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RESEARCH PAPER

Chronic blockade of CB_1 receptors reverses startle gating deficits and associated neurochemical alterations in rats reared in isolation

E Zamberletti[1], F Piscitelli[3], F Cadeddu[4], T Rubino[1], W Fratta[4], P Fadda[4], V Di Marzo[3] and D Parolaro[1,2]

[1]*Department of Theoretical and Applied Sciences, Biomedical Division and Center of Neuroscience, University of Insubria, Busto Arsizio (VA), Italy,* [2]*Zardi-Gori Foundation, Milan, Italy,* [3]*Endocannabinoid Research Group, Institute of Biomolecular Chemistry, CNR, Pozzuoli (NA), Italy, and* [4]*B.B. Brodie Department of Neuroscience, University of Cagliari, Cagliari, Italy*

Correspondence
Daniela Parolaro, DiSTA and Neuroscience Center, University of Insubria, Busto Arsizio (VA), Italy. E-mail: daniela.parolaro@uninsubria.it

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BACKGROUND AND PURPOSE
Pharmacological interventions aimed at restoring the endocannabinoid system functionality have been proposed as potential tools in the treatment of schizophrenia. Based on our previous results suggesting a potential antipsychotic-like profile of the CB_1 receptor inverse agonist/antagonist, AM251, here we further investigated the effect of chronic AM251 administration on the alteration of the sensorimotor gating functions and endocannabinoid levels induced by isolation rearing in rats.

EXPERIMENTAL APPROACH
Using the post-weaning social isolation rearing model, we studied its influence on sensorimotor gating functions through the PPI paradigm. The presence of alterations in the endocannabinoid levels as well as in dopamine and glutamate receptor densities was explored in specific brain regions following isolation rearing. The effect of chronic AM251 administration on PPI response and the associated biochemical alterations was assessed.

KEY RESULTS
The disrupted PPI response in isolation-reared rats was paralleled by significant alterations in 2-AG content and dopamine and glutamate receptor densities in specific brain regions. Chronic AM251 completely restored normal PPI response in isolated rats. This behavioural recovery was paralleled by the normalization of 2-AG levels in all the brain areas analysed. Furthermore, AM251 partially antagonized isolation-induced changes in dopamine and glutamate receptors.

CONCLUSIONS AND IMPLICATIONS
These results demonstrate the efficacy of chronic AM251 treatment in the recovery of isolation-induced disruption of PPI. Moreover, AM251 counteracted the imbalances in the endocannabinoid content, specifically 2-AG levels, and partially reversed the alterations in dopamine and glutamate systems associated with the disrupted behaviour. Together, these findings support the potential antipsychotic-like activity of CB_1 receptor blockade.

LINKED ARTICLES
This article is part of a themed section on Cannabinoids. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.167.issue-8

Abbreviations
2-AG, 2-arachidonoylglycerol; AEA, anandamide; Amy, amygdala; CPu, caudate putamen; ECS, endocannabinoid system; Hippo, hippocampus; NAc, nucleus accumbens; OEA, oleoylethanolamide; PCP, phencyclidine; PEA, palmitoylethanolamide; PFC, prefrontal cortex; PND, post-natal day; PPI, pre-pulse inhibition; Thal, thalamus

Introduction

Considerable evidence from animal and human studies suggests a role for the endocannabinoid system (ECS) in the pathophysiology of schizophrenia (Rubino *et al.*, 2012). Indeed, dysfunctions in several components of the ECS have been reported in schizophrenic patients (Leweke *et al.*, 1999; Dean *et al.*, 2001; De Marchi *et al.*, 2003; Giuffrida *et al.*, 2004; Zavitsanou *et al.*, 2004; Dalton *et al.*, 2011) as well as in different animal models of schizophrenia-like behaviours (Malone *et al.*, 2008; Viganò *et al.*, 2009; Eisenstein *et al.*, 2010; Guidali *et al.*, 2010; Robinson *et al.*, 2010; Sciolino *et al.*, 2010; Seillier *et al.*, 2010; Zamberletti *et al.*, 2012a), thus suggesting their possible involvement in the pathogenesis of the disease. Further, experimental data has been obtained suggesting that antagonism of CB_1 receptors could represent a promising pharmacological tool for novel approaches in the treatment of schizophrenia. Accordingly, we recently demonstrated that chronic administration of the CB_1 receptor antagonist, AM251, exerts a beneficial action in terms of reversing the behavioural alterations induced by either chronic-intermittent phencyclidine (PCP) administration or an isolation rearing procedure, two established animal models of schizophrenia-like symptoms in rats (Guidali *et al.*, 2010; Zamberletti *et al.*, 2012a). More intriguingly, in both models, behavioural recovery was paralleled by normalization of PCP- and isolation-induced alterations in CB_1 receptor density and efficiency (Guidali *et al.*, 2010; Zamberletti *et al.*, 2012a). Furthermore, in the PCP model, AM251 significantly increased AEA levels in the prefrontal cortex (PFC), without affecting the concentrations of two cannabinoid receptor-inactive congeners of this endocannabinoid, oleoylethanolamide (OEA) or palmitoylethanolamide (PEA) content, while simultaneously counteracting the PCP-induced increase in the levels of the other major endocannabinoid, 2-arachidonoylglycerol (2-AG) (Guidali *et al.*, 2010). Consistent with a potential antipsychotic action, AM251 normalized the reduction in neuronal activity induced by either PCP treatment or isolation rearing procedure (Guidali *et al.*, 2010; Zamberletti *et al.*, 2012a), showing an effect similar to that of the atypical antipsychotic, clozapine.

One important endophenotype of schizophrenia is the impairment of sensorimotor gating (Gotesman and Gold, 2003). The most reliable paradigm for measuring abnormalities of sensorimotor gating is the pre-pulse inhibition (PPI) of the acoustic startle reflex. Notably, PPI can be assessed across species, using similar stimuli to elicit comparable response characteristics, and changes in this paradigm have been used to predict antipsychotic efficacy (Swerdlow and Geyer, 1998). Since PPI deficits observed in schizophrenic patients can be modelled in rats by the post-weaning social isolation procedure and are reversed by conventional neuroleptics (Bakshi *et al.*, 1998), in the present study, we examined the potential antipsychotic effect of chronic AM251 administration on PPI responses in rats reared in isolation from weaning. Moreover, to clarify a possible contribution of imbalances in endocannabinoid content to the development of schizophrenia-like traits in isolated animals, we checked for possible changes in AEA, 2-AG, PEA and OEA levels following the isolation rearing procedure in specific brain regions [PFC, NAc, CPu and hippocampus (Hippo)], where alterations in CB_1 receptors occur (Zamberletti *et al.*, 2012a). The same biochemical analyses were performed following chronic AM251 treatment, in order to further our understanding of the molecular mechanisms responsible for the recovery observed both at the behavioural level and with regard to CB_1 receptor alterations. Finally, to assess whether biochemical changes in the ECS could influence other neurocircuits and information processing within the CNS, changes in glutamate NMDA as well as dopamine D_1 and D_2 receptor densities were also investigated following isolation rearing and AM251 treatment.

Methods

Animals and isolation rearing procedure

Lister Hooded dams were received from Harlan (S. Pietro al Natisone, Italy) each one with eight pups aged 14 days on arrival. At weaning (PND 21), male rats 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, kept on a 12 h light–dark cycle (lights on 08:00 h) and in a temperature- (22 ± 2°C) and humidity-controlled environment (50 ± 10%). All animals had free access to food and water. The isolated animals were left undisturbed in their cages and received the minimal handling associated with husbandry (cage and bedding changed weekly).

All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All experiments were carried out during the light phase and 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.

