

Title Page

Antidepressant-like effect induced by systemic and intra-hippocampal administration of DNA methylation inhibitors.

Amanda J. Sales^{1#}, Caroline Biojone^{2#}, Mateus S. Terceti², Francisco S. Guimarães², Marcus V. M. Gomes^{3*}; Sâmia R. L. Joca^{1*}

¹Laboratory of Pharmacology, Department of Physics and Chemistry, School of Pharmaceutical of Ribeirão Preto, University of São Paulo, Brazil

²Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

³Laboratory of Epigenetics, Centre of Biological and Health Sciences, University of Northern Paraná (UNOPAR), Londrina-PR, Brazil

Authors equally contributed to this work.

*Correspondence to:

- S. Joca. Department of Physics and Chemistry, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, 14040-903, Ribeirão Preto, SP, Brazil, Fax: +55-16-36023080, Phone: +55-16-36024705, Email: samia@usp.br.

This is an Accepted Article that has been peer-reviewed and approved for publication in the *British Journal of Pharmacology*, but has yet to undergo copy-editing and proof correction. Please cite this article as an "Accepted Article"; doi: 10.1111/j.1476-5381.2011.01489.x

- M.V. Gomes. Laboratory of Epigenetics, Centre of Biological and Health Sciences, University of Northern Paraná (UNOPAR), Londrina-PR, Brazil, Fax:+55-43-33717725, Phone: +55-43-33717993, Email: mvmgomes@gmail.com

Abstract

Background and purpose: epigenetic modifications are thought to play an important role in the neurobiology of depression. For example, antidepressant treatment induces histone acetylation in the hippocampus, which is associated with transcriptional activation, whereas stress increases DNA methylation, which is associated with transcriptional repression. Since the specific involvement of DNA methylation in the regulation of depressive-like behaviours is not yet known, we aimed at investigating the effects induced by systemic or intra-hippocampal administration of DNA methyltransferase (DNMT) inhibitors in rats submitted to the forced swimming test (FST).

Experimental approach: Rats received i.p. injections of 5-aza-2-deoxycytidine (5-azaD, 0.1-0.8 mg.Kg⁻¹), 5-azacytidine (5-azaC, 0.4-3.2 mg.Kg⁻¹), imipramine (15 mg.Kg⁻¹) or vehicle and were submitted to the FST or open field test. Independent group of rats received intra-hippocampal injection of DNMT inhibitors.

Key results: systemic administration of DNMT inhibitors induced a dose-dependent antidepressant-like effect, which was followed by decreased DNA methylation and increased BDNF levels, in the hippocampus. Hippocampal inhibition of DNA methylation induced similar behavioural effects. No treatment induced any locomotor effects in the open field test. The antidepressant-like effects of 5-azaD were confirmed in mice submitted to the FST or the tail suspension test.

Conclusions and implications: this is the first report to suggest that global, as well as hippocampal, inhibition of DNA methylation induces antidepressant-like effects. The results also indicate that these effects could be associated to increased hippocampal BDNF expression. In

summary, the data give further support to the hypothesis that DNA methylation is an important epigenetic mechanism involved in the development of depressive-like behaviours.

Keywords: epigenetic, 5-azacytidine, 5-aza-2-deoxy-cytidine, RG-108, DNA methylation, hippocampus, forced swimming test, tail suspension test.

List of Abbreviations

- 5-azaC: 5-azacytidine
- 5-azaD: 5-aza-2'-deoxy-cytidine
- BDNF: brain derived neurotrophic factor
- CpGs: cytosine guanine dinucleotides
- DNMT: DNA methyltransferase
- FST: forced swimming test
- HDAC: histone deacetylase
- IA: interaural distance
- IMI: imipramine
- MAOI: monoamine oxidase inhibitors
- OFT: open field test
- TST: tail suspension test

Introduction

Depression is a common and serious illness with the potential of becoming the leading cause of disability worldwide (Kessler *et al.*, 2003; Kessler *et al.*, 2008). Its lifetime prevalence rate is in the range of 8-20% throughout different populations, and it is expected to increase in the next years (Kessler *et al.*, 2003; Kessler *et al.*, 2008). These facts contribute to the high economic burden of depression, including direct treatment costs, lost earnings due to depression-related suicide and indirect workplace costs (Greenberg *et al.*, 2003).

The earliest treatments for depression were based upon the serendipitous discovery of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA), which inhibit the metabolism and the reuptake of monoamines, respectively (Castren, 2005); (Krishnan *et al.*, 2008). Since then, the pathophysiology of depression has been dominated by the monoamine hypothesis and newer antidepressants have been developed based on the aforementioned mechanisms of action (Castren, 2005; Hindmarch, 2002; Racagni *et al.*, 2008). However, despite the advances in drug discovery and therapeutic options to treat depression, there are still some shortcomings that need to be improved. For example, a significant symptom improvement is only observed after 2-4 weeks of treatment and only 60–65% of patients respond to the initial regimen, with less than half of these patients reaching remission or becoming symptom-free (Rosenzweig-Lipson *et al.*, 2007). Therefore, the study of the neurobiology of depression could lead to the discovery of new targets, alternative to the biogenic-amine-based, for the development of more effective therapies for depression.

The findings that chronic antidepressant treatment induces neuronal plastic changes, most likely as an attempt to counteract stress-induced effects in brain circuitry, have supported a new hypothesis about the neurobiology of this disorder, the so called “Neurotrophic (or Molecular)

Hypothesis of Depression” (Castren, 2005; Duman *et al.*, 2006; Krishnan *et al.*, 2008). It is mainly based on observations that exposure to stress decreases the expression of brain derived neurotrophic factor (BDNF), induces dendritic atrophy and reduces neurogenesis, particularly in the hippocampus, neuroplastic changes that can be attenuated by chronic antidepressant treatment (Castren, 2005; Duman *et al.*, 2006; Krishnan *et al.*, 2008). Moreover, antidepressant-induced hippocampal neurogenesis and BDNF expression are thought to be necessary for their behavioural effects (Adachi *et al.*, 2008; Saarelainen *et al.*, 2003).

