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Effects of DNA methylation inhibitors and conventional antidepressants on mice behaviour and brain DNA methylation levels

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Objective: Stress increases DNA methylation and decreases the expression of genes involved in neural plasticity, while treatment with DNA methyltransferase inhibitors (DNMTi) increases gene expression and induces antidepressant-like effects in preclinical models. Therefore, the aim of the present work was to further investigate the potential antidepressant-like effect induced by DNMTi by evaluating the behavioural effects induced by associating DNMTi treatment with conventional antidepressant drugs in mice submitted to the forced swimming test (FST). In addition, brain levels of DNA methylation were also investigated.
Methods: Mice received systemic injections of 5-aza-2'-deoxycytidine (5-AzaD, 0.1, 0.2 mg/kg), RG108 (0.1, 0.2, 0.4 mg/kg), desipramine (DES, 2.5, 5, 10 mg/kg) or fluoxetine (FLX, 5, 10, 20, 30 mg/kg) and were submitted to the FST or to the open field test (OFT). Additional groups received a combination of subeffective doses of 5-AzaD or RG108 (DNMTi) with subeffective doses of DES or FLX (antidepressants).
Results: Subeffective doses of RG108 (0.1 mg/kg) or 5-AzaD (0.1mg/kg) in association with subeffective doses of DES (2.5 mg/kg) or FLX (10 mg/kg) induced significant antidepressant-like effects. Effective doses of RG108 (0.2 mg/kg), 5-AzaD (0.2 mg/kg), DES (10 mg/kg) and FLX (20 mg/kg) atenuated stress-induced changes in DNA methylation levels in the hippocampus and prefrontal cortex. None of the treatments induced locomotor effects in the OFT.
Conclusion: These results suggest that DNMTi potentiate the behavioural effects of antidepressant drugs in the FST and that antidepressants, as well as DNMTi, are able to modulate stress-induced changes in DNA methylation in brain regions closely associated with the neurobiology of depression.

Keywords: Antidepressant; DNA methylation; RG108; 5-AzaD

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Significant outcomes

The results as given below further support a possible antidepressant-like profile for drugs that inhibit DNA methyltransferases.

- Systemic administration of different classes of DNA methyltransferase inhibitors induces antidepressant-like effects.
- The association of subeffective doses of DNA methyltransferase inhibitors with subeffective doses of conventional antidepressant drugs induces antidepressant-like effects.
- DNA methyltransferase inhibitors and antidepressant drugs induces similar changes in stress-induced DNA methylation in the hippocampus and prefrontal cortex.

Limitations

- The present work measured only global levels of DNA methylation, not representing individual changes that would have occurred in candidate genes, which makes it difficult to draw further conclusions on the molecular effects of the drugs used.
- Lack of cell-type specificity and anatomical subdivisions of the prefrontal cortex and the hippocampus for dissecting tissues where DNA methylation levels were analysed.
- Only acute effect of the treatments was investigated in animals submitted to the forced swimming test. Similar analysis conducted in the brains of animals submitted to other animal models as well as the effect of prolonged treatment would add important additional information to the data presented herein.

Introduction

Depression is a psychiatric disorder that encompasses a number of signs and symptoms among which depressed mood and anhedonia are highlighted. In addition, depression is often associated with poor quality of life, disability and increased suicide risk, thus representing a serious health problem (1,2). Monoaminergic antidepressants have been used to treat depression worldwide for >60 years. Although these drugs have revolutionised psychiatry and provided significant mood improvement effects in depressed patients, symptom remission is only achieved after chronic treatment, and even then, about 60% of the individuals do not fully respond to treatment (3). Therefore, the search for a better understanding of the neurobiology of depression, as well as the development of faster-acting and more effective antidepressant treatments have been of great importance.

The mechanism of action of classic antidepressants is primarily based on the inhibition of monoamine uptake or metabolism in the central nervous system (CNS) (4–6). After repeated administration, other molecular mechanisms would be observed, including changes in gene expression and synthesis of proteins that are important to neural plasticity, such as brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate responsive element-binding protein (CREB; 7,8). It has been widely accepted that depression would result from imbalances on monoamines and/or neurotrophin levels in limbic regions as the result of the interaction of genetic and environmental factors, such as exposure to stress (9). In this scenario, antidepressant effects would result from their ability to restore monoamine and/or neurotrophin levels in the brain after repeated treatment (10–12).

It has been recently proposed that epigenetic mechanisms, which involve experience-induced modifications in chromatin structure and gene expression without changing DNA sequence (13), are related to CNS disorders such as schizophrenia, Alzheimer and epilepsy (14–17). These changes in chromatin structure may facilitate or hinder the access of transcriptional machinery, thus altering gene expression and leading to different cellular phenotypes (18). Several studies have shown that mechanisms such as DNA methylation and histone acetylation are involved in the regulation of BDNF expression, plasticity and adult neurogenesis (5,19,20). DNA methylation corresponds to the transfer of a methyl group, catalysed by DNA methyltransferase (DNMT) enzymes to the 5-position cytosine residue in DNA in regions where cytosine–guanine dinucleotide sequences are present (CpG islands), usually resulting in the repression of gene transcription and consequent decrease in protein synthesis (21).

A large number of evidence has shown that stress exposure is able to induce epigenetic changes in the brain, including DNA methylation, which results in reduced expression of several genes that are important for stress coping and resilience, such as BDNF, P11, corticotrophin release factor and glucocorticoid receptors (GR), among others (reviewed in 22). Further corroborating the involvement of these mechanisms in depression neurobiology, increased DNMT expression (23) and increased DNA methylation in specific genomic loci have been reported in the brain of depressed individuals (24–28). Moreover, our research group has recently shown that decreasing DNA methylation by means of pharmacological treatment with 5-AzaD, a DNMT inhibitor (DNMTi) induces antidepressant-like effects in different preclinical models (29). This treatment induced an overall decrease in DNA methylation and increase in BDNF expression in the hippocampus (HPC), the only region investigated in that study.

Zimmermann et al. (30) reported that long-term incubation of cortical cultured astrocytes with selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants decreased DNMT activity by an indirect mechanism. These data support the idea that antidepressant drugs could interfere with DNA methylation as part of their mechanism of action. However, this was not investigated *in vivo*, thus lacking information regarding the effect of different antidepressant drugs in DNA methylation in limbic regions of animals submitted to stress models predictive of antidepressant effects.

