocannabinoid system (Malone *et al*. 2008; Robinson *et al*. 2010), thus in these animals, using autoradiographic techniques, we explored the levels of CB_1Rs and observed no changes in CB_1R binding sites in all brain regions considered. Our data appear in contrast to the results reported in recent papers that demonstrated a reduction in CB_1 immunoreactivity restricted to CPu and amygdala (Malone *et al*. 2008) or, more recently, an increase of the CB_1R mRNA expression (Robinson *et al*. 2010). The discrepancies between our study and those of Malone and Robinson can be due to the different techniques used to evaluate CB_1R density (autoradiographic binding assay or immunohistochemistry), the different rat species (Lister Hooded or Sprague–Dawley) and the duration of the isolation-rearing protocol (5 wk or 8 wk), in fact it is possible that down-regulation in CB_1R could appear after longer periods of isolation.

Despite the results regarding CB_1R density, our data show widely diffused alterations in CB_1R/G protein coupling in isolation-reared rats. In particular, it was significantly reduced in the PFC (−35%), CPu (−45%), NAc (−52%), Hippo (−39%) and VTA (−58%).

We can speculate that an increase in the endocannabinoid levels could underlie the reduction in CB_1 functionality observed in isolation-reared rats. In line with this, Sciolino *et al*. (2010) demonstrated alterations in anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in socially isolated rats: they found an increase in 2-AG in the PFC and an increase of 2-AG and AEA in the piriform cortex. Moreover, Robinson *et al*. (2010) observed an increase in mRNA levels of the enzymes responsible for the synthesis of AEA and 2-AG, accompanied by a reduction in FAAH mRNA levels in rats reared in isolation (Robinson *et al*. 2010), thus suggesting the presence of alterations in endocannabinoid content. Taken together, all these observations suggest that the widely diffused desensitization of CB_1R we observed might be ascribed to elevation of endocannabinoid level induced by isolation.

It is well established that schizophrenia and other psychiatric conditions are associated with specific alterations in the dopaminergic (Howes & Kapur, 2009) and endocannabinoid (Parolaro *et al*. 2010) systems. Interestingly, an overlapping distribution of cannabinoid receptors with dopaminergic receptors has been demonstrated in most brain areas where we found alterations in CB_1R functionality (Hermann *et al*. 2002). Cannabinoid receptors have been shown to modulate dopaminergic transmission through transsynaptic mechanisms, involving GABAergic and glutamatergic synapses (Chiu *et al*. 2010; van der Stelt & Di Marzo, 2003). In our model, the alteration in the endocannabinoid system observed in isolation-reared rats may reflect a homeostatic mechanism to hyperdopaminergic transmission produced by isolation or, alternatively, be a direct cause of the psychosis through a reduction of the endocannabinoid inhibitory control on dopaminergic transmission (Pistis *et al*. 2002; Robbe *et al*. 2002). In line with this, the reduction in CB_1R functionality may enhance dopamine transmission which may, at least in part, account for the behavioural alterations observed in isolation-reared rats. It is worth noting that all brain regions showing altered CB_1R functionality (PFC, CPu, NAc, Hippo, VTA) are part of a complex circuitry whose alterations may lead to the abnormal behaviour observed in isolated rats. Specifically, the reduction observed in CB_1R functionality in the PFC and Hippo might contribute to the cognitive impairment observed in this animal model (Bilkei-Gorzo *et al*. 2005; Eggan *et al*. 2010; Hill *et al*. 2005). Reduced CB_1R functionality in the CPu may account for the hyperlocomotion observed in isolation-reared rats (Marcellino *et al*. 2008) and the mesolimbic dopaminergic pathways, originating from the VTA and projecting to forebrain nuclei such as NAc may be involved in aggressive behaviours (Fone & Porkess, 2008) as well as psychotic symptoms (Laviolette & Grace, 2006; Watanabe *et al*. 1998).

Atypical antipsychotic drugs are effective in alleviating isolation rearing-induced cognitive impairment and social withdrawal (Feifel *et al*. 2004; Li *et al*. 2007*a*; Toua *et al*. 2010) and recent findings suggest that CB_1R antagonists possess a pharmacological profile reminiscent of atypical antipsychotics (Guidali *et al*. 2010). In fact, our previous work demonstrated the ability of

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chronic treatment with the CB_1R antagonist AM251 to reverse psychotic-like symptoms in a pharmacological model of aspects of schizophrenia based on chronic-intermittent PCP injections (Guidali *et al*. 2010), suggesting a potential antipsychotic action of this compound.

In the present work, we further investigated the potential antipsychotic effect of acute and chronic AM251 treatment on isolation rearing-induced behavioural and neurochemical alterations. Acute AM251 administration did not reverse isolation-induced cognitive impairment and social withdrawal, suggesting that a single AM251 administration may not be sufficient to reach a beneficial effect. The results obtained with chronic AM251 treatment appear more interesting. To the best of our knowledge, this is the first study evaluating the effect of a chronic manipulation of the endocannabinoid system in rats reared in isolation. Rats were chronically administered with AM251 (or vehicle) daily for 3 wk, as reported previously, for the atypical antipsychotic clozapine (Li *et al*. 2007*b*). Interestingly, the alterations observed in rats reared in isolation have been reported to be particularly influenced by chronic handling (Sciolino *et al*. 2010) and this might be taken into consideration when performing a chronic pharmacological treatment in socially isolated rats. In our model, chronic handling did not affect the cognitive impairment induced by isolation whereas the aggressive behaviours were reduced by handling but still remained significantly elevated when compared to group-housed controls. Intriguingly, chronic AM251 alone did not affect the cognitive functions in group housed controls. However, chronic treatment of AM251 to isolation-reared rats significantly improved the performance in the NOR test and reduced the aggressive behaviours in the social interaction test. It is worth noting that this recovery persisted up to 10 d after discontinuing the treatment, indicating a long-lasting effect of the cannabinoid antagonist on psychotic-like symptoms. This is an intriguing property of AM251 since, following treatment with antipsychotic drugs, patients have shown a relapse of psychotic symptoms when taken off the drug (Li *et al*. 2007*a*) and, moreover, antipsychotics have been associated with untoward effects upon withdrawal (Lee & Robertson, 1997).