New insights about the neurobiology of depression have arisen based on recent evidence that gene expression changes related to neural plasticity processes, such as adult hippocampal neurogenesis (Covic *et al.*) and activity-driven long-lasting plasticity (Levenson *et al.*, 2006a), are modulated by complex ‘epigenetic’ mechanisms (Krishnan *et al.*, ; Krishnan *et al.*, 2008; Tsankova *et al.*, 2006). Epigenetic refers to changes in DNA packing and chromatin structure that control gene expression without changing the original DNA sequence, thus leading to different cellular phenotypes without a change in genotype (Jones *et al.*, 2001). Therefore, epigenetic modifications are important mechanisms by which environmental experience can modify gene function in the absence of DNA sequence changes and produce long-lasting changes in protein availability and brain function (Tsankova *et al.*, 2007).

The epigenetic modifications include covalent changes to DNA (methylation) and post-translational modifications of histone N-terminal tails (acetylation, methylation, phosphorylation and ubiquitinylation), as well as non-transcriptional gene silencing mechanisms (micro-RNAs, for example) (Kim *et al.*, 2009).

Amongst the processes that modulate chromatin structure and gene expression, modifications in histone tails have been the most studied in relation to the neurobiology of

depression. For example, histone acetylation of lysines, which is most often associated with transcription activation through its ability to relax condensed areas of chromatin, is induced by chronic antidepressant treatment. This mechanism is thought to account to its behavioural effects (Tsankova *et al.*, 2006). In addition, inhibition of histone deacetylase (HDAC) induces antidepressant-like effects in animals (Covington *et al.*, 2009; Schroeder *et al.*, 2007). On the other hand, histone methylation, depending on the number of methyl groups and position of lysine residues, could be involved with reduced gene expression and stress-induced behavioural consequences (Hunter *et al.*, 2009).

DNA (cytosine-5) methylation is another important epigenetic mechanism for gene transcription regulation in the nervous system. DNA methylation is a process accomplished by DNA methyltransferases (DNMTs), which catalyze a covalent addition of a methyl group to the 5' position of cytosine residue almost exclusively at cytosines within cytosine guanine dinucleotides (CpGs) (Weber *et al.*, 2007). Methylated sites can be targeted by methylated DNA-binding proteins, triggering additional phenomena that result in condensed chromatin state and transcriptional repression (Weber *et al.*, 2007).

Besides its recognized role in development and differentiation (Kim *et al.*, 2009), recent evidence has suggested that DNA methylation is also an important epigenetic mechanism that modulates synaptic plasticity in the adult brain (Covic *et al.*, ; Levenson *et al.*, 2006a; Miller *et al.*, 2007). This assumption is further supported by evidence that exposure to different stressors, such as social defeat (LaPlant *et al.*, 2010), footshocks (Miller *et al.*, 2007) and low maternal care (Zhang *et al.*, 2010), increases the expression of DNMTs in different brain regions, what is accompanied by increased DNA methylation and decreased expression of genes that regulates synaptic plasticity and neurotransmission. On the other hand, there is evidence that stress

decreases DNA methylation of specific genes involved in stress response, indicating that DNA methylation could be a dynamic process involved in stress-induced behavioural and physiological outcomes (Murgatroid *et al.*, 2009; Elliot *et al.*, 2010).

Despite the aforementioned pieces of evidence, little is known about the role played by DNA methylation in the aetiology of psychiatric disorders such as depression. However, considering that stress increases DNA methylation in the hippocampus and other brain regions (Miller *et al.*, 2007; Zhang *et al.*, 2010) and that higher levels of DNA methylation have also been described in specific genomic loci at the hippocampus of suicide victims (McGowan *et al.*, 2009; McGowan *et al.*, 2008; Poulter *et al.*, 2008), it is plausible to suggest that stress-induced DNA methylation could contribute to the pathophysiology of depression. In this regard, apart from the work recently reported by (LaPlant *et al.*, 2010), where it is described that inhibition of DNA methylation in the nucleus accumbens induces antidepressant-like effects in rodents, there is no other work studying the involvement of DNA methylation in the modulation of depressive-like behaviours.

Therefore, we aimed at investigating the effects induced by single or repeated systemic administration of 5-aza-2'-deoxycytidine and 5-azacytidine, two potent inhibitors of DNMTs (Stresemann *et al.*, 2008; Yoo *et al.*, 2004), in rats and mice submitted to the forced swimming or tail suspension tests, two widely used animal model predictive of antidepressant activity (Cryan *et al.*, 2002). Besides, we also investigated a possible site of action for these drugs by injecting them into the dorsal hippocampus of rats submitted to the aforementioned model, since neural plasticity in this brain region has been related to the behavioural effects induced by antidepressant drugs (Adachi *et al.*, 2008; Saarelainen *et al.*, 2003; Santarelli *et al.*, 2003). In addition, considering that BDNF is proposed to mediate the behavioural effects of antidepressant

drugs (Saarelainen *et al.* 2003) and its gene expression is regulated by methylation (Roth *et al.*, 2011), the effects of DNMT inhibition on the hippocampal levels of this neurotrophin were also investigated.

Material and Methods

Animals

Male Wistar rats weighing 200-220 g at the beginning of each experiment were housed in pairs in a temperature-controlled room ($24 \pm 1^\circ\text{C}$) under standard laboratory conditions with free access to food and water and a 12h light/12h dark cycle (lights on at 06:30h a.m.). Male Swiss mice weighing 25-30 g at the beginning of each experiment were housed in 6-10 per cage (570 cm^2) under the same conditions. Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behaviour guidelines for the care and use of laboratory animals, which are in compliance with international laws and politics. The local Ethical Committee approved the experimental procedures (protocol number 10.1.136.53.2) and all efforts were made to minimize animal suffering.