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Therefore, this work aimed to further investigate the potential antidepressant-like effects induced by DNA methylation inhibitors as well as the involvement of DNA methylation changes in response to antidepressant treatment. In order to do that, we tested the effects induced by combining subeffective doses of different classes of DNMTi (nucleoside and non-nucleoside) with serotonergic (fluoxetine) and noradrenergic (desipramine) antidepressant drugs, in mice submitted to the forced swimming test (FST), an animal model predictive of antidepressant effects (31). In addition, we evaluated the effects induced by DNMTi and antidepressant drugs on DNA methylation levels in the prefrontal cortex (PFC) and HPC, brain regions related to the neurobiology of depression (5).

Aims of the study

The aim of the present study was to investigate the behavioural effects induced by the combination of subeffective doses of different DNMTi with subeffective doses of antidepressant drugs in animals submitted to the FST. In addition, DNA methylation levels were investigated in the PFC and HPC of treated animals submitted to FST.

Materials and methods

Animals

This study was performed in male Swiss mice, 7 weeks old. The animals were housed in groups of 10 animals/cage (1147 cm^2) in a climate-controlled room with constant temperature (24 ± 1°C) under standard laboratory conditions (12-h light/12-h dark, lights on at 06:30 a.m.) with food and water available *ad libitum*. Procedures were conducted in accordance with the guidelines of the Brazilian Council (COBEA) for care and use of laboratory animals, which are in compliance with international laws and policies. All efforts were made to minimise animal suffering and to reduce the number of animals used. All experiments were conducted between 12 and 17 h. The protocols described in the present study were approved by our local Ethical Committee (CETEA, protocol number 072/2014).

Drugs and treatment

The following drugs were used: desipramine hydrochloride (DES, tricyclic antidepressant; Sigma-Aldrich, St. Louis, MO, USA): 2.5, 5 and 10 mg/kg (32); fluoxetine hydrochloride (FLX, SSRI; Sigma-Aldrich): 5, 10, 20 and 30 mg/kg (33); 5-aza-2'-deoxycytidine (5-AzaD, nucleoside DNMT inhibitor; Sigma-Aldrich): 0.1 and 0.2 mg/kg (29); RG108 (non-nucleoside DNMT inhibitor; Tocris Biosciences, Bristol, UK): 0.1, 0.2 and 0.4 mg/kg (19). All drugs, except FLX and RG108, were dissolved in sterile isotonic saline and administered intraperitoneally (i.p.). FLX was dissolved in Tween 80 2%/sterile isotonic saline and RG108 in dimethyl sulfoxide (DMSO) 10%/sterile isotonic saline (29,33).

FST

Animals were individually submitted to 6 min of forced swimming in glass cylinders (height 25 cm, diameter 17 cm) containing 10 cm of water (31). The test was videotaped and the immobility time (characterised by slow movements needed to avoid drowning) was measured during the last 4 min period by a trained observer that was blind to the treatment condition. The water was changed after each trial to maintain the temperature at 23–25°C and to prevent the influence of alarm substances (34).

Open field test (OFT)

The OFT was used to measure the locomotor activity of the animals (35). Mice were placed individually in a circular open-field arena (40 cm in diameter with a 50 cm high Plexiglas wall) for 6 min. The exploratory activity was videotaped and the number of crossings between the quadrants of the arena was measured afterwards by an observer that was blind to the treatment condition. After each test, the arena was cleaned with 70% alcohol solution.

DNA methylation analysis

The animals were deeply anesthetised with 5% chloral hydrate (10 ml/kg) and decapitated. Brain structures (HPC and PFC) were dissected and the tissues stored at −80°C until analysis. DNA was extracted using the AxyPrep Blood Genomic DNA Mini-prep Kit (Axygen Biosciences, NY, USA) according to the manufacturer's instructions. The purified DNA was digested with Nuclease P1 (#P2640; Sigma-Aldrich, 2 U/mg of DNA, 4 h at 65°C in acetate buffer 20 mM pH 5.3) and with alkaline phosphatase (#N8630; Sigma-Aldrich, 1 U/mg of DNA, 2 h at 65°C in Tris-HCl 20 mM pH 7.5). The digested DNA was precipitated in pure ethanol and NaCl 5 M at −20°C and centrifuged at 20 000 g for 15 min. The pellet was resuspended in TE buffer (Tris-HCl 5 mM, ethylenediaminetetraacetic acid 0.1 mM, pH 8.5) and the methylated DNA was quantified using the DNA Methylation EIA kit (#589324; Cayman Chemicals, Ann Arbor, Michigan, USA), according to the manufacturer's instructions. The absorbance produced in the assay was measured by SpectraMax 190 plate reader (Version 6.2.1, Molecular Devices, Sunnyvale, CA, USA; absorbance of 280/260 nm).

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Experimental design

Experiment 1 Effects of different vehicles (saline, saline/DMSO 10% and saline/Tween 2%) in mice submitted to the FST and OFT.

Mice received i.p. injections of saline (10 ml/kg), saline/DMSO 10% (10 ml/kg), saline/Tween 2% (10 ml/kg) and were submitted to the FST or OFT 30 min later.

Experiment 2 Effects of 5-AzaD, RG108, desipramine and fluoxetine treatment in mice submitted to the FST.

Mice received i.p. injections of 5-AzaD (0.1 and 0.2 mg/kg), RG108 (0.1, 0.2 and 0.4 mg/kg), DES (2.5, 5 and 10 mg/kg), FLX (5, 10, 20 and 30 mg/kg) or vehicle (10 ml/kg) and were submitted to the FST 30 min later.

Experiment 3 Effects of associating subeffective doses of 5-AzaD or RG108 with subeffective doses of desipramine or fluoxetine in mice submitted to the FST.

Independent groups of mice received i.p. injections of 5-AzaD (0.1 mg/kg), RG108 (0.1 mg/kg) or vehicle, followed by a second i.p. injection of DES (2.5 mg/kg), FLX (10 mg/kg) or vehicle (10 ml/kg), 5 min later. The animals were submitted to the FST 30 min after the last drug injection.