Drug treatment

AM251 (Tocris Bioscience, Bristol, UK) was dissolved in DMSO, Tween-80 and saline (1:1:8). The drug was administered at 0.5 $mg \cdot kg^{-1}$ i.p. (5 $mL \cdot kg^{-1}$). After 5 weeks of isolation or group rearing, animals were treated with AM251 or its vehicle daily for 3 weeks. Behavioural and biochemical tests were performed 24 h after the last injection (Figure 1).

Pre-pulse inhibition of startle reflex apparatus

The startle reflex system is composed of four standard cages each placed in a sound-attenuated and ventilated chamber (Med Associates Inc., St. Albans, VT, USA). Plexiglas cylinders (diameter 9 cm), closed by two doors to restrict the animals, were mounted on a piezoelectric accelerometer platform connected to an analogue–digital converter. Background noise and acoustic bursts were conveyed through two speakers placed in proximity to the startle cage so as to produce a variation in sound intensity within 1 dB. On the test day, each rat was placed in the experimental cage for a 5 min acclimatization period with a 70 dB white noise background; this was continued for the remainder of the session. Animals were then tested on three consecutive trial blocks. The first

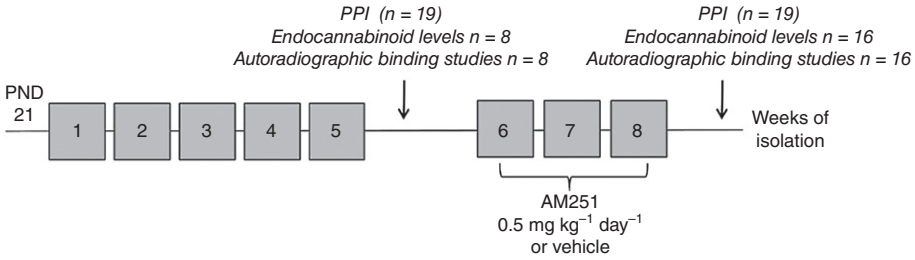

Figure 1
Treatment schedule.

and the third blocks consisted of five pulse-alone trials of 40 ms at 115 dB, while the second block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 74, 78 or 82 dB pre-pulses (10 for each level of pre-pulse loudness) and eight no-stimulus trials (where only the background noise was delivered). The % PPI was calculated based only on the values relative to the second block, and using the following formula: 100 – [(mean startle amplitude for pre-pulse + pulse trials/ mean startle amplitude for pulse-alone trials) × 100].

Endocannabinoid levels

Separate animals not subjected to the PPI procedure were used for the dissection of PFC, CPu, NAc and Hippo after 5 weeks of isolation or social rearing and 24 h after the last AM251 (or vehicle) injection. The samples collected were immediately frozen in liquid nitrogen and stored at –80°C.

Extraction and quantification of endocannabinoids

Frozen tissue samples were homogenized in chloroform/ methanol/Tris–HCl 50 mM pH 7.4 (2:1:1, v v^{-1}) containing 10 pmol of [^{2}H]-8-AEA, [^{2}H]-4-PEA and [^{2}H]-4-OEA, and 50 pmol of [^{2}H]-5-2-AG as internal deuterated standards (purchased from Cayman Chemicals, Ann Arbor, MI). The extract was purified by means of silica gel mini-columns as described in Bisogno *et al*. (1997), and the eluted fraction containing AEA and 2-AG was analysed by means of liquid chromatography-atmospheric pressure-MS (LC-APCI-MS) conducted as described previously (Marsicano *et al*., 2002). Analyses were carried out in the selected ion-monitoring mode using *m*/*z*-values of 356 and 348 (molecular ions +1 for deuterated and undeuterated AEA), 304 and 300 (molecular ions +1 for deuterated and undeuterated PEA), 330 and 326 (molecular ions +1 for deuterated and undeuterated OEA), and 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG). AEA, OEA, PEA and 2-AG concentrations were calculated by isotope dilution and are expressed as pmol g^{-1} or mg^{-1} of wet tissue weight. The concentrations of 2-AG were obtained by adding up to the amounts of the 2-isomer also those of the 1(3)-isomer, which mostly originates from the isomerization of the former during work-up.

Autoradiographic binding studies

Rats were decapitated; and brains were rapidly removed, frozen in liquid nitrogen and stored at –80°C. Coronal sections (20 µm thick) were cut on a cryostat, mounted on gelatin-coated slides and stored at –80°C until processing.

[^{3}H]-SCH23390 and [^{3}H]-raclopride receptor autoradiographic binding

The experiments were performed as previously described by Zamberletti *et al*. (2012b).

[^{3}H]-MK-801 receptor autoradiographic binding

The experiment was performed as previously described by Newell *et al*. (2007). Sections were incubated at room temperature for 2.5 h in 30 mM HEPES buffer containing 20 nM [^{3}H]-MK801 (Perkin Elmer Life Sciences, Milan, Italy) and exposed to Kodak Biomax MR films for 5 weeks.

Image analysis

The intensity of the autoradiographic films was assessed as previously reported in Rubino *et al*. (2000). Data are expressed as fmol·mg^{-1} of tissue.

Statistical analysis

PPI data were analysed by use of Student's unpaired *t*-test or two-factor ANOVA with rearing condition or drug treatment as between-subjects factor and trial type (pre-pulse intensity) as repeated measure (within-subjects factor) followed by Bonferroni's *post hoc* test. Biochemical results were analysed by Student's unpaired *t*-test or a two-way ANOVA with housing conditions and drug treatment as independent variables, followed up by Bonferroni's *post hoc* test. All data are expressed as mean ± SEM. The level of statistical significance was set at $P < 0.05$.

Results

Behavioural and neurochemical characterization after 5 weeks of isolation rearing

PPI response. Figure 2 represents the effect of rearing condition on % pre-pulse inhibition startle magnitude (% PPI).

Both housing condition and AM251 treatment did not alter the magnitude of the startle response (data not shown).

At a pre-pulse intensity of 74 dB, isolation-reared rats showed a significant lower %PPI than group-housed controls

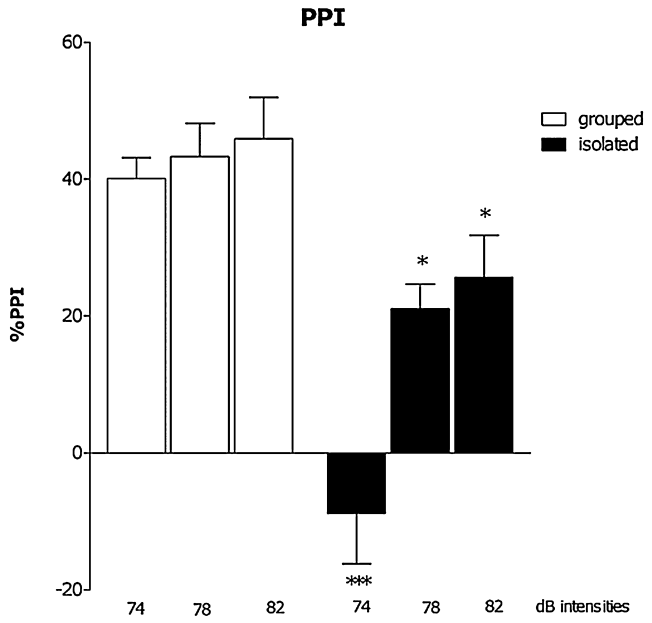

Figure 2

The %PPI after 5 weeks of isolation rearing. Data are expressed as the average PPI response of 10 animals per group over the three pre-pulse intensities. ***$P < 0.001$; *$P < 0.05$ versus grouped at the respective dB intensity (Student's unpaired *t*-test).

($t = 6.756$; $P < 0.0001$). A significant disruption in the PPI response, although less intense, was still evident in isolated rats at a pre-pulse intensity of 78 dB ($t = 3.466$; $P = 0.0026$) and 82 dB ($t = 2.491$; $P = 0.0221$) compared with social controls.