Drugs

Imipramine hydrochloride (Sigma-Aldrich[®], USA) was dissolved in sterile isotonic saline and administered i.p. at the dose of 15 mg/kg (according to Joca and Guimarães, 2006). 5-Aza-2'-deoxycytidine (5-azaD or Decitabine, Sigma-Aldrich[®], USA), a drug that inhibits DNA methylation (Christman, 2002; Oki *et al.*, 2007), was dissolved in sterile isotonic saline and administered i.p. at the doses of 0.1, 0.2, 0.3, 0.4, 0.6 or 0.8 mg.Kg^{-1} (Pereira *et al.*, 2004). 5-azacytidine (5-azaC, Sigma-Aldrich[®], USA), another DNA methylation inhibitor (Christman, 2002),

was administered i.p. at the doses 0.4, 1.6 or 3.2 mg.Kg⁻¹ according to its relative potency in inhibiting DNA methylation compared to 5-azaD (Stresemann *et al.*, 2006). For intra-hippocampal injections, 5-azaD (50, 100 or 200 nmol.0.5µl⁻¹) or the non-nucleoside DNMT inhibitor RG-108 (Tocris Biosciences, USA, 100 or 200 nmol/0. 5µl⁻¹) were dissolved in sterile isotonic saline or DMSO, respectively, and administered bilaterally, according to (Miller *et al.*, 2007). All drugs were freshly prepared and protected from light during the experimental session.

Open Field Test (OFT)

Independent groups of animals were submitted to the open field test in order to investigate if the treatments used could induce any significant exploratory/motor effect, which would interfere in the FST results. The protocol was done as previously described (Crestani *et al.*, 2010; Scopinho *et al.*, 2010). Briefly, the animals were placed individually in the centre of an open circular arena (diameter: 72 or 40 cm in diameter, for rats or mice, respectively, with a 50 cm high Plexiglas wall) located in a sound-attenuated, temperature-controlled room, illuminated with three 40W fluorescent bulbs. The animals were left in the arena for five (rats) or six (mice) minutes. Their exploratory activity was videotaped and the behavioural analysis was blindly performed with the help of the Any-Maze software (Stoelting, USA). This software detects the position of the animal in the open arena and calculates the distance moved.

Stereotaxic Surgery and intracerebral administration

Stereotaxic surgery was performed as described before (Joca *et al.*, 2003). Briefly, rats were anaesthetized with 2,2,2-tribromoethanol (10 mg.Kg⁻¹, i.p., Aldrich Chemical USA) and fixed in a stereotaxic frame. Stainless steel guide cannulae (0.7 mm OD) were implanted

bilaterally aimed at the dorsal hippocampus (coordinates: AP = -4.0 mm from lambda, L = 2.8 mm, D = 2.1 mm), according to the Paxinos and Watson (1997) atlas. The cannulae tips were located 1.5 mm above the site of injection and the cannulae were attached to the skull bone with stainless steel screws and acrylic cement. An obturator inside the guide cannulae prevented obstruction.

Five to seven days after the surgery intracerebral injections were performed with a thin dental needle (0.3 mm OD) that was introduced bilaterally through the guide cannula until its tip was 1.5 mm below the cannulae end. A volume of 0.5 μ L/side was injected in 1 minute using a microsyringe (Hamilton) controlled by an infusion pump (Insight Equipamentos Científicos, Brazil). A polyethylene catheter (PE10) was interposed between the upper end of the dental needle and the microsyringe. The movement of an air bubble inside the polyethylene catheter confirmed drug flow.

Forced swimming test (FST)

The FST procedure for rats was similar to that first described by (Porsolt *et al.*, 1978), with minor modifications according to (Joca *et al.*, 2007b). Animals were initially placed individually to swim in plastic cylinders (30 cm of diameter by 40 cm in height containing 25 cm of water at 24 \pm 1 $^{\circ}$ C) for 15 min (pretest). They were then removed and allowed to dry in a separate cage before returning to their home cages. Twenty-four hours later the animals were submitted to a 5 min session of forced swim. The procedure for mice consisted in placing the animals into glass cylinders (height 25 cm, diameter 17 cm) containing 10 cm of water maintained at 23–25 $^{\circ}$ C. The animals were left in the cylinder for 6 min and the total duration of immobility was measured during the last 4-min period. During the test session the total amount of time in which animals

remained immobile (except for small limb movements necessary for floating) was recorded. The water was changed after each trial to avoid the influence of alarm substances.

Tail suspension test (TST)

The animals were suspended by the tail and fixed using a tape in a wood platform elevated 50 cm of the floor, according to the protocol described by (Viana *et al.*, 2010). Their behaviour was videotaped and the immobility time was recorded during 6 min by a trained observer. The mice were considered immobile when they remained suspended passively.

Histology

After the behavioural tests the rats were sacrificed under deep urethane anaesthesia and perfused through the left ventricle of the heart with isotonic saline followed by 10% formalin solution. After that, a dental needle was inserted through the guide cannula and 0.5 μ L of fast green was injected. The brains were removed and, after a minimum period of 3 days immersed in a 10 % formalin solution, 40 μ m sections were obtained in a Cryostat (Cryocut 1800). The injection sites were identified on diagrams from the Paxinos and Watson's atlas (Paxinos and Watson 1997). Rats that received injections outside the aimed area were excluded from analysis.

DNA Methylation analysis

The animals were deeply anesthetized with chloral hydrate (500 mg/kg) and decapitated. The hippocampus was dissected and the tissue stored at -80°C until the analysis. DNA was extracted using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, USA) according to the manufacturer's instructions. The purified DNA was digested with Nuclease P1 (#P2640,

Sigma) (2U/1 μg of DNA, 4h at 65°C in acetate buffer 20mM pH 5.3) and with alkaline phosphatase (#N8630, Sigma) (1U/1 μg of DNA, 2h at 65°C in Tris-HCl 20 mM pH 7.5). The digested DNA was precipitated in pure cold ethanol and NaCl 5M at -20°C for 18 h and centrifuged at 20000g for 15 min. The pellet was resuspended in TE buffer (5 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) and the methylated DNA was quantified using the DNA Methylation EIA kit (#589324, Cayman Chemical), according the manufacturer's instructions. The absorbance produced in the assay was measured by VictorX3 plate reader and software (Perkin Elmer). Several concentrations of purified 5-methyl-2-deoxy cytidine (provided by the kit) were used to construct the standard curve. The concentration of the sample (ng/ml) was calculated based on the 4-parameter logistic equation of the standard curve.