Experiment 4 Effects of 5-AzaD, RG108, desipramine and fluoxetine in mice submitted to the OFT.

Independent groups of mice received i.p. injections of 5-AzaD (0.1 mg/kg), RG108 (0.1 mg/kg) or vehicle followed by a second i.p. injection of DES (2.5 mg/kg), FLX (10 mg/kg) or vehicle (10 ml/kg) 5 min later. The animals were submitted to the OFT 30 min after the last drug injection.

Experiment 5 Effects of drug administration on behaviour and levels of methylated DNA in the HPC and PFC of the mice submitted to the FST.

Mice received i.p. injections of 5-AzaD (0.2 mg/kg), RG108 (0.4 mg/kg), DES (10 mg/kg), FLX (30 mg/kg) or vehicle (saline, saline/DMSO 10% or saline/Tween 2%; 10 ml/kg) and were submitted to the FST 30 min later. Immediately after the test, the animals were anesthetised, sacrificed and their HPC and PFC were dissected for further analysis of DNA methylation. An independent group of naïve animals (no stress and no treatment) was sacrificed at the same moment to have their HPC and PFC dissected and analysed for global levels of DNA methylation. The samples were stored at −80°C until use.

Data analysis

The immobility time in the FST and the distance moved in the OFT were analysed using one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's test. In the case of animals that received two injections, the results were analysed using two-way ANOVA followed by Bonferroni's test (factors: first injection and second injection) and one-way ANOVA followed by Tukey's test or Kruskal–Wallis followed by Dunn's test. The results were expressed as mean ± SEM. Statistical differences were considered significant when $p < 0.05$.

Results

Experiment 1 Effects of different vehicles (saline, saline/DMSO 10% and saline/Tween 2%) in mice submitted to the FST and OFT.

Systemic treatment with different vehicles (saline, saline/DMSO or saline/Tween; 10 ml/kg) did not induce any significant difference in the immobility time in the FST [$F(3,20) = 0.51$, $p > 0.05$; Fig. 1a] and in the number of crossings in the OFT [$F(3,20) = 0.27$, $p > 0.05$; Fig. 1b] when compared with not injected animals (naïve).

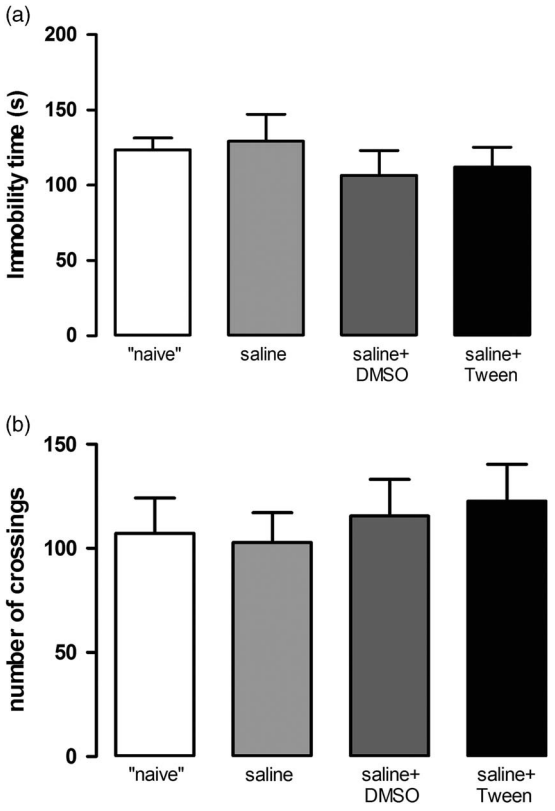

Fig. 1. Systemic injection of different vehicles (saline, saline/DMSO 10% or saline/Tween 2%; 10 ml/kg) did not induce any significant changes in the immobility time of mice submitted to the FST ($n = 6$/group; ANOVA, $p > 0.05$; a) and in the locomotor activity of mice submitted to the OFT ($n = 6$/group; ANOVA, $p > 0.05$; b). Data are expressed as mean ± SEM 'naïve' indicate no injected group and tested behaviourally. ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; FST, forced swimming test; OFT, open field test.

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Experiment 2 Effects of 5-AzaD, RG108, desipramine and fluoxetine treatment in mice submitted to the FST.

Immobility time was significantly reduced by desipramine [5 and 10 mg/kg; $F(3,28) = 6.04$, $p < 0.05$; Fig. 2a], fluoxetine [20 and 30 mg/kg; $F(3,27) = 4.36$, $p < 0.05$, Fig. 2b], 5-AzaD [0.2 mg/kg; $F(2,19) = 8.52$, $p < 0.05$; Fig. 2c] and RG108 [0.2 mg/kg; $F(4,33) = 12.04$, $p < 0.05$; Fig. 2d]. *Post hoc* analysis indicated that DES (2.5 mg/kg), FLX (10 mg/kg), 5-AzaD (0.1 mg/kg) and RG108 (0.1 mg/kg) did not significantly reduce immobility time when compared with their respective vehicle group (Dunnett's test, $p > 0.05$; Fig. 2). Therefore, those doses were chosen for the next experiments as the subeffective doses of each respective treatment.

Experiment 3 Effects of associating subeffective doses of 5-AzaD or RG108 with subeffective doses of desipramine or fluoxetine in mice submitted to the FST.