Endocannabinoid levels. Figure 3 shows the effect of 5 weeks of social isolation on endocannabinoid levels in the PFC, NAc, CPu and Hippo.

In the PFC, social isolation induced a significant reduction in 2-AG levels by about 52% ($t = 2.525$; $P = 0.0450$) compared with group-reared controls. Moreover, a significant increase in PEA content (+59%) was evident in isolated rats ($t = 3.827$; $P = 0.0187$). No changes were found in AEA and OEA levels following 5 weeks of isolation rearing (AEA: $t = 0.3344$; $P = 0.7494$; OEA: $t = 0.9077$; $P = 0.3990$).

Similarly, in the NAc, a significant reduction in 2-AG content (–23%) was present following the isolation rearing procedure ($t = 2.744$; $P = 0.0336$). No changes in AEA, PEA and OEA levels were observed (AEA: $t = 0.4032$; $P = 0.7035$; PEA: $t = 0.1621$; $P = 0.8765$; OEA: $t = 1.183$; $P = 0.2814$).

Finally, isolated animals showed a significant increase in 2-AG levels in both the CPu ($t = 3.051$; $P = 0.0284$) and hippocampus ($t = 2.957$; $P = 0.0316$) by about 170% and 67% respectively. AEA, PEA and OEA levels did not differ from controls in these two brain areas (CPu: AEA: $t = 0.3681$; $P = 0.7254$; PEA: $t = 0.2863$; $P = 0.7843$; OEA: $t = 1.091$; $P = 0.3250$. Hippo: AEA: $t = 1.106$; $P = 0.3112$; PEA: $t = 0.2914$; $P = 0.7805$; OEA: $t = 1.166$; $P = 0.2877$).

Dopamine D_1 and D_2 receptor densities. The effect of social isolation on dopamine D_1 and D_2 receptor densities in the PFC, CPu and NAc is illustrated in Figure 4A.

Isolated animals showed a significant reduction in D_1 receptor density in the PFC by about 71% compared with group-reared controls ($t = 6.680$; $P < 0.0001$). No significant changes in [^{3}H]-SCH23390 binding were observed in the CPu ($t = 1.651$; $P = 0.1130$) and NAc ($t = 1.480$; $P = 0.1529$) following the isolation procedure.

In contrast, D_2 receptor density was significantly increased in the PFC of isolated rats by about 82% ($t = 5.181$; $P = 0.0001$). Isolation rearing did not alter [^{3}H]-raclopride binding in the CPu ($t = 2.045$; $P = 0.0601$) and NAc ($t = 0.3168$; $P = 0.7560$). Representative autoradiograms of the effect of social isolation on dopamine D_1 and D_2 receptor densities in the PFC are shown in Figure 5.

Glutamate NMDA receptor density. Figure 4B shows the effect of isolation rearing on glutamate NMDA receptor binding in the PFC, CPu, NAc, Hippo, amygdala (Amy) and thalamus (Thal).

A significant decrease in NMDA receptor density was present in the CPu following 5 weeks of isolation rearing ($t = 2.766$; $P = 0.0113$). Isolated animals also displayed a significant increase in [^{3}H]-MK-801 binding in the NAc compared with group-housed controls ($t = 2.566$; $P = 0.0184$). No changes were observed in the PFC ($t = 1.819$; $P = 0.0825$), Hippo ($t = 0.5691$; $P = 0.5751$), Amy ($t = 0.7774$; $P = 0.4452$) and Thal ($t = 0.4034$; $P = 0.6905$) following isolation rearing.

Behavioural and neurochemical characterization after AM251 (or vehicle) treatment in isolated and group-housed rats

PPI response. Figure 6 shows the effect of chronic AM251 (or vehicle) treatment on the PPI response in isolation- and group-reared rats.

Two-way ANOVA revealed a significant effect of rearing conditions on PPI response at 74 and 78 dB intensities (74 dB: $F_{1-15} = 13.41$; $P = 0.0023$; 78 dB: $F_{1-15} = 16.15$; $P = 0.0011$), PPI disruption being still evident in isolation-reared animals compared with group-housed controls following vehicle treatment. The effect of rearing conditions on PPI response was not present at 82 dB intensities ($F_{1-15} = 2.471$; $P = 0.1368$).

AM251 treatment did not affect PPI response in control animals at any of the dB intensities tested (74 dB: $F_{1-15} = 2.263$; $P = 0.1533$; 78 dB: $F_{1-15} = 0.4114$; $P = 0.5309$; 82 dB: $F_{1-15} = 0.02212$; $P = 0.8837$).

In contrast, AM251 administration completely counteracted PPI disruption in isolated rats at 74 dB as well as 78 dB intensities (74 dB: $F_{1-15} = 5.750$; $P = 0.0299$; 78 dB: $F_{1-15} = 4.562$; $P = 0.0496$).

Endocannabinoid levels. Figure 7 represents endocannabinoid levels following chronic AM251 (or vehicle) administration with respect to housing conditions.

Effect of chronic handling on endocannabinoid content. Endocannabinoid levels appeared to be particularly influenced by chronic handling due to vehicle/drug administration in isolated animals. Thus, we first checked whether the handling