Quantification of BDNF levels

The animals were deeply anesthetized with chloral hydrate 500 mg/kg and decapitated. The hippocampus was dissected and homogenized in lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol supplemented with protease inhibitor cocktail, P2714, Sigma) and centrifuged at 10000g 4°C for 15 min. The supernatant was collected and BDNF measured with the BDNF Emax® ImmunoAssay System (#G7610, Promega, USA), according with the manufacturer's instructions. The concentration of the samples was calculated based on the standard curve, constructed with purified recombinant human BDNF. The levels of total protein content were determined by the reaction with Bradford reagent (#B6916, Sigma), following the manufacturer's instructions, and used to normalize the BDNF results. The final result was expressed as pg BDNF/ μg total proteins.

Experimental design

Experiment one: Effects of 5-azaD, 5-azaC or imipramine systemic administration in rats submitted to the FST. Rats were submitted to the pretest and afterwards received two injections 5-azaD, 5-azaC, imipramine or respective vehicles (0 and 5h later). The third injection was given 1h prior to the test session.

Experiment two: Effects of 5-azaD or 5-azaC systemic administration in rats submitted to the OFT. Rats received three injections 5-azaD, 5-azaC, or respective vehicles, 1h, 19h and 24h before the test session.

Experiment three: Effects of single systemic administration of 5-azaD in rats submitted to the FST. In order to investigate whether a single injection of the drug would be able to induce antidepressant-like effects, independent groups of rats were submitted to the pretest and received a single injection of 5-azaD immediately or 5h after the pretest, or 1h before the test.

Experiment four: Effects of 5-azaD in the levels of methylated DNA and BDNF in the hippocampus of rats submitted to the FST. Independent groups of animals were submitted to the experimental protocol as described in Experiment 1 and were sacrificed immediately after or immediately before the test, in order to have their hippocampus dissected and the levels of methylated DNA and of BDNF, respectively, analysed. The levels of BDNF were measured in animals that underwent pretest 24h before but were not submitted to the test in order to avoid that the protein levels could be influenced by the exposure to the test session. Therefore, since we wanted to know how BDNF levels would be at the moment that the animal performed the test, we believe that this experimental design would avoid false-negative results and allow us to identify even modest differences upon BDNF protein levels.

Experiment five: Effects of intra-hippocampal administration of 5-azaD or RG-108 in rats submitted to the FST. Rats were submitted to the pretest and, immediately after, received a bilateral injection of 5-azaD (50, 100 or 200 nmol.0.5 μ l⁻¹), or vehicle and were tested 24h later. An independent group of animals was submitted to the same experimental protocol but received intra-hippocampal injections of RG-108 (100 or 200 nmol.0.5 μ l⁻¹), a non nucleoside inhibitor of DNMTs which, unlike 5-azaD, does not need to be incorporated into DNA to inhibit DNMTs (Szyf, 2009).

Experiment six: Effects of 5-azaD systemic administration in mice submitted to the FST, TST or OFT. Independent groups of mice received a single i.p. injection of 5-azaD or vehicle and were submitted to the behavioural tests 1 h later.

Statistical analysis

The treatment effects were compared using one-way Analysis of Variance followed by the Dunnet's test for pos-hoc comparisons. Data from Experiment 2 were analyzed by a two-way Analysis of Variance with the factors being treatment and time. Probability less than 0.05 was accepted as significant.

Results

Experiment one: Effects of 5-azaD, 5-azaC or imipramine systemic administration in rats submitted to the FST.

Systemic treatment with 5-azaD, at the dose of 0.4 mg.Kg⁻¹, or with imipramine significantly reduced immobility time ($F_{7,40} = 8.97$, $P < 0.0001$; Dunnet's, $P < 0.05$; Figure 1). Systemic injection of 5-azaC induced similar effects, with the dose of 3.2 mg.Kg⁻¹ being the

most effective in reducing immobility time ($F_{4,33} = 10.86$, $P < 0.0001$; Dunnett's, $P < 0.05$; Figure 1).

Experiment two: Effects of 5-azaD or 5-azaC systemic administration in rats submitted to the OFT.

Two-way ANOVA indicated that the distance moved in the OFT decreased along time but it did not differ among groups treated with 5-azaD or vehicle (treatment factor: $F_{4,100} = 1.63$, $P > 0.05$; time factor: $F_{4,100} = 65.14$, $P < 0.001$; interaction: $F_{16,100} = 2.31$, $P > 0.05$). Similar effects were observed for the groups treated with 5-azaC or vehicle (treatment factor: $F_{3,76} = 1.63$, $P > 0.05$; time: factor $F_{4,76} = 48.33$, $P < 0.001$). Although there was an interaction between factors ($F_{12,76} = 8.92$, $P < 0.05$), post-hoc analysis indicated that there were no significant difference between treatments at any time of the test (5-azaC vs. vehicle, Bonferroni, $P > 0.05$). None of the treatments induced any significant difference in the total distance moved in the OFT (5-azaD: $F_{4,25} = 0.54$, $P > 0.05$; 5-azaC: $F_{3,20} = 0.57$, $P > 0.05$; Figure 2).

Experiment three: Effects of single systemic administration of 5-azaD in rats submitted to the FST.

5-azaD reduced immobility time when injected immediately or 5h after pretest ($F_{3,24} = 17.25$, $P < 0.01$, Dunnett's, $P < 0.05$, Figure 3), but not when administered 1h before the test (Dunnett's, $P > 0.05$, Figure 5).

Experiment four: Effects of 5-azaD in the levels of methylated DNA and BDNF in the hippocampus of rats submitted to the FST.

Similar to experiment one, systemic treatment with 5-azaD, at the dose of 0.4 mg.Kg⁻¹ significantly reduced immobility time (mean±epm of saline and 5-azaD respectively: 135±20 and 47.8±31, $t_{10}=2.434$, $p<0.05$). This same treatment reduced hippocampal levels of methylated DNA ($t_{10}=3.10$, $p<0.05$, Figure 4) and BDNF protein ($t_{11}=2.35$, $p<0.05$, Figure 4).

Experiment five: Effects of intra-hippocampal administration of 5-azaD or RG-108 in rats submitted to the FST.