The administration of 5-AzaD (0.1 mg/kg) in combination with desipramine [2.5 mg/kg; two-way ANOVA, interaction: $F(1,28) = 1.542$, $p > 0.05$; injection 1: $F(1,28) = 9.172$, $p < 0.05$; injection 2: $F(1,28) = 10.98$, $p < 0.05$; one-way ANOVA followed Tukey's test, $F(3,28) = 7.231$, $p < 0.05$; Fig. 3a] or with fluoxetine [10 mg/kg, two-way ANOVA, interaction: $F(1,24) = 13.43$, $p < 0.05$; injection 1: $F(1,24) = 18.16$, $p < 0.05$; injection 2: $F(1,24) = 8.119$, $p < 0.05$; Kruskal–Wallis followed Dunn's test, $H = 13.12$, $p < 0.05$; Fig. 3b] induced a significant reduction in the immobility time in the FST, an antidepressant-like effect. The same occurred with the combination of RG108 (0.1 mg/kg) with DES [2.5 mg/kg, two-way ANOVA, interaction: $F(1,27) = 2.922$, $p > 0.05$; injection 1: $F(1,27) = 9.196$, $p < 0.05$; injection 2: $F(1,27) = 8.319$, $p < 0.05$; one-way ANOVA followed Tukey's, $F(3,27) = 6.408$, $p < 0.05$; Fig. 4a] or with FLX [10 mg/kg, two-way ANOVA followed Bonferroni's test, interaction: $F(1,26) = 5.981$, $p > 0{,}05$; injection 1: $F(1,26) = 6.558$, $p < 0.05$; injection 2: $F(1,26) = 8.467$, $p < 0.05$; one-way ANOVA followed Tukey's, $F(3,26) = 6.741$, $p < 0.05$; Fig. 4b].

Experiment 4 Effects of 5-AzaD, RG108, desipramine and fluoxetine in mice submitted to the OFT.

The administration of DNMTi (5-AzaD and RG108, dose of 0.1 mg/kg) associated with desipramine (2.5 mg/kg) did not induce any significant difference

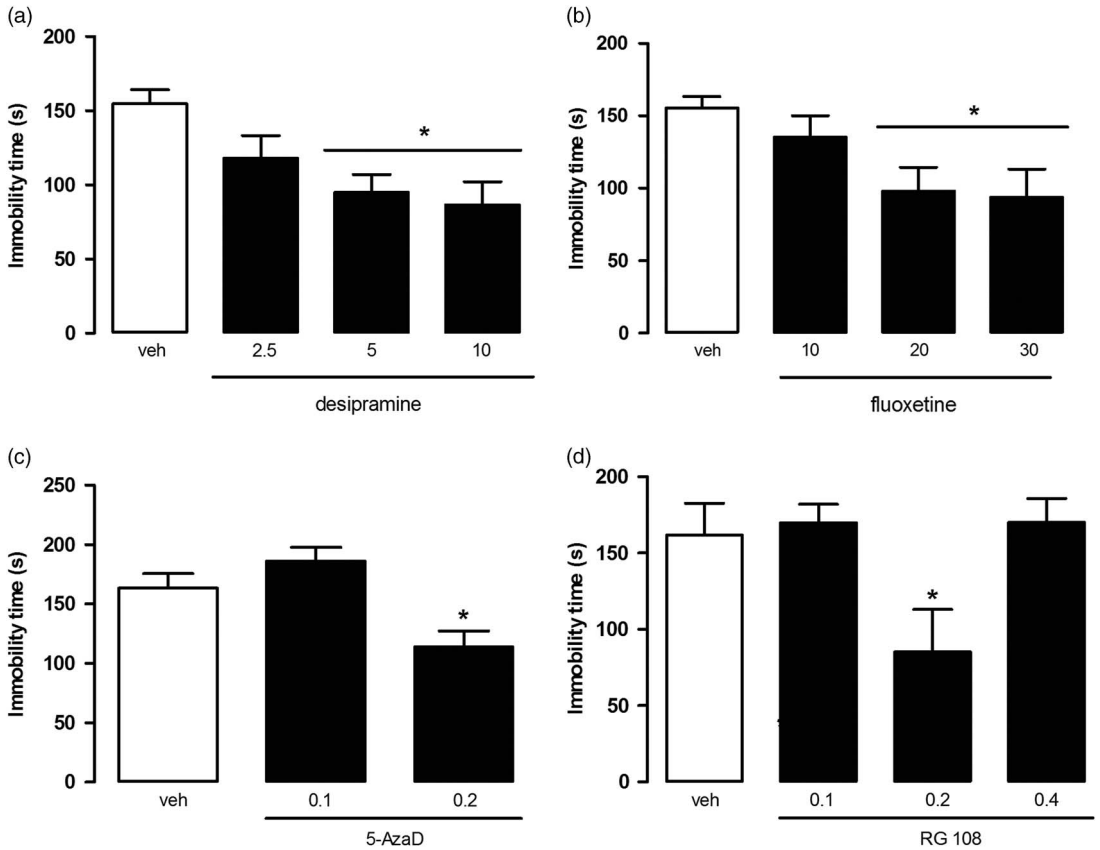

Fig. 2. Desipramine (5 and 10 mg/kg; a), fluoxetine (20 and 30 mg/kg; b), 5-AzaD (0.2 mg/kg; c) and RG108 (0.2 mg/kg; d) treatment reduced immobility time of mice submitted to the FST. Data are expressed as mean ± SEM (n = 7–10/group). *Indicate significant difference from the vehicle-treated group (one-way ANOVA followed by Dunnet's, $p < 0.05$). ANOVA, analysis of variance; FST, forced swimming test; veh, vehicle; 5-AzaD, 5-aza-2'-deoxycytidine.

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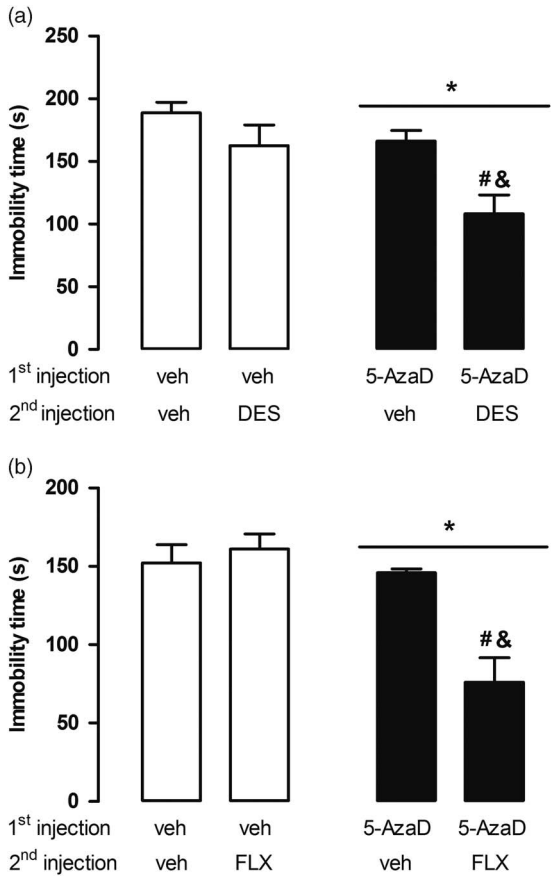