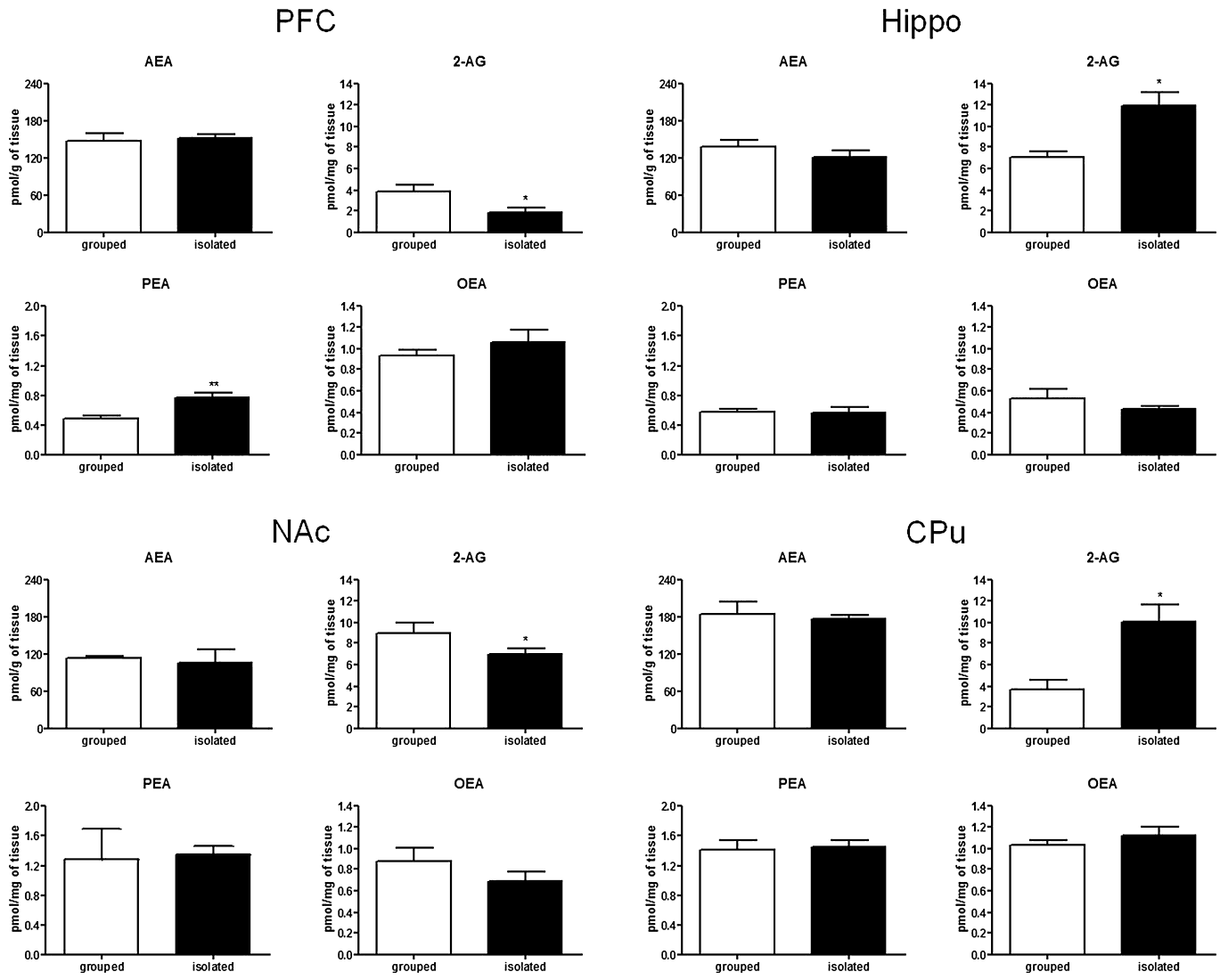

Figure 3

Brain tissue concentrations of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) after 5 weeks of isolation rearing. Brain areas: PFC, CPu, NAc,; Hippo. Data are expressed as the mean ± SEM of four animals per group. $^{**}P < 0.01$; $^{*}P < 0.05$ versus grouped (Student's unpaired *t*-test).

procedure *per se* further modified the isolation rearing-induced alteration in endocannabinoid levels.

In the PFC, a significant effect of handling was evident on OEA content in isolated rats ($F_{1-12} = 9.588$; $P = 0.0092$), OEA levels being reduced in both vehicle- and AM251-treated animals. AM251 administration did not affect OEA content in group-housed controls ($F_{1-12} = 0.08697$; $P = 0.7731$). Moreover, in the same brain regions, handling counteracted isolation-induced increase in PEA levels ($F_{1-12} = 4.83$; $P = 0.036$).

Similarly, in the NAc, a significant handling effect was found on OEA levels ($F_{1-12} = 12.38$; $P = 0.0042$), with a reduction in OEA content in both the vehicle and AM251 treated rats.

Finally, in the CPu and hippocampus, two-way ANOVA revealed a significant main effect of handling on endocannabinoid content ($F_{1-12} = 6.57$; $P = 0.017$). Specifically, chronic handling completely counteracted the isolation-induced increase in 2-AG in the CPu but caused an increase in AEA levels in the hippocampus.

In summary, 3 weeks of handling induced a significant reduction in OEA levels in the PFC and NAc, which were resistant to AM251 treatment, completely reversed the 5 week isolation-induced increase in 2-AG in the CPu and induced an increase in AEA levels in the hippocampus. In contrast, handling did not affect the significant alterations in 2-AG levels measured in the PFC, NAc and hippocampus after 5 weeks of isolation.

Effect of AM251 administration on endocannabinoid content. In the PFC, the social isolation procedure significantly reduced 2-AG levels in vehicle-treated rats ($F_{1-12} = 6.637$; $P = 0.0258$). Interestingly, AM251 administration did not alter 2-AG levels in control animals ($F_{1-12} = 1.472$; $P = 0.2504$) but com-

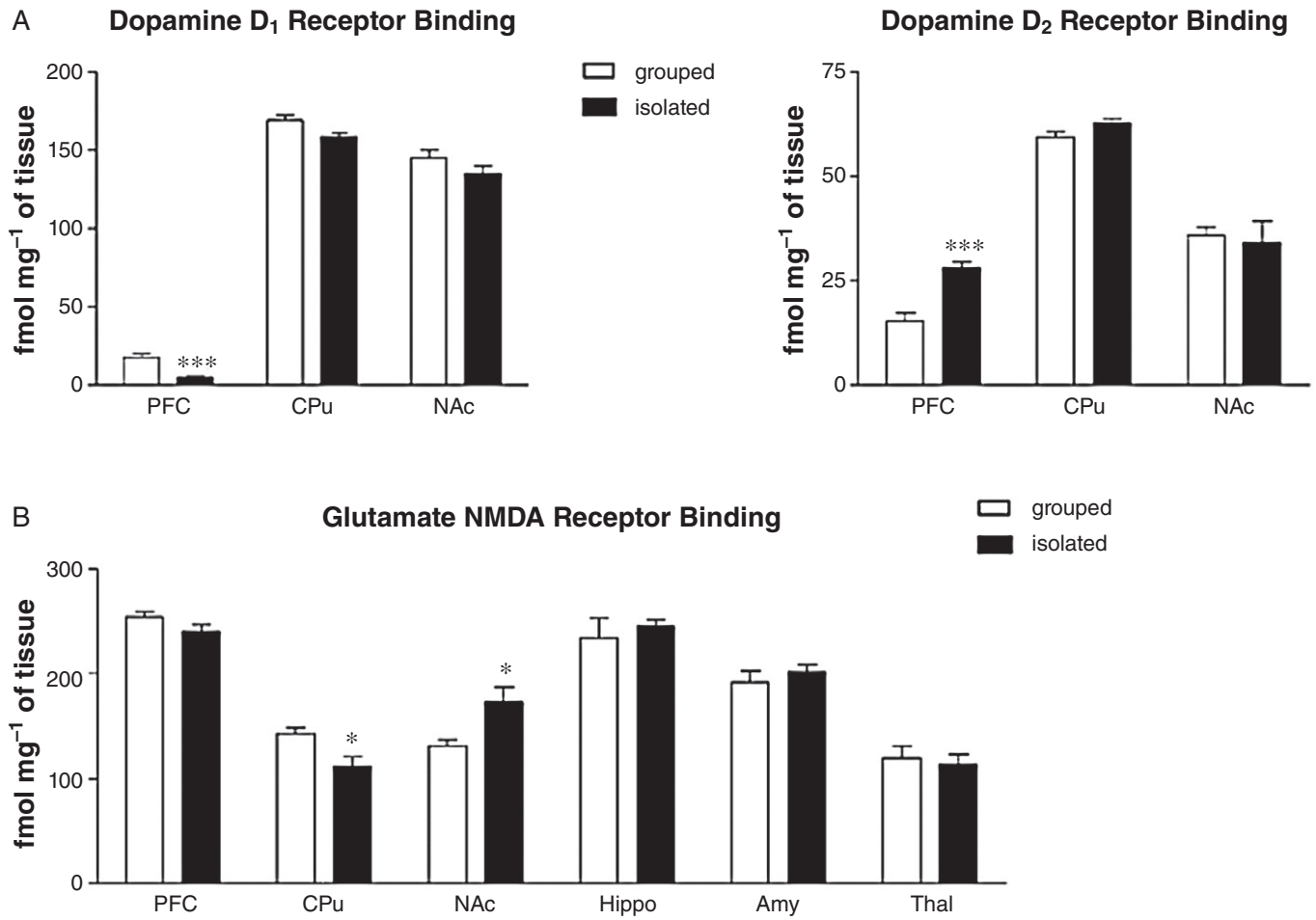

Figure 4

Effect of 5 weeks of isolation rearing on (A) D_1 and D_2 receptor density and (B) NMDA receptor density. D_1 and D_2 receptor densities were assessed through [^{3}H]-SCH23390 and [^{3}H]-raclopride receptor binding respectively. NMDA receptor density was assessed through [^{3}H]-MK801 receptor binding. Results are expressed as fmol·mg^{-1} of tissue. Brain areas: PFC, CPu, NAc, Hippo, Amy, Thal. Data are expressed as mean ± SEM of four animals per group. ***$P < 0.001$; *$P < 0.05$ versus grouped (Student's unpaired *t*-test).

pletely restored 2-AG levels in isolated rats ($F_{1-12} = 10.72$; $P = 0.0074$).