Microinjection sites in the dorsal hippocampus can be seen in Figure 5. Intra-hippocampal injection of 5-azaD significantly reduced immobility time at the dose of 100 nmol.0.5μL⁻¹ ($F_{3,28} = 3.59$, $P<0.05$; Dunnet's, $P<0.05$; Figure 8). RG-108 produced similar effects at the dose of 200 nmol.0.5μL⁻¹ ($F_{2,18}= 3.42$, $P<0.05$; Dunnet's, $P<0.05$; Figure 5).

Experiment six: Effects of 5-azaD systemic administration in mice submitted to the FST, TST or OFT. 5-azaD, at the doses of 0.2 and 0.8 mg.Kg⁻¹ for the FST and TST, respectively, decreased immobility time. Similar effects were found for imipramine (FST: $F_{4,25} = 2.85$; TST: $F_{4,31} = 6.96$, $P<0.05$; Dunnet's, $P<0.05$; Figure 9). The drug also decreased the total distance moved in the OFT ($F_{3,20} = 8.10$, $P<0.05$; Dunnet's, $P<0.05$; Figure 6).

Discussion

The results of the present study showed that systemic administration of DNA methylation inhibitors decreased immobility time in the forced swimming test, an antidepressant-

like effect. These effects are not likely to be related to unspecific motor changes, since the same treatments did not modify locomotor activity of rats in the open field test. Although 5-azaD did induce a decrease in distance moved by mice in this model, this effect would, by favouring increased immobility, actually prevent rather than induce antidepressant-like activity in the FST. 5-azaD also decrease immobility time in mice submitted to the tail suspension test, another widely employed animal model of depression (Cryan *et al.*, 2002), thus confirming the antidepressant-like effects observed in the FST. The behavioural effects induced by 5-azaD was accompanied by decreased DNA methylation and increased BDNF levels in the dorsal hippocampus. Moreover, intra-hippocampal injections of 5-azaD induced antidepressant-like effects in the FST. Together the results show that prevention of DNA methylation induces antidepressant-like effects and suggest that the dorsal hippocampus could be an important site of action for such effects.

Several DNMTs have been identified, and grouped into three major classes (DNMT1, DNMT3A and DNMT3B) depending on their substrate preference and resulting function (Klose *et al.*, 2006). DNMT1 has a preference for hemimethylated substrates and has been designated as a maintenance methyltransferase that copies the pattern of methylation from the paternal strand to the nascent strand during cell division, whereas DNMT3A and DNMT3B show equal preference for unmethylated and hemimethylated DNA *in vitro*, and were proposed as *de novo* methyltransferases (Kim *et al.*, 2009; Klose *et al.*, 2006).

DNA methylation can be prevented by Decitabine (5-azaD), a unique cytosine analog that inhibits DNMT1, reverses methylation and reactivates silenced genes (Oki *et al.*, 2007). It does so by being incorporated into DNA in place of cytosine, which covalently trap DNA methyltransferases and eventually leads to degradation of DNMTs (Szyf, 2009). 5-azaD has shown therapeutic activity in patients with myelodysplastic syndrome (MDS) or with acute and chronic

leukemias, effects which are likely to be related to its hypomethylating activity of tumor-suppressor genes (Oki *et al.*, 2007).

In the present study, the involvement of DNA methylation in the modulation of stress-induced depressive-like behaviour was investigated by systemic injection of 5-azaD to animals submitted to the FST. The results showed that this drug treatment induced an antidepressant-like effect which was significant at the dose of mg.Kg^{-1} , what is in agreement with a previous report that the dose of 0.4 mg/Kg is able to induce a significant decrease in DNA methylation in rats (Pereira *et al.*, 2004). However, higher doses produced no significant effect in the FST. This U-shape profile could be attributed to the fact that while smaller doses of 5-azaD inhibits DNMTs, higher doses can induce toxic effects due to inhibition of DNA synthesis and cell cycle arrest (Oki *et al.*, 2007).

5-azaC is another cytidine analog which, although less potent than 5-azaD (Stresemann *et al.*, 2006; Stresemann *et al.*, 2008), can also induce DNMT inhibition (Christman, 2002). Therefore, we used this drug as an attempt to further strengthen the possibility that the effects induced by 5-azaD would be due to DNMT inhibition, since these two drugs share this common mechanism of action. The results showed that systemic administration of 5-azaC to rats also induced antidepressant-like effects, in a dose-dependent manner. It is noteworthy that the effective dose of 5-azaC was 4 times smaller than the effective dose of 5-azaD, what is in agreement with their relative potency to inhibit DNA methylation *in vitro* (Stresemann *et al.*, 2006; Stresemann *et al.*, 2008).

In the FST performed in rats the immobility time is responsive to systemic antidepressant treatment only after repeated drug administration over a period of at least 24h (Cryan *et al.*, 2002; Porsolt *et al.*, 1978). However, the results presented here suggest that DNMT inhibitors might have a different pharmacological profile, with a faster onset of action, since a single administration of the drug within a short time window after the pretest session significantly reduced

the immobility time in the FST. This suggests that in rats the increased gene and protein expression that follow the pretest swimming session is necessary to counteract stress-induced behavioural consequences or mediate behavioural adaptation during the test session. The involvement of DNA methylation in the modulation of depressive-like behaviours was also supported by our findings that a single injection of 5-azaD induced antidepressant-like effects in mice submitted to the FST or the TST. In this case, however, the drug was effective when administered before the test. However, different from rats, the FST and TST in mice are known to be sensitive to pre-test acute antidepressant treatment (Cryan *et al.*, 2002). The reasons for this species difference is unknown, but the results suggest that at least in mice DNA methyltransferases are involved in the acute stress response.

Since the drugs used in this study can readily cross the blood brain barrier (Chabot *et al.* 1983), it is probable that these effects depend on DNA methylation inhibition in brain regions related to stress coping responses. Considering that DNMTs are constitutively expressed in the adult hippocampus (Brown *et al.*, 2008), an important brain region involved in behavioural adaptation to stress and in depression physiopathology (Castren, 2005; Duman *et al.*, 2006; Graeff *et al.*, 1996; Joca *et al.*, 2007a; Krishnan *et al.*, 2010), we hypothesized that the hippocampus could be an important site of action for the antidepressant-like effects induced by systemic administration of DNMT inhibitors. Corroborating this proposal, intra-hippocampal administration of 5-azaD immediately after stress induced antidepressant-like effect in the rat FST. In addition, the administration of the nonnucleoside DNMT inhibitor RG1-108 that, unlike 5-azaD, does not need to be incorporated into DNA to inhibit DNMTs (Szyf, 2009), induced similar results. This further confirms that DNA methylation in the hippocampus modulates depressive-like behaviour and suggests that this effect is not restricted to cells undergoing mitosis in this brain structure.