Fig. 3. Systemic injection of 5-AzaD (0.1 mg/kg) and DES (2.5 mg/kg; a) or FLX (10 mg/kg; b) significantly reduced the immobility time of mice submitted to the FST. Data are expressed as mean ± SEM (n = 7–8/group). *Indicate significant difference from veh versus 5-AzaD; [&]indicate significant difference from 5-AzaD/veh versus 5-AzaD/DES or 5-AzaD/FLX group; [#]indicate significant difference from veh/DES or veh/FLX versus 5-AzaD/DES or 5-AzaD/FLX group (a: ANOVA followed Tukey's test and b: Kruskal–Wallis followed Dunn's test). ANOVA, analysis of variance; DES, desipramine; FLX, fluoxetine; FST, forced swimming test; veh, vehicle; 5-AzaD, 5-aza-2'-deoxycytidine.

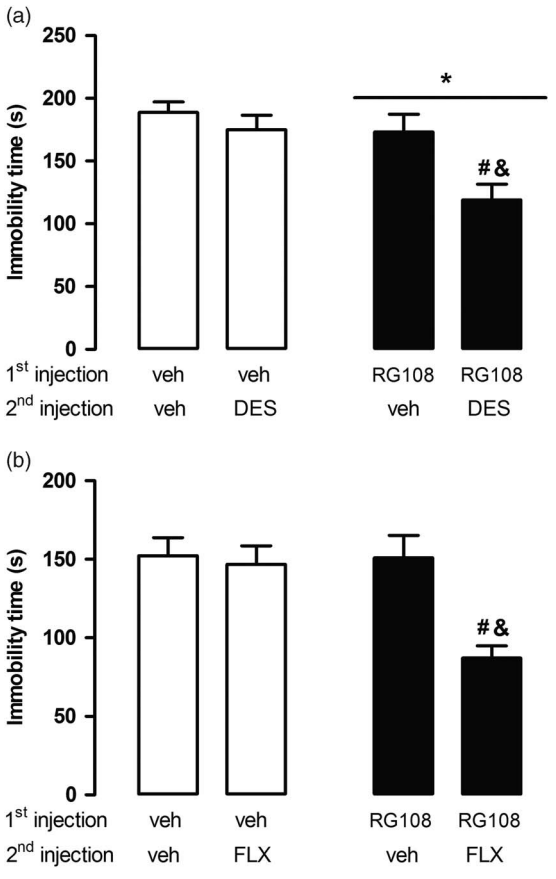

Fig. 4. Systemic injection of RG108 (0.1 mg/kg) and DES (2.5 mg/kg; a) or FLX (10mg/kg; b) significantly reduced the immobility time of mice submitted to the FST. Data are expressed as mean ± SEM (n = 7–8/group). *Indicate significant difference from veh versus RG108; [&]indicate significant difference from RG108/veh versus RG108/DES or RG108/FLX group; [#]indicate significant difference from veh/DES or veh/FLX versus RG108/DES or RG108/FLX group (ANOVA followed Tukey's test). ANOVA, analysis of variance; DES, desipramine; FLX, fluoxetine; FST, forced swimming test; veh, vehicle.

in the number of crossings in the OFT when compared with the vehicle [5-AzaD: interaction: $F(1,21) = 0.281$, $p > 0.05$; injection 1: $F(1,21) = 0.238$, $p > 0.05$; injection 2: $F(1,21) = 0.339$, $p > 0.05$; Fig. 5a; RG108: interaction: $F(1,22) = 0.297$, $p > 0.05$; injection 1: $F(1,22) = 0.414$, $p > 0.05$; injection 2: $F(1,22) = 2.465$, $p > 0.05$; Fig. 5c; two-way ANOVA]. Similarly, DNMTi (5-AzaD and RG108, dose of 0.1 mg/kg) associated with fluoxetine did not change the locomotor activity of the animals when compared with the vehicle [5-AzaD: interaction: $F(1,20) = 0.051$, $p > 0.05$; injection 1: $F(1,20) = 0.954$, $p > 0.05$; injection 2: $F(1,20) = 0.026$, $p > 0.05$; Fig. 5b; RG108: interaction: $F(1,21) = 2.119$, $p > 0.05$; injection 1: $F(1,21) = 0.132$, $p > 0.05$; injection 2: $F(1,21) = 0.599$, $p > 0.05$; Fig. 5d; two-way ANOVA]. The 5-AzaD (0.2 mg/kg), RG108 (0.2 mg/kg), desipramine (10 mg/kg) and fluoxetine (30 mg/kg) also did not induce any significant difference in the total distance travelled in the open field when compared with the vehicle [one-way ANOVA, $F(4,39) = 0.27$, $p > 0.05$; Fig. 6b].

Experiment 5 Effects of drug administration on behaviour and levels of methylated DNA in the HPC and PFC of the mice submitted to the FST.

Systemic treatment with DES (10 mg/kg), FLX (30 mg/kg), 5-AzaD (0.2 mg/kg) or RG108 (0.2 mg/kg) significantly reduced immobility time [one-way ANOVA followed by Dunnett, $F(4,35) = 4.71$, $p < 0.05$; Fig. 6a]. The analysis of methylated DNA indicated that stress increased DNA methylation