Neither isolation rearing nor AM251 treatment altered AEA and PEA levels in this brain area (AEA: housing condition: $F_{1-12} = 1.732$; $P = 0.2120$; drug: $F_{1-12} = 0.00007932$; $P = 0.9930$. PEA: housing condition: $F_{1-12} = 0.2793$; $P = 0.6068$; drug: $F_{1-12} = 0.06082$; $P = 0.8094$).

Similarly, in the NAc, two-way ANOVA revealed a significant effect of housing conditions on 2-AG content ($F_{1-12} = 10.23$; $P = 0.0095$), its levels being significantly reduced in isolation-reared rats chronically treated with vehicle. AM251 administration counteracted the isolation-induced reduction of 2-AG ($F_{1-12} = 5.167$; $P = 0.0463$) without affecting 2-AG content in control animals ($F_{1-12} = 3.944$; $P = 0.0751$).

No effects of isolation rearing were observed on AEA and PEA content (AEA: $F_{1-12} = 0.2595$; $P = 0.6205$; PEA: $F_{1-12} = 3.298$; $P = 0.0944$); and AM251 treatment did not affect AEA, PEA and OEA levels in this brain region (AEA: $F_{1-12} = 0.6064$; $P = 0.4526$; PEA: $F_{1-12} = 0.2132$; $P = 0.6526$; OEA: $F_{1-12} = 1.103$; $P = 0.3143$).

In the Hippo, AEA ($F_{1-12} = 5.112$; $P = 0.0450$) and 2-AG ($F_{1-12} = 7.271$; $P = 0.0224$) levels were significantly increased in isolated rats compared to group-housed controls; although, as mentioned above, the effect on AEA was probably due to handling. Interestingly, AM251 administration completely reversed the isolation-induced increase in 2-AG ($F_{1-12} = 7.061$; $P = 0.0240$) and the handling-induced increase in AEA ($F_{1-12} = 38.99$; $P < 0.0001$), without affecting per se the AEA and 2-AG levels in group-housed controls (AEA: $F_{1-12} = 2.438$; $P = 0.1467$; 2-AG: $F_{1-12} = 4.493$; $P = 0.0601$).

Neither social isolation nor AM251 administration altered PEA (housing condition: $F_{1-12} = 0.7320$; $P = 0.4123$; Drug: $F_{1-12} = 0.1241$; $P = 0.7319$) or OEA (housing condition: $F_{1-12} = 0.7937$; $P = 0.3921$; Drug: $F_{1-12} = 1.530$; $P = 0.2419$) levels in this brain area.

Finally, in the CPu, the isolation rearing procedure did not alter AEA, 2-AG, PEA or OEA levels (AEA: $F_{1-12} = 0.5577$; $P = 0.4696$; 2-AG: $F_{1-12} = 0.07726$; $P = 0.7862$; PEA: $F_{1-12} = 0.1744$; $P = 0.6836$; OEA: $F_{1-12} = 1.262$; $P = 0.2833$). In contrast, two-way ANOVA revealed a significant effect of drug

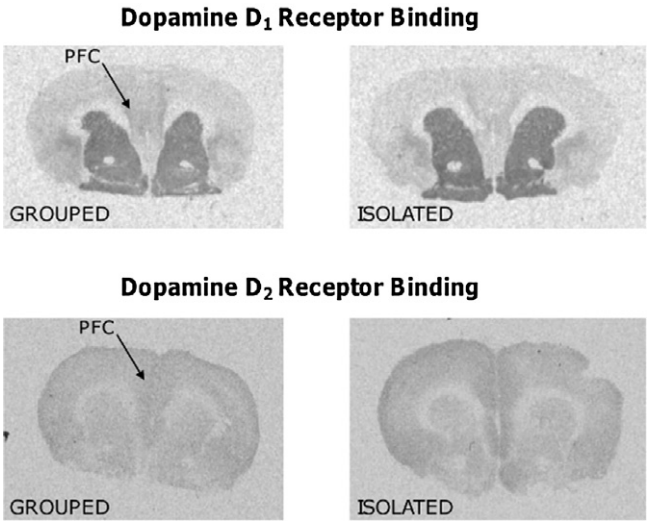

Figure 5

Representative autoradiograms showing the effect of 5 week isolation rearing on D_1 and D_2 receptor density in the PFC.

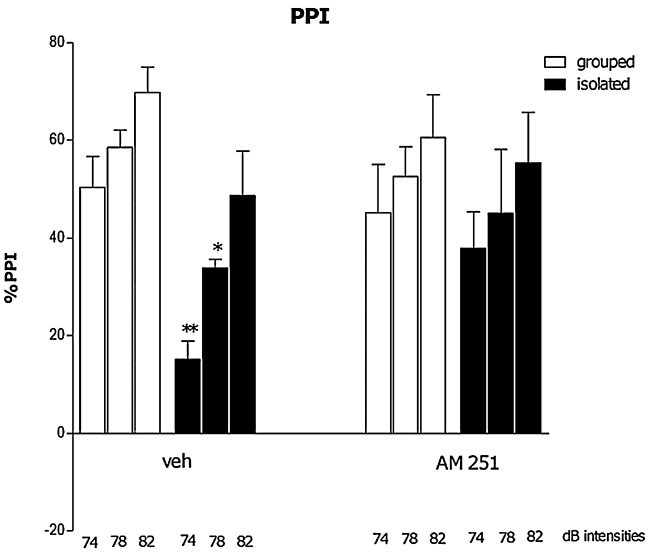

Figure 6

%PPI after chronic AM251 (or vehicle) treatment in isolated and group-housed rats. Data are expressed as the average PPI response of five animals per group over the three pre-pulse intensities. $^{**}P < 0.01$; $^{*}P < 0.05$ versus grouped at the respective dB intensity (Bonferroni's *post hoc* test).

treatment on PEA levels ($F_{1-12} = 10.61$; $P = 0.0069$), with AM251 treatment slightly increasing PEA content in both isolated and group-housed rats. AM251 administration did not alter AEA ($F_{1-12} = 0.03262$; $P = 0.8597$), 2-AG ($F_{1-12} = 1.120$; $P = 0.3127$) or OEA ($F_{1-12} = 0.3095$; $P = 0.5882$) levels in this brain region.

Effect of AM251 administration on dopamine D_1 and D_2 receptor densities. Figure 8A shows the effect of AM251 (or vehicle) administration on dopamine D_1 and D_2 receptor densities in isolated and group-reared animals.

Isolation rearing significantly reduced D_1 receptor density in the PFC ($F_{1-12} = 46.50$; $P < 0.0001$), the effect being evident both in AM251- and vehicle-treated rats. No effect of isolation rearing on [^{3}H]-SCH23390 binding was observed in the CPu ($F_{1-12} = 0.1546$; $P = 0.6970$) and NAc ($F_{1-12} = 2.577$; $P = 0.1193$).

AM251 treatment did not affect [^{3}H]-SCH23390 binding in the PFC ($F_{1-12} = 0.04747$; $P = 0.8291$) or CPu ($F_{1-12} = 0.7729$; $P = 0.3866$). In contrast, two-way ANOVA showed a significant effect of drug treatment in the NAc ($F_{1-12} = 5.439$; $P = 0.0268$), D_1 receptor density being significantly down-regulated in isolation-reared rats ($F_{1-12} = 8.015$; $P = 0.0083$).