Altogether, these data suggest that increased DNA methylation in specific genomic loci within the hippocampus could reduce the expression of genes that would be important to regulate its functioning under aversive situations and, therefore, predispose to the development of stress-induced behavioural/emotional outcomes. This hypothesis is supported by evidence that exposure to social adversity early in life (Brown *et al.*, 2008; Zhang *et al.*, 2010) or to stressful situations in adulthood (Miller *et al.*, 2007) increases DNA methylation in the rat hippocampus. Moreover, hypermethylation of ribosomal DNA promoter sequences have been found in the hippocampus of suicide victims with a history of childhood abuse or neglect (McGowan *et al.*, 2008). Since ribosomal RNAs encode the building blocks for the protein synthesis machinery of the cell, its reduced expression is likely to impair cellular functions in the affected hippocampi (Akbarian, 2008).

Impairments in hippocampal structure, such as atrophy of apical dendrites, increased cell death and decreased neurogenesis, have been consistently found in stressed animals and humans (Lucassen *et al.*, 2010; McEwen, 2010; Pittenger *et al.*, 2008), and are suggested to contribute to the hippocampal dysfunction described in depressed humans (Sheline *et al.*, 1999; Sheline *et al.*, 1996). Since DNA methylation of genes involved in structural and neurotrophic functions seems to affect adult neurogenesis (Covic *et al.*), as well as activity-driven neuroplasticity in the hippocampus (Levenson *et al.*, 2006a; Levenson *et al.*, 2006b), it is possible that increased hippocampal DNA methylation of such genes could contribute to the stress-induced neuroplasticity within the region and its functional consequences. In line with this view, it has been reported that stress exposure increases DNA methylation and decreases the expression of BDNF in the hippocampus (Roth *et al.*, 2009), an important neurotrophin for cell survival and proliferation (Arancio *et al.*, 2007) and for antidepressant-induced behavioural effects in the FST (Adachi *et al.*, 2008; Duman *et al.*, 2006;

Saarelainen *et al.*, 2003). Corroborating this idea, in the present work the antidepressant-like effect induced by 5-azaD was accompanied by decreased global methylation in cytosine residues of DNA and increased BDNF levels in the hippocampus. It is possible that this latter effect is contributing for the antidepressant-like effects induced by 5-azaD. However, in addition to BDNF, methylation is known to regulate the expression of other genes involved in the neurobiology of depression, such as those that code for the glucocorticoid (McGowan *et al.*, 2009) and the GABA_A receptors (Poulter *et al.*, 2008). The involvement of these genes in the present results remains to be investigated. Moreover, increased DNA methylation in other brain structures could also be involved in the development of stress-induced depressive-like behaviours. It has been recently shown, for example, that DNMT3a inhibition in the nucleus accumbens induces antidepressant-like effects in the chronic social defeat stress model (LaPlant *et al.* 2010), suggesting that inhibition of DNA methylation in this nucleus could also have contribute to the antidepressant-like effects induced by systemic administration of 5-azaD and 5-azaC.

In conclusion, the present results suggest that systemic or hippocampal administration of DNA methylation inhibitors induces antidepressant-like effects and gives further support for the involvement of DNA methylation in the neurobiology of depression.

Disclosure/Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge Flávia Salata, Juliana Vercesi and José Carlos de Aguiar for their helpful technical assistance. We are also thankful to Dr L. Resstel for his helpful

discussions of this manuscript. This research was supported by grants from FAPESP (2009/18372-6; 2007/03685-3) and CNPq.

References

- Adachi M, Barrot M, Autry AE, Theobald D, Monteggia LM (2008). Selective loss of brain-derived neurotrophic factor in the dentate gyrus attenuates antidepressant efficacy. *Biol Psychiatry* 63(7): 642-649.
- Akbarian S (2008). Approaching the molecular pathology of suicide. *Biol Psychiatry* 64(8): 643-644.
- Arancio O, Chao MV (2007). Neurotrophins, synaptic plasticity and dementia. *Curr Opin Neurobiol* 17(3): 325-330.
- Brown SE, Weaver IC, Meaney MJ, Szyf M (2008). Regional-specific global cytosine methylation and DNA methyltransferase expression in the adult rat hippocampus. *Neurosci Lett* 440(1): 49-53.
- Castren E (2005). Is mood chemistry? *Nat Rev Neurosci* 6(3): 241-246.
- Christman JK (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21(35): 5483-5495.
- Covic M, Karaca E, Lie DC (2010). Epigenetic regulation of neurogenesis in the adult hippocampus. *Heredity* 105(1): 122-134.
- Covington HE, 3rd Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O et al. (2009). Antidepressant actions of histone deacetylase inhibitors. *J Neurosci* 29(37): 11451-11460.
- Crestani CC, Alves FH, Correa FM, Guimaraes FS, Joca SR (2010). Acute reversible inactivation of the bed nucleus of stria terminalis induces antidepressant-like effect in the rat forced swimming test. *Behav Brain Funct* 6: 30.
- Cryan JF, Markou A, Lucki I (2002). Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* 23(5): 238-245.
- Duman RS, Monteggia LM (2006). A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 59(12): 1116-1127.

Graeff FG, Guimaraes FS, De Andrade TG, Deakin JF (1996). Role of 5-HT in stress, anxiety, and depression. *Pharmacol Biochem Behav* 54(1): 129-141.

Greenberg PE, Kessler RC, Birnbaum HG, Leong SA, Lowe SW, Berglund PA et al (2003). The economic burden of depression in the United States: how did it change between 1990 and 2000? *J Clin Psychiatry* 64(12): 1465-1475.