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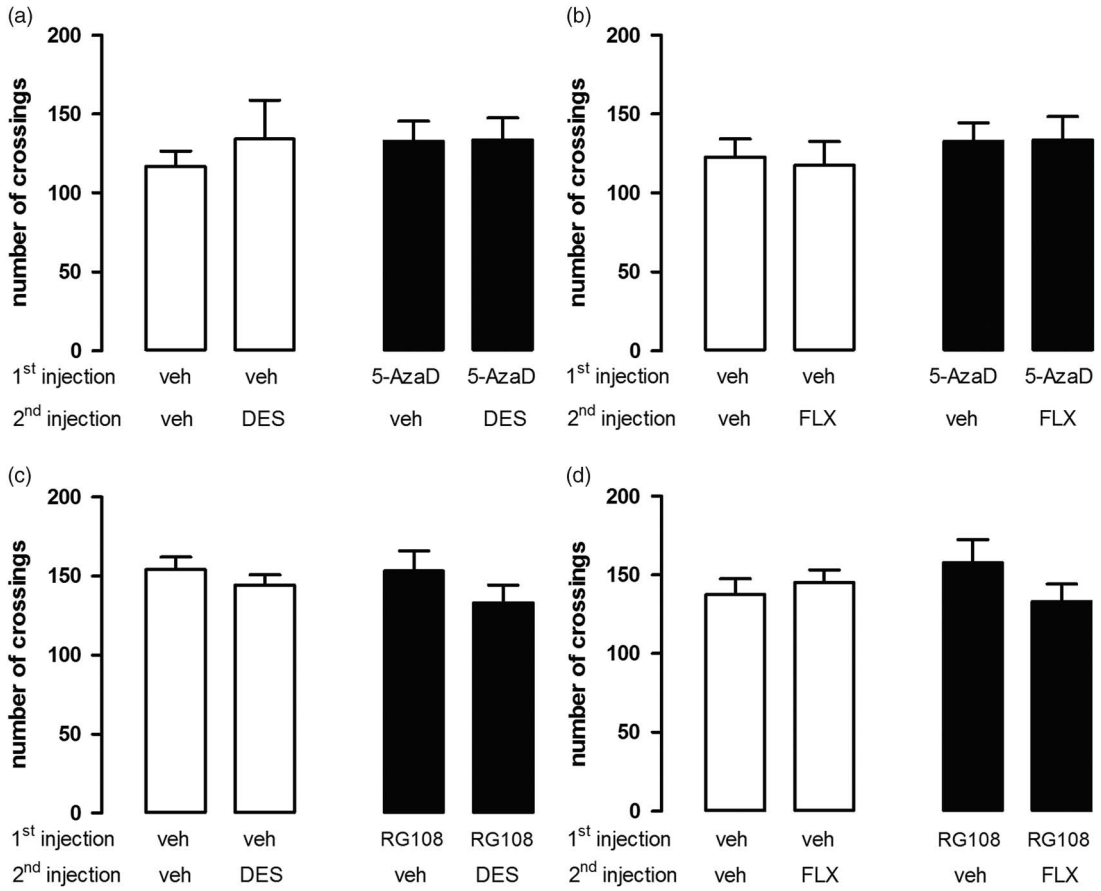

Fig. 5. Systemic injection of 5-AzaD (0.1 mg/kg) or RG108 (0.1 mg/kg) in association with DES (2.5 mg/kg; a and c, respectively) or FLX (10 mg/kg; b and d, respectively) did not induce any significant changes in the locomotor activity of mice submitted to the OFT. Data are expressed as mean ± SEM (n = 6–7/group, two-way ANOVA, $p > 0.05$). ANOVA, analysis of variance; DES, desipramine; FLX, fluoxetine; OFT, open field test; veh, vehicle; 5-AzaD, 5-aza-2'-deoxycytidine.

levels in the HPC, which was attenuated by all treatments [one-way ANOVA followed by Tukey, $F(5,37) = 6.53$, $p < 0.05$; Fig. 7c]. In the PFC, stress reduced DNA methylation levels and all treatments reversed this effect [one-way ANOVA followed by Tukey, $F(5,40) = 28.67$, $p < 0.05$; Fig. 7d]. No alteration was observed in animals treated with different vehicles [HPC: $F(3,20) = 0.74$, $p > 0.05$; Fig. 7a and PFC: $F(3,20) = 0.46$, $p > 0.05$; Fig. 7b].

Discussion

The results of the present study revealed for the first time that systemic administration of a non-nucleoside DNMTi (RG108) induces antidepressant-like effects in the FST, as previously reported for the treatment with a nucleoside inhibitor (5-AzaD). In addition, it was also shown that the association with subeffective doses of those compounds with subeffective doses of conventional and chemically unrelated antidepressants (fluoxetine and desipramine) induced similar effects, thus indicating a synergistic effect of the association. Furthermore, these effects were not related to unspecific motor changes, since the same treatments did not modify locomotor activity of mice exposed to the OFT. Finally, it was also observed that DNMTi and antidepressant drugs attenuated stress-induced DNA methylation changes in the HPC and PFC in a similar way.

The conventional antidepressants available act via monoaminergic mechanisms, mainly by inhibiting monoamine reuptake or its metabolism, thus increasing monoamine availability at the synaptic cleft (4,36,37). It has been suggested that molecular mechanisms, downstream the monoaminergic ones, would also contribute to the antidepressant effect (4,38). For instance, it has been shown that acute, as well as repeated treatment with antidepressant alters the expression of several genes, including those that code for receptors and trophic factors (10,39,40). Moreover, a large number of data shows that treatment with antidepressants restores stress-induced effects on gene expression and cellular plasticity (41) and that the behavioural effects of

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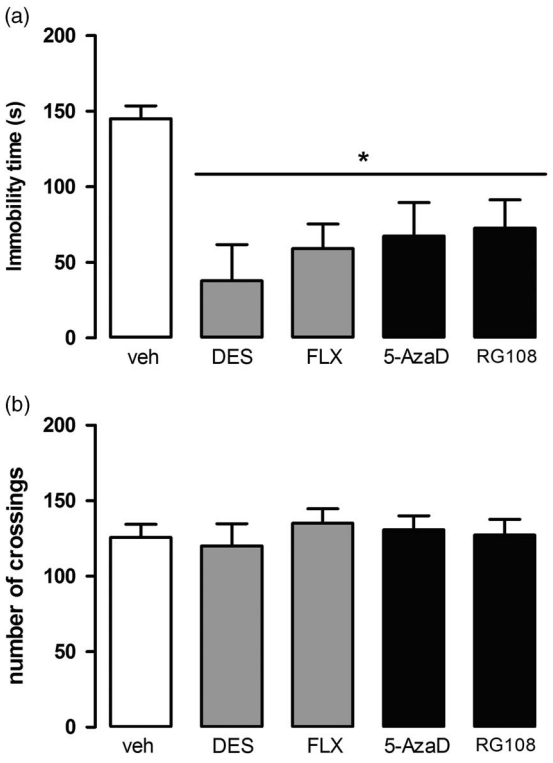