In the PFC, statistical analysis revealed a significant effect of housing conditions ($F_{1-12} = 5.360$; $P = 0.0264$), drug treatment ($F_{1-12} = 8.387$; $P = 0.0064$) and housing condition × drug treatment interaction ($F_{1-12} = 20.50$; $P < 0.0001$) on dopamine D_2 receptor density. Isolation rearing significantly increased [^{3}H]-raclopride binding in this brain area, and AM251 administration completely antagonized the isolation-induced alteration.

Neither isolation rearing nor AM251 treatment altered D_2 receptor density in the CPu and NAc (CPu: housing conditions: $F_{1-12} = 0.01988$; $P = 0.8887$; drug treatment: $F_{1-12} = 1.858$; $P = 0.1813$; NAc: housing conditions: $F_{1-12} = 0.1370$; $P = 0.7140$; drug treatment: $F_{1-12} = 0.2175$; $P = 0.6446$).

Effect of AM251 administration on glutamate NMDA receptor density. Figure 8B represents the effect of AM251 (or vehicle) treatment on NMDA receptor density in respect to the different housing conditions.

A significant down-regulation of NMDA receptor density was evident in isolated rats in the CPu ($F_{1-12} = 9.408$; $P = 0.0050$). Moreover, isolation rearing induced a significant up-regulation of the receptor in the NAc ($F_{1-12} = 23.32$; $P < 0.0001$). However, no changes were observed in the PFC ($F_{1-12} = 0.8202$; $P = 0.3734$), Hippo ($F_{1-12} = 3.485$; $P = 0.0728$), Amy ($F_{1-12} = 0.8727$; $P = 0.3599$) or Thal ($F_{1-12} = 0.4155$; $P = 0.5248$) following isolation rearing.

AM251 treatment did not affect [^{3}H]-MK-801 binding in group-reared controls in any of the brain areas analysed but completely restored isolation rearing-induced alterations in the CPu (drug: $F_{1-12} = 6.718$; $P = 0.01550$; housing conditions x drug interaction: $F_{1-12} = 4.404$; $P = 0.0457$) and NAc (drug: $F_{1-12} = 6.254$; $P = 0.0190$; housing conditions x drug interaction: $F_{1-12} = 14.01$; $P = 0.0009$). Moreover, in the Hippo two-way ANOVA revealed a significant housing conditions × drug treatment interaction ($F_{1-12} = 20.38$; $P = 0.0001$), NMDA receptor binding being significantly down-regulated in isolated rats following AM251 treatment.

Discussion and conclusions

Rearing rats in isolation from weaning results in long-term abnormalities on brain structure, neurotransmitter function and behaviour compared with group-housed controls (Lapiz *et al.*, 2003). We previously reported that rats that underwent

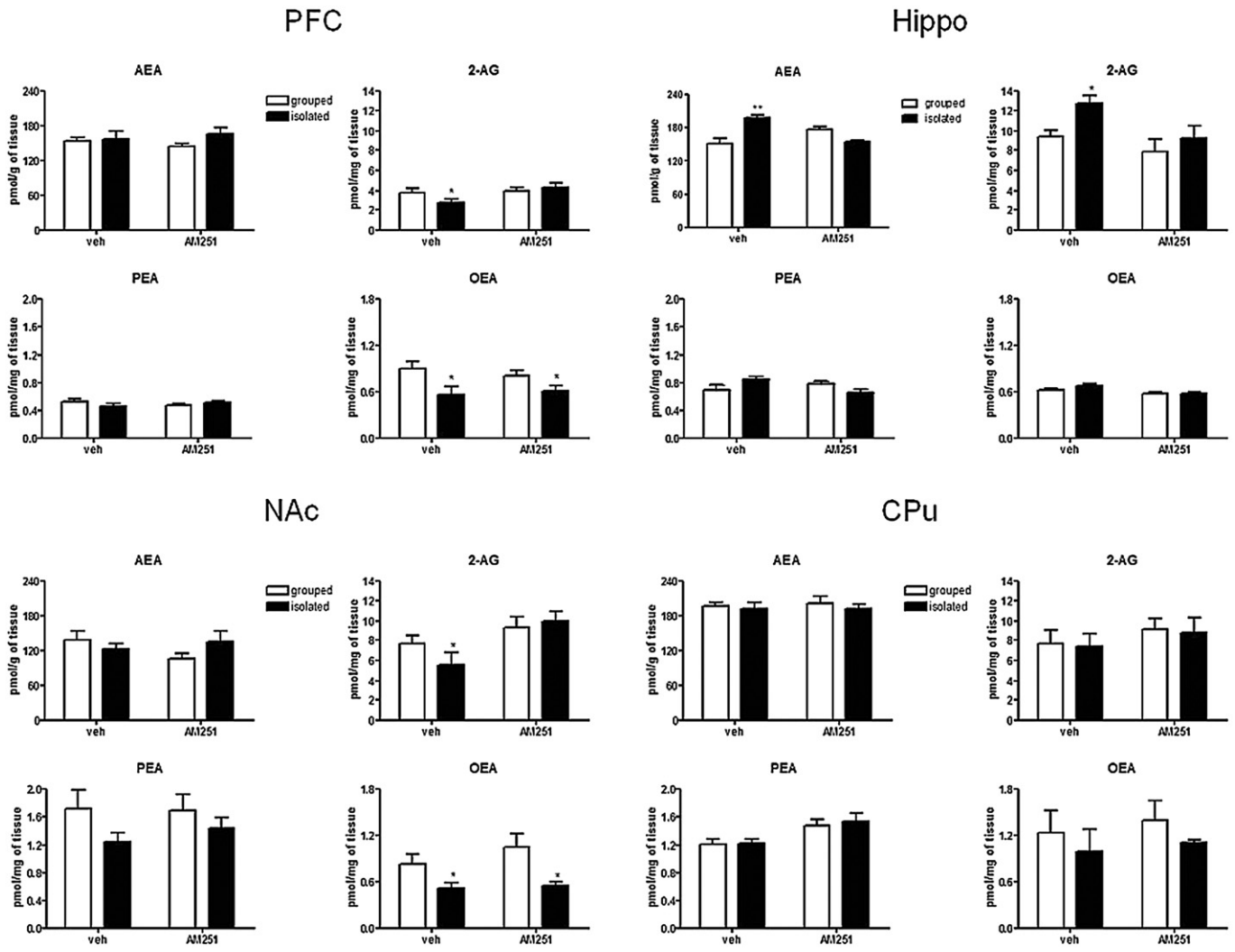

Figure 7

Brain tissue concentrations of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) after chronic AM251 or vehicle treatment in isolated and group-housed rats. Brain areas: PFC, NAc, CPu, Hippo. Data are expressed as the mean ± SEM of four animals per group. $^{**}P < 0.01$; $^{*}P < 0.05$ versus grouped (Bonferroni's *post hoc* test).

5 weeks of isolation rearing showed a marked increase in total horizontal locomotor activity, a cognitive impairment in the novel object recognition test and a significant increase in the number of aggressive behaviours in the social interaction test (Zamberletti *et al.*, 2012a). Consistent with the presence of a schizophrenia-like phenotype, rats reared in social isolation from weaning throughout adulthood showed PPI deficits, which are thought to model the sensorimotor gating deficits seen in schizophrenia (Fone and Porkess, 2008). In line with previous reports, we demonstrated here the presence of robust deficits in PPI response in isolated rats compared with socially reared littermates, thus reinforcing the original findings that this environmental manipulation provides a viable, non-pharmacological model of impaired PPI.