Hindmarch I (2002). Beyond the monoamine hypothesis: mechanisms, molecules and methods. *Eur Psychiatry* 17 Suppl 3: 294-299.

Hunter RG, McCarthy KJ, Milne TA, Pfaff DW, McEwen BS (2009). Regulation of hippocampal H3 histone methylation by acute and chronic stress. *Proc Natl Acad Sci U S A* 106(49): 20912-20917.

Joca SR, Ferreira FR, Guimaraes FS (2007a). Modulation of stress consequences by hippocampal monoaminergic, glutamatergic and nitrenergic neurotransmitter systems. *Stress* 10(3): 227-249.

Joca SR, Guimaraes FS, Del-Bel E (2007b). Inhibition of nitric oxide synthase increases synaptophysin mRNA expression in the hippocampal formation of rats. *Neurosci Lett* 421(1): 72-76.

Joca SR, Padovan CM, Guimaraes FS (2003). Activation of post-synaptic 5-HT(1A) receptors in the dorsal hippocampus prevents learned helplessness development. *Brain Res* 978(1-2): 177-184.

Jones, PA, Takai, D (2001) The role of DNA methylation in mammalian epigenetics. *Science* 293(5532): 1068-1070.

Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR et al (2003). The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA* 289(23): 3095-3105.

Kessler RC, Gruber M, Hettema JM, Hwang I, Sampson N, Yonkers KA (2008). Co-morbid major depression and generalized anxiety disorders in the National Comorbidity Survey follow-up. *Psychol Med* 38(3): 365-374.

Kim JK, Samaranyake M, Pradhan S (2009). Epigenetic mechanisms in mammals. *Cell Mol Life Sci* 66(4): 596-612.

Klose RJ, Bird AP (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* 31(2): 89-97.

Krishnan V, Nestler EJ (2010). Linking Molecules to Mood: New Insight Into the Biology of Depression. *Am J Psychiatry* 167(11): 1305-20.

- Krishnan V, Nestler EJ (2008). The molecular neurobiology of depression. *Nature* 455(7215): 894-902.
- LaPlant Q, Vialou V, Covington HE 3rd, Dumitriu D, Feng J, Warren BL et al (2010). Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat Neurosci* 13(9): 1137-1143.
- Levenson JM, Roth TL, Lubin FD, Miller CA, Huang IC, Desai P et al (2006a). Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *J Biol Chem* 281(23): 15763-15773.
- Levenson JM, Sweatt JD (2006b). Epigenetic mechanisms: a common theme in vertebrate and invertebrate memory formation. *Cell Mol Life Sci* 63(9): 1009-1016.
- Lucassen PJ, Meerlo P, Naylor AS, van Dam AM, Dayer AG, Fuchs E et al (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *Eur Neuropsychopharmacol* 20(1): 1-17.
- McEwen BS (2010). Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Ann N Y Acad Sci* 1204 Suppl: E38-59.
- McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, Turecki G, Meaney MJ (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 12(3): 342-348.
- McGowan PO, Sasaki A, Huang TC, Unterberger A, Suderman M, Ernst C et al (2008). Promoter-wide hypermethylation of the ribosomal RNA gene promoter in the suicide brain. *PLoS One* 3(5): e2085.
- Miller CA, Sweatt JD (2007). Covalent modification of DNA regulates memory formation. *Neuron* 53(6): 857-869.
- Oki Y, Aoki E, Issa JP (2007). Decitabine--bedside to bench. *Crit Rev Oncol Hematol* 61(2): 140-152.
- Pereira MA, Wang W, Kramer PM, Tao L (2004). DNA hypomethylation induced by non-genotoxic carcinogens in mouse and rat colon. *Cancer Lett* 212(2): 145-151.
- Pittenger C, Duman RS (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology* 33(1): 88-109.
- Porsolt RD, Anton G, Blavet N, Jalfre M (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47(4): 379-391.
- Porsolt RD, Le Pichon M, Jalfre M (1977). Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266(5604): 730-732.

Poulter MO, Du L, Weaver IC, Palkovits M, Faludi G, Merali Z et al (2008). GABAA receptor promoter hypermethylation in suicide brain: implications for the involvement of epigenetic processes. *Biol Psychiatry* 64(8): 645-652.

Racagni G, Popoli M (2008). Cellular and molecular mechanisms in the long-term action of antidepressants. *Dialogues Clin Neurosci* 10(4): 385-400.

Rosenzweig-Lipson S, Beyer CE, Hughes ZA, Khawaja X, Rajarao SJ, Malberg JE et al (2007). Differentiating antidepressants of the future: efficacy and safety. *Pharmacol Ther* 113(1): 134-153.

Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009). Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biol Psychiatry* 65(9): 760-769.

Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E et al (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J Neurosci* 23(1): 349-357.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S et al (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301(5634): 805-809.

Schroeder FA, Lin CL, Crusio WE, Akbarian S (2007). Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. *Biol Psychiatry* 62(1): 55-64.

Scopinho AA, Scopinho M, Lisboa SF, Correa FM, Guimaraes FS, Joca SR (2010). Acute reversible inactivation of the ventral medial prefrontal cortex induces antidepressant-like effects in rats. *Behav Brain Res* 214(2): 437-442.

Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999). Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. *J Neurosci* 19(12): 5034-5043.

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996). Hippocampal atrophy in recurrent major depression. *Proc Natl Acad Sci U S A* 93(9): 3908-3913.

Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F (2006). Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res* 66(5): 2794-2800.

Stresemann C, Lyko F (2008). Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* 123(1): 8-13.

Szyf M (2009). Epigenetics, DNA methylation, and chromatin modifying drugs. *Annu Rev Pharmacol Toxicol* 49: 243-263.

Tsankova N, Renthal W, Kumar A, Nestler EJ (2007). Epigenetic regulation in psychiatric disorders. *Nat Rev Neurosci* 8(5): 355-367.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9(4): 519-525.

Weber M, Schubeler D (2007). Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr Opin Cell Biol* 19(3): 273-280.

Yoo CB, Cheng JC, Jones PA (2004). Zebularine: a new drug for epigenetic therapy. *Biochem Soc Trans* 32(Pt 6): 910-912.