Fig. 6. Systemic injection of DES (10 mg/kg), FLX (30 mg/kg), 5-AzaD (0.2 mg/kg) or RG108 (0.2 mg/kg) significantly reduced immobility time of mice submitted to the FST (a; $n = 8$/group, one-way ANOVA, $p < 0.05$) and it did not induce any significant changes in the locomotor activity of mice submitted to the OFT (b). Data are expressed as mean ± SEM ($n = 8$–9/group, one-way ANOVA, $p > 0.05$). *Indicate significant difference from vehicle versus treated group (ANOVA followed by Dunnett's test). ANOVA, analysis of variance; DES, desipramine; FLX, fluoxetine; FST, forced swimming test; OFT, open field test; veh, vehicle; 5-AzaD, 5-aza-2'-deoxycytidine.

conventional antidepressant drugs rely on those changes (10,11,42). Although these changes are more pronounced
after chronic treatment with antidepressants, evidence suggests that some changes can be observed after acute injection and play important role in the establishment of their behavioural effects (43,44). In fact, signalling mechanisms that ultimately mediate gene expression changes and cellular plasticity may also take part in the acutely induced antidepressant-like effects in the FST (45,46). For instance, it was shown that different antidepressants (fluoxetine and imipramine) enhanced tyrosine kinase receptor type 2 (TrkB) activation in the PFC, within 30 min of drug injection, what was required for the activation of the transcription factor (CREB) and, ultimately, for the behavioural effects of antidepressants in mice exposed to the FST (45).

The regulation of gene expression occurs either by genetic or epigenetic mechanisms (47). The major epigenetic mechanisms include DNA methylation, post-translational modifications (histone phosphorylation and acetylation), micro-RNA and histone variants (48). DNA methylation refers to the transfer of a methyl radical to 5' position carbon of cytosine in regions of the DNA under the action of the enzymes called DNA methyltransferases. This chemical change turns chromatin into a more condensed state, thus hindering the access of transcriptional machinery and resulting in the gene silencing (49). Recent evidence has suggested that abnormal patterns of DNA methylation could be involved in the aberrant gene expression observed in stressed animals as well as in patients with mood disorders (22,25,50). In addition, it was shown that blocking DNA methylation by means of pharmacological treatment with 5-AzaD, a DNMTi, induces antidepressant-like effect in preclinical models (29). Therefore, the results of the present study are in agreement with previous data showing antidepressant-like effect in response to treatment with different DNA methylation inhibitors (29,51,52).

The molecular mechanisms involved in the behavioural effects of DNMTi are not completely understood. However, it has been shown that decitabine (5-AzaD) decreases DNA methylation and increases BDNF expression in the HPC (29), an effect that has been recognised as necessary for the behavioural effect of conventional antidepressant drugs (41,45). Several other genes involved in cellular plasticity and neurotransmission can be regulated by DNA methylation, such as TrkB (BDNF receptor), GAD65 and GR, among others (for review see 22). However, it is not known if these mechanisms would be involved in the antidepressant-like effects induced by DNMTi in the results presented herein, since mRNA expression and protein levels were not measured. Although 30 min is a short time to consider that mRNA and protein expression would have happened, previous work indicates that it may happen in a very short time-window (19,53).

Considering that antidepressants are able to indirectly modulate DNMT activity *in vitro* (30) as well as acutely promote TrkB activation (43), we hypothesised that the combination of subeffective doses of antidepressants with subeffective doses of DNMTi would promote synergistic behavioural effects in mice submitted to the FST. As an attempt to avoid the interference of any unspecific and unrelated effect induced by the drugs, we combined different classes of DNMTi (5-AzaD, nucleoside inhibitor; RG108, non-nucleoside inhibitor) with different classes of antidepressants (desipramine, tricyclic noradrenergic antidepressant; fluoxetine, SSRI) to evaluate possible synergic antidepressant-like effects.

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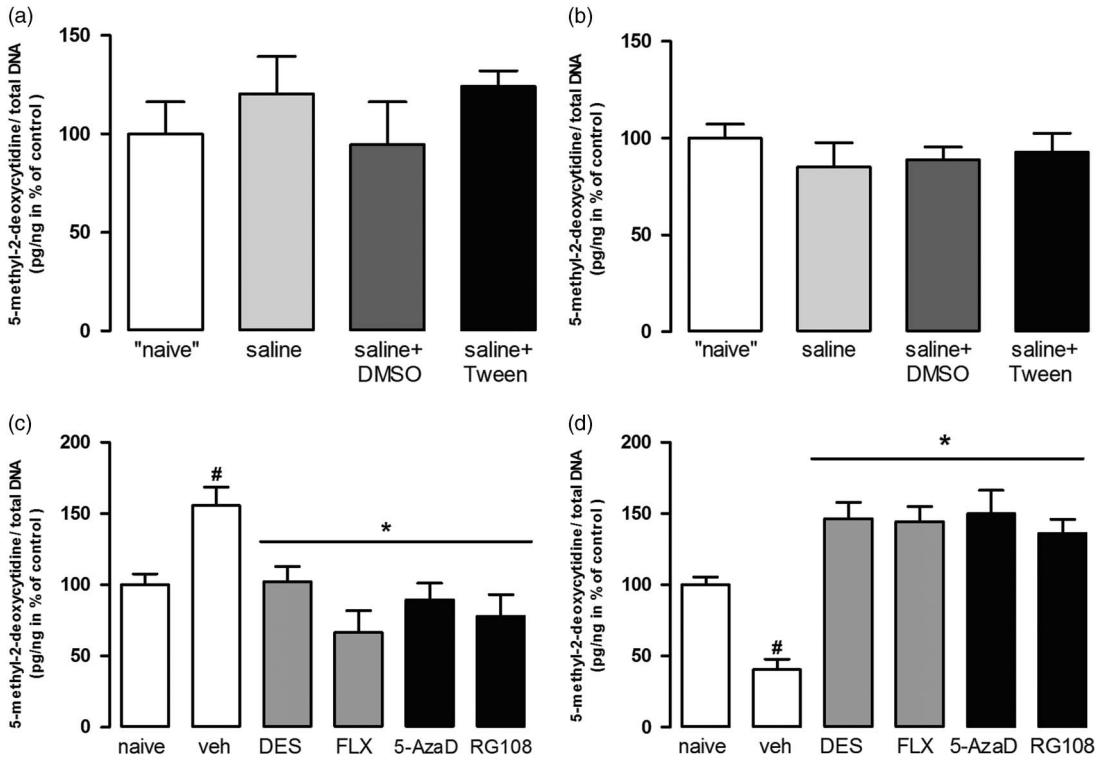