Several lines of evidence suggest that dysregulation of endocannabinoid signalling might be specifically implicated in the behavioural phenotype exhibited by isolated animals (Malone *et al.*, 2008; Robinson *et al.*, 2010). Accordingly, our previous study highlighted the presence of a widespread desensitization of CB_1 receptors in specific brain regions of isolated rats (Zamberletti *et al.*, 2012a), suggesting the possibility of an enhanced endocannabinoid tone in isolation-reared rats. In the present study, significant differences in the endocannabinoid content were evident in isolated rats compared with group-housed controls in all the brain regions analysed. The most intriguing finding was that these alterations mainly involved the endocannabinoid 2-AG, the levels of which were differentially regulated in isolated rats depending on the brain area considered. Particularly, 2-AG levels were significantly higher in the CPu and Hippo, whereas significant decreases were present in the PFC and NAc following 5 weeks of isolation rearing. No changes in the other endocannabinoid, AEA, or in the AEA-related mediators, OEA and PEA, occurred in any of the brain regions under investigation, except for a significant increase in PEA levels in the PFC.

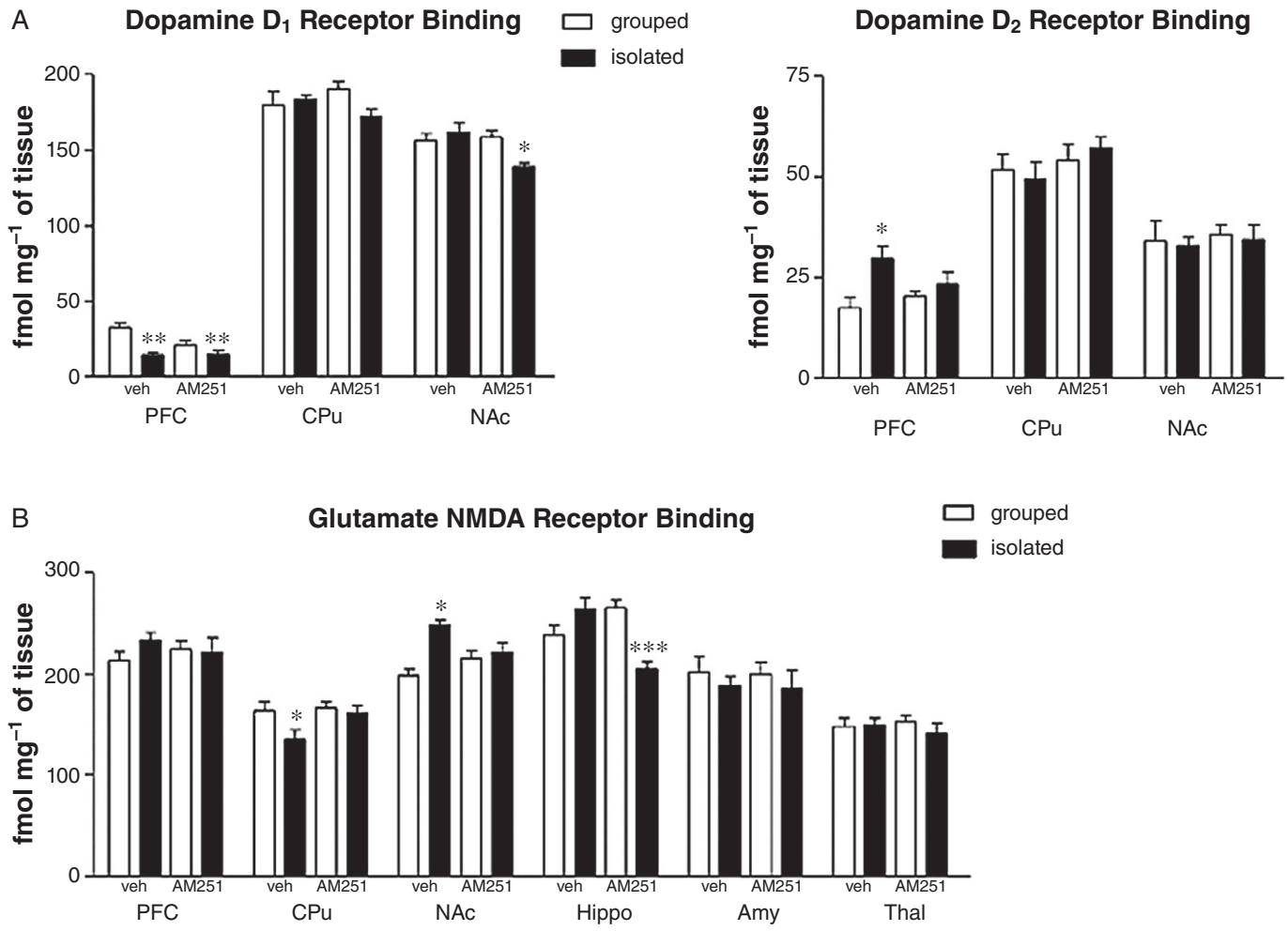

Figure 8

Effect of chronic AM251 (or vehicle) treatment on (A) D_1 and D_2 receptor density and (B) NMDA receptor density. D_1 and D_2 receptor densities were assessed through [^{3}H]-SCH23390 and [^{3}H]-raclopride receptor binding respectively. NMDA receptor density was assessed through [^{3}H]-MK801 receptor binding. Results are expressed as fmol·mg^{-1} of tissue. Brain areas: PFC, CPu, NAc, Hippo, Amy, Thal. Data are expressed as mean ± SEM of four animals per group. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ versus grouped (Bonferroni's *post hoc* test).

Previous studies demonstrated that environmental stressors alter the expression of enzymes involved in 2-AG biosynthesis and degradation in brain regions controlling emotion and motor behaviour (Sutt *et al.*, 2008; Suarez *et al.*, 2010). Moreover, the presence of alterations in 2-AG content following the isolation rearing procedure is further supported by the recent findings of Robinson *et al.* (2010), demonstrating the presence of widespread alterations in DAGLα and DAGLβ mRNA as well as MAGL mRNA in isolation-reared rats. In line with these data, we speculate that the alteration in 2-AG tissue concentrations reported here might be due to an alteration in the enzymes involved in 2-AG synthesis or degradation. To date, only the work of Sciolino *et al.* (2010) directly investigated the presence of alterations in the endocannabinoid levels in isolated rats. In this study, increases in 2-AG levels were present in the PFC, while no changes were found in the NAc and Hippo. This discrepancy with our present findings could be ascribed to the different isolation rearing protocol as well as to the different rat strain used, since strain differences in the isolation-induced alterations have already been reported for other behavioural parameters (Weiss *et al.*, 2000).

As a whole, these data clearly indicate that post-weaning social isolation procedures in rats induce marked alterations in specific components of the ECS, suggesting that abnormal endocannabinoid signalling could represent one of the molecular underpinnings of isolation-induced behavioural deficits. Inasmuch as the endocannabinoid system plays a homeostatic role during brain development, it is tempting to speculate that disrupted endocannabinoid signalling could negatively affect the maturation of other neurotransmitter systems within the CNS, leading to abnormal neurotransmission. For this reason, we also investigated the possible effect of altered endocannabinoid neuromodulatory function on the interface between glutamatergic and dopaminergic function by assessing the PPI response and dopamine D_1 and D_2 as well as NMDA receptor densities in isolated and group-housed rats. Interestingly, a significant increase in D_1 receptor

and a significant reduction in D_2 receptor density were observed in the PFC of isolated rats compared with group-reared controls. An imbalance between D_1 and D_2 receptors has been suggested to contribute to the symptoms of schizophrenia (Scott and Aperia, 2009). In agreement with our present findings, a PET study showed that schizophrenic patients have reduced dopamine D_1 receptor binding in the PFC, which is related to the severity of the negative symptoms (Sedvall and Farde, 1996; Okubo *et al.*, 1997), and, recently, a reduction in D_1 receptor density was reported in the PFC of isolated rats (Toua *et al.*, 2010). Moreover, the increase in D_2 receptors we observed in the PFC of isolated animals agrees with first PET studies suggesting that dopamine D_2 receptors are indeed up-regulated in schizophrenia patients (Wong *et al.*, 1986). Based on the suggestion of a strong interaction between D_1 and D_2 second messenger systems (Seeman *et al.*, 1989; Strange, 1991), it is possible that the opposite effect of isolation rearing on D_1 and D_2 receptor densities might reflect a compensatory mechanism. Furthermore, both D_1 and D_2 receptors are involved in the regulation of PPI in rats (Geyer *et al.*, 2001), with most of the evidence indicating a major contribution of D_2 rather than D_1 receptors to this behaviour. However, a role for D_1 receptors in the modulation of PPI has been also highlighted (Ralph-Williams *et al.*, 2003); thus, it is more likely that D_1 and D_2 receptors work together to modulate PPI in rats. As a whole, these data suggest the presence of altered dopamine transmission in the PFC following isolation rearing procedure.