The mechanisms underlying the beneficial effects of AM251 on psychotic-like symptoms are still unclear. We have clearly demonstrated that psychotic symptoms in isolation-reared rats are accompanied by alterations in the endocannabinoid system, thus we first investigated a direct effect of AM251 treatment on CB_1R functionality. Chronic AM251 had no effect on CB_1R density in grouped or isolated rats. The alterations in CB_1R/G protein coupling reported in isolated rats in the PFC, NAc, Hippo, and VTA were still evident after 3 wk of chronic vehicle treatment, except for the reduction in the CPu that seemed to have been counteracted by chronic handling. Interestingly, chronic AM251 completely restored CB_1R functionality in the PFC, NAc, Hippo, and VTA in isolated rats without having *per se* any effect in all brain areas analysed. Since a decreased CB_1R functionality in the PFC and Hippo has been associated with cognitive impairment (Bilkei-Gorzo *et al*. 2005; Eggan *et al*. 2010; Hill *et al*. 2005), we can speculate that the ability of AM251 to normalize the CB_1R functionality in these areas may underlie the restoration of cognitive functions in isolation-reared rats. Similarly, if a reduction in CB_1 functionality in the mesolimbic pathway could account for the increased dopamine in the NAc responsible for aggressive behaviours (van Erp & Miczek, 2000), the ability of AM251 to restore normal CB_1R functionality in this pathway may, at least in part, contribute to the recovery of aggressiveness.

Finally, we investigated c-Fos immunoreactivity in the PFC, CPu, and NAc of isolation-reared rats to identify activated neurons and extended circuits since c-Fos is the most widely used functional anatomical marker of activated neurons within the central nervous system (Kovacs, 2008). Isolation significantly reduced c-Fos immunoreactivity in the PFC and CPu without affecting it in the NAc. It has previously been demonstrated that a decrease in c-Fos expression in the PFC after social isolation (Levine *et al*. 2007) and reduced neuronal activity in this area may be associated with negative symptoms of schizophrenia such as social withdrawal observed after isolation (Perlstein *et al*. 2003; Weinberger & Berman, 1988). In this paper, we extended the analyses of c-Fos immunoreactivity to other forebrain regions and our results contribute to the improvement of knowledge on the effects of isolation rearing on neuronal activity. However, further investigations are needed to clarify the functional significance of the alterations observed.

To further support the hypothesized antipsychotic properties of AM251, we investigated its effects on c-Fos protein expression. Chronic AM251 treatment counteracted the reduction in c-Fos expression in the PFC and CPu of isolated rats without altering it in group-housed controls. In the NAc, AM251 *per se* significantly increased c-Fos expression in group- and isolation-reared rats. These areas are reported to be fundamental for determining the therapeutic outcome of antipsychotic drugs. As demonstrated previously, both typical and atypical antipsychotics increase c-Fos

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levels in the NAc and this effect appears to be predictive of an antipsychotic action (Fujimura *et al*. 2000; Oka *et al*. 2004; Wan *et al*. 1995). Moreover, AM251 increased c-Fos in the NAc, thus suggesting its potential antipsychotic action. Additionally, a boost in c-Fos expression in the PFC is correlated to a superior efficacy against negative symptoms of schizophrenia (Deutch & Duman, 1996; Robertson & Fibiger, 1996), thus the increase in c-Fos immunoreactivity due to AM251 treatment in this area may account for the reduction of aggressiveness observed in isolated rats and further support the antipsychotic potential of this cannabinoid antagonist. Finally, several studies have shown that c-Fos expression in rat CPu is a reliable index of the extrapyramidal symptom liability of antipsychotic drugs (Marchese *et al*. 2008; Robertson *et al*. 1994; Wan *et al*. 1995). In our model, AM251 re-established a normal striatal neuronal activity without further enhancement of c-Fos over the control levels rather than as observed for typical antipsychotics (Marchese *et al*. 2008; Robertson *et al*. 1994; Wan *et al*. 1995). This might be suggestive of a drug nearly devoid of extrapyramidal side-effects, as already demonstrated for clozapine (Marchese *et al*. 2008; Robertson *et al*. 1994; Wan *et al*. 1995).

In conclusion, our results suggest a potential antipsychotic role of the cannabinoid antagonist AM251 in a neurodevelopmental model of psychotic-like symptoms. Acute AM251 administration is not sufficient to reach a therapeutic effect whereas chronic treatment counteracts both aggressive behaviour and cognitive impairment induced by isolation. The behavioural recovery appears to be mediated by the rescue of CB_1R functionality altered by isolation in specific brain areas that may impact neuronal activation, as demonstrated by c-Fos immunoreactivity, as well as other neurotransmitter systems.

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Statement of Interest

None.

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