Fig. 7. Systemic injection of DES (10 mg/kg), fluoxetine (30 mg/kg), 5-AzaD (0.2 mg/kg) or RG108 (0.2 mg/kg) significantly changed DNA methylation in HPC (c) and PFC (d). No difference was observed in the animals injected with different vehicles (HPC; a and PFC; b). Data are expressed as mean ± SEM (n = 6–10/group). *Indicate significant difference from vehicle versus treated group; #indicate significant difference from naïve versus treated or vehicle group (ANOVA followed by Tukey's test). ANOVA, analysis of variance; DES, desipramine; DMSO, dimethyl sulfoxide; FLX, fluoxetine; FST, forced swimming test; HPC, hippocampus; OFT, open field test; PFC, prefrontal cortex; veh, vehicle; 5-AzaD, 5-aza-2'-deoxycytidine.

The behavioural results obtained are in agreement with that hypothesis, since all combinations tested induced significant antidepressant-like effects. The molecular mechanisms involved in those effects are not yet clear and warrants further investigation. However, it is possible to hypothesise that DNMTi and antidepressants could share similar or convergent molecular mechanisms that would contribute to their behavioural effects.

Based on that, we investigated the levels of global DNA methylation in the PFC and in the HPC, given their proposed involvement in the behavioural effect of antidepressant drugs (54). Surprisingly, we found that stressed animals had increased DNA methylation levels in the HPC and decreased levels in the PFC. Both effects were attenuated by antidepressant drugs as well as by DNMTi. Since DNA methylation is site (within a single gene) and region specific, this bidirectional effects induced by stress on HPC and PFC are not completely unexpected. Corroborating that assumption, it was shown that psychosocial stress regimen significantly increased BDNF DNA methylation in the dorsal HPC, whereas it significantly decreased or induced no change in DNA methylation in the ventral HPC and the medial PFC, respectively (55). These data highlights the complexity involved in the regulation of stress-induced DNA-methylation changes.

The mechanisms underlying antidepressant-induced effects in DNA methylation are not yet clear. It was reported that the incubation of cortical astrocytes with different antidepressants (paroxetine, amitriptyline and imipramine) decreased DNMT activity through an indirect mechanism that involved reduced expression of the histone methyltransferase G9a, a known modulator of DNMT1 activity (30). However, this effect was not achieved acutely, but only after 72 h of incubation with the drugs. Thus, this mechanism could not explain the results of the present study, since the changes in DNA methylation were found 30 min after drug injection. An alternative explanation for such acute effects of antidepressants on DNA methylation levels could be the modulation of intracellular signalling cascades that ultimately lead to changes in DNMT activity. For instance, antidepressants are able to modulate glutamatergic neurotransmission (56), which is capable of activating intracellular mechanisms that culminate with the modulation of the epigenetic machinery (57,58). In fact, it was reported that exposure to stressful events and consequent increased release of glucocorticoids and

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glutamate (and NMDA activation) can result in the phosphorylation of ERK1/2 which form a complex with GR and induce transcription factors that culminate with the modulation of the epigenetic machinery (57,58). Therefore, antidepressants could ultimately target DNMT activity acutely by modulating glutamate-induced levels upon stress exposure. This hypothesis, however, should be further investigated.

Evidence has suggested that active DNA methylation as well as DNA demethylation are likely to happen in response to stress-induced and neurotransmitter-mediated neuronal activation (19,59). Upon intense neuronal activation (such as under stress exposure) these could be important mechanisms to allow the dynamic control of the expression of genes that would be required or not at that situation, thus favoring or inhibiting their expression (19,60). Therefore, either increases or decreases in DNA methylation can be observed in the brain of stressed animals, as we observed herein (HPC vs. PFC). Likewise, by modulating stress-induced changes in neuronal activation, antidepressant drugs could be able to indirectly affect DNA methylation in both directions, as also observed in the present study.

Similarly to the effects induced by FLX, DES, 5-AzaD and RG108 treatment were also able to bidirectionally modulate stress-induced DNA methylation in the HPC and PFC. RG108 and 5-AzaD reduced stress-induced DNA methylation in the HPC while attenuated stress-induced decrease in DNA methylation in the PFC. Since the only mechanism described for these drugs is DNMT inhibition, albeit through different mechanims (for review see 61), it is possible to speculate that both effects could result from inhibiting DNMT activity. In agreement with that possibility, the infusion of 5-azacytidine (5-AzaC) attenuated the DNA demethylation induced by the intense stimulation of PFC slices (62). Even thought DNMTs are commonly known for their ability to catalyse DNA methylation, they may also act as DNA demethylases (for review see 61). Considering that DNMTi could block overall DNMT enzymatic activity, significant effects on methylation and demethylation could be observed as the result of inhibiting DNMTs, as observed herein (HPC vs. PFC). These could provide reasonable explanation for our data and also for the data described by Sui et al. (62).

Altogether, the present results indicate that antidepressants and DNMTi are able to bidirectionally modulate stress-induced DNA methylation/demethylation in limbic regions. Additional experiments would be necessary to investigate which genes could be rapidly expressed in the PFC and HPC that could be related to the behavioural responses induced by 5-AzaD and RG108. In addition, the present study shows that systemic DNMT inhibition administered alone or in combination with antidepressants induces an antidepressant-like effect associated with changes in stress-induced DNA methylation in mice submitted to FST.

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Conflicts of Interest

The authors have no competing interests to declare. S.J. is Associate Editor in Acta Neuropsychiatrica. However, S.J. did not handle the manuscript or was involved in any decisions related to the present work.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The protocols were approved by our local ethical committee (CETEA, protocol number 072/2014).

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