Intriguingly, changes in NMDA receptor density were also present in rats reared in isolation. In particular, we found a significant reduction in NMDA receptor binding in the CPu paralleled by a significant increase in the NAc. Functional interactions involving dopamine and NMDA receptors have been documented in several forebrain regions and associated with the modulation of locomotor activity and memory processes (Adriani *et al.*, 1998; Tseng and O'Donnell, 2003). Moreover, interactions at the dopamine–glutamate interface have been implicated in the regulation of sensorimotor gating (Wan and Swerdlow, 1996). Therefore, altered dopamine and glutamate transmission in the forebrain regions might underlie the disrupted behaviour we observed in isolated rats.

Although a causal link cannot be established based uniquely on our present data, it is tempting to hypothesize that aberrant endocannabinoid signalling, such as that observed here in isolation-reared rats, could negatively impact on normal behaviour and neurotransmission in these animals and contribute, through this mechanism, to the behavioural alterations that accompany this condition. In this scenario, pharmacological interventions aimed at restoring normal endocannabinoid transmission could be effective in reversing isolation-induced PPI deficits as well as the related neurochemical alterations. Intriguingly, chronic administration of the CB_1 receptor inverse agonist/antagonist, AM251, was effective at reversing aggressive behaviours and cognitive impairments induced by isolation rearing (Zamberletti *et al.*, 2012a). In this study, AM251 did not affect PPI responses in group-reared animals but completely counteracted PPI deficits in isolation-reared rats. Since both typical and atypical antipsychotic treatments are effective in normalizing PPI responses in schizophrenic patients as well as in isolated rats (Kumari and Sharma, 2002; Powell and Geyer, 2002), the beneficial effect of AM251 is consistent with a potential antipsychotic-like profile of this compound. However, the mechanisms underlying this antipsychotic effect of AM251 are still unclear. We previously demonstrated that chronic administration of this compound completely restored CB_1 receptor functionality in isolated rats (Zamberletti *et al.*, 2012a); therefore, in the present work, we investigated a possible effect of AM251 administration on endocannabinoid levels.

Indeed, the ECS appears to be particularly sensitive to environmental stimuli, although not all the isolation-induced alterations in endocannabinoid content are stable and long-lasting. In line with previous reports (Sciolino *et al.*, 2010), there are some isolation-induced changes in endocannabinoid levels that seem to be transient and can be therefore reversed by even a mild environmental manipulation, such as, as shown here, daily handling. In fact, a handling effect was clearly evident in the CPu of isolated rats, where chronic handling completely counteracted the isolation-induced increase in 2-AG content. Since in this same brain area, handling also counteracted the isolation-induced desensitization of CB_1 receptors (Zamberletti *et al.*, 2012a), this latter effect might be ascribed to the normalization of 2-AG levels in this brain area. Consistent with a role for the CPu in the development of aggressiveness, the above-mentioned handling effects might also have contributed to the partial recovery of aggressive behaviours observed in isolated rats following vehicle administration (Zamberletti *et al.*, 2012a).

On the other hand, some of the other isolation-induced changes observed here were less sensitive to chronic handling and might therefore play a more relevant role in the development of the psychotic-like phenotype. Indeed, the reductions in 2-AG levels in the PFC and NAc, as well as the increase of 2-AG in the Hippo, were still evident in isolated rats undergoing 3 weeks of vehicle treatment. More importantly, these persistent alterations in 2-AG contents were completely counteracted by chronic AM251 administration in all the corresponding brain regions. Therefore, given the observation that AM251 concomitantly also reversed the behavioural and schizophrenia-like consequences of isolation, it seems reasonable to suggest that these specific long-lasting changes in endocannabinoid levels may account for the behavioural recovery observed in isolation-reared rats, although further studies are needed to clarify the underlying mechanisms. Even allowing for the many differences between our present model of some symptoms of schizophrenia and the human disorder, our data appear to contrast with the results of a very recent clinical trial by Leweke *et al.* (2012), who showed that a non psychotropic cannabinoid with little direct activity on CB_1 receptors, cannabidiol, produces clinical antipsychotic actions and, concomitantly, elevates AEA serum levels. The authors proposed that the beneficial effect of cannabidiol was due to its previously demonstrated ability to inhibit the enzyme fatty acid amide hydrolase (FAAH) (Bisogno *et al.*, 2001) and hence elevate AEA serum levels thereby indirectly activating CB_1 receptors. However, the authors produced no evidence that cannabidiol was in fact acting via CB_1 receptors, and AEA is also known to act on other molecular

targets. We can reconcile these two sets of data by suggesting the following: if increased 2-AG levels contribute to the development of the pathology, their 'neutralization', either by blocking 2-AG effect at cannabinoid receptors (with AM251) or by inducing a compensative increase in AEA levels (with cannabidiol), which, in turn, may inhibit 2-AG production in the brain (Maccarrone *et al.*, 2008), would result in the observed antipsychotic effects.

Although they were not affected by chronic handling, AM251 administration also partially counteracted the isolation-induced changes in dopamine and glutamate receptors, possibly by restoring normal ECS functionality. It is possible that only persistent alterations in endocannabinoid levels induced by isolation can affect to some extent dopamine and glutamate receptor expression. However, AM251 restored D_2 receptor binding in the PFC of isolated rats but failed to normalize D_1 receptor density in the same brain area. While much attention has been focused on striatal D_2 receptors in schizophrenia, recent evidence has implicated cortical D_2 receptors as the important sites of action of antipsychotic drugs and changes in the dopamine receptors in the cerebral cortex need to be taken into account when evaluating the regulatory actions of neuroleptics (Lidow *et al.*, 1998). Therefore, the observed recovery of the D_2 receptor in the PFC following AM251 treatment could be one of the molecular underpinnings of the antipsychotic-like profile of this compound. Furthermore, a role for the glutamatergic system in the behavioural recovery induced by AM251 administration cannot be ruled out, since AM251 treatment completely restored NMDA receptor binding in the CPu and NAc of isolated animals. Finally, AM251 administration significantly reduced D_1 receptor density in the NAc as well as NMDA receptor binding in the Hippo of isolated rats, without altering *per se* either D_1 or NMDA receptors in group-housed animals. The functional meaning and the possible involvement of these changes in the beneficial effect of AM251 administration are still unclear and need further investigation.

In conclusion, our present results demonstrate that chronic AM251 administration is effective at reversing disrupted PPI in isolation-reared rats, thus providing further evidence for the antipsychotic potential for antagonists of CB_1 receptors. Imbalances in endocannabinoid content, specifically 2-AG levels, could represent one of the molecular abnormalities related to the disrupted behaviour observed in isolated rats, and their normalization may account for AM251-induced recovery of psychotic-like symptoms in this animal model. Furthermore, the present findings indicate the occurrence, following isolation rearing procedure, of dopamine and glutamate disturbances in brain regions relevant to schizophrenia-like behaviours. The ability of AM251 to partially normalize these disturbances may also participate in its antipsychotic action.

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Conflicts of interest

VD is a consultant for GW Pharmaceuticals, UK.

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