Zhang TY, Hellstrom IC, Bagot RC, Wen X, Diorio J, Meaney MJ (2010). Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. *J Neurosci* 30(39): 13130-13137.

LEGENDS OF FIGURES

Figure 1. Systemic injection of 5-aza-2-deoxycytidine (5-azaD), 5-azacytidine (5-azaC) or imipramine (IMI) in rats reduced the immobility time in the forced swimming test. Animals were submitted to the pretest session and received three i.p. injections (0, 5 and 23h after) of vehicle, 5-azaD (0.1-0.8 mg.Kg⁻¹) or 5-azaC (0.4-3.2 mg.Kg⁻¹). The immobility time was recorded 1h after the last injection in a 5 min test session. Data are expressed as mean ± S.E.M. (n=5-8/group). * indicates significant difference from vehicle-treated group (P<0.05, ANOVA followed by Dunnet's test).

Figure 2. Systemic injection with 5-aza-2-deoxycytidine (5-azaD) or 5-azacytidine (5-azaC) in rats did not modify locomotor activity in the open field test. Animals received 3 i.p. injections of 5-azaD (0.1-0.8 mg.Kg⁻¹), 5-azaC (0.4-3.2 mg.Kg⁻¹) or vehicle (24, 19 and 1h before the test) and had their locomotor activity evaluated during 5 min. Data are expressed as mean ± S.E.M. (n=6-10/group). No significant differences between groups were detected (P>0.05, two-way ANOVA).

Figure 3. Single systemic injection of 5-aza-2-deoxycytidine (5-azaD) in rats, immediately or 5h after the pretest, reduced the immobility time in the forced swimming test. Animals were submitted to the pretest session and received an i.p. injection (0, 5 or 23h after) of vehicle or drug. The immobility time was recorded in a 5 min test session, 24h after pretest. Data are expressed as mean ± S.E.M. (n=5-7/group). * indicates significant difference from vehicle-treated group (P<0.05, Student-t test).

Figure 4. Systemic injection of 5-azaD in rats reduced global DNA methylation and increased BDNF levels, in the hippocampus. Animals were submitted to the pretest session and received three i.p. injections (0, 5 and 23h after) of vehicle or drug and were sacrificed 1h after the last injection, immediately after (DNA methylation) or before (BDNF levels) the test session. Data are expressed as mean \pm S.E.M. (n=5-7/group). * indicates significant difference from vehicle-treated group ($P < 0.05$, Student-t test).

Figure 5. Post-stress intra-hippocampal administration in rats of 5-aza-2-deoxycytidine (5-azaD) or RG-108 reduced the immobility time in the forced swimming test. Animals received a bilateral intra-hippocampal injection (0.5 μ l) of drug or vehicle immediately after the pretest session and were tested 24h later. A) Diagram representing injection sites aimed at the dorsal hippocampus (dots indicate the localization of the injection site visualized by the dye injection). IA: interaural distance. B) Effects of 5-azaD (50-100 nmol.0.5 μ l⁻¹) or RG-108 (100, 200 nmol.0.5 μ l⁻¹) on the immobility time in the FST. Data are expressed as mean \pm S.E.M. (n=6-9/group). * indicates significant difference from vehicle-treated group ($p < 0.05$, ANOVA followed by Dunnet's test).

Figure 6. Single systemic injection of 5-azaD (0.2-0.8 mg.Kg⁻¹) in mice decreased immobility time in the forced swimming (FST, upper panel) and tail suspension tests (TST, middle panel). The drug also decrease the total distance travelled in the open field test (OFT, lower panel). Experiments were conducted with independent groups of animals that received i.p. injections of 5-azaD, imipramine or vehicle and were submitted to the behavioural tests 1 h later. Data are

expressed as mean \pm S.E.M. (n=5-9/group). * indicates significant difference from vehicle-treated group (p<0.05, ANOVA followed by Dunnet's test).

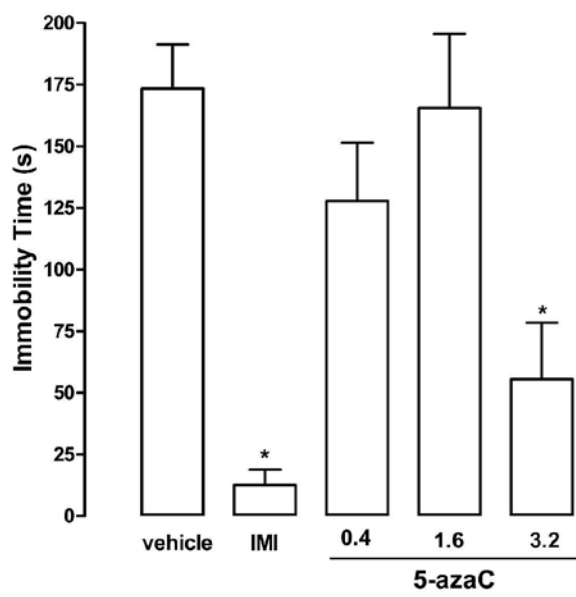
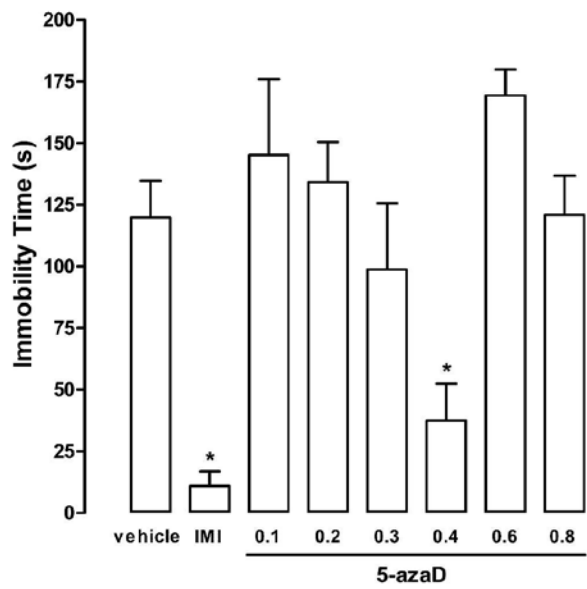


figure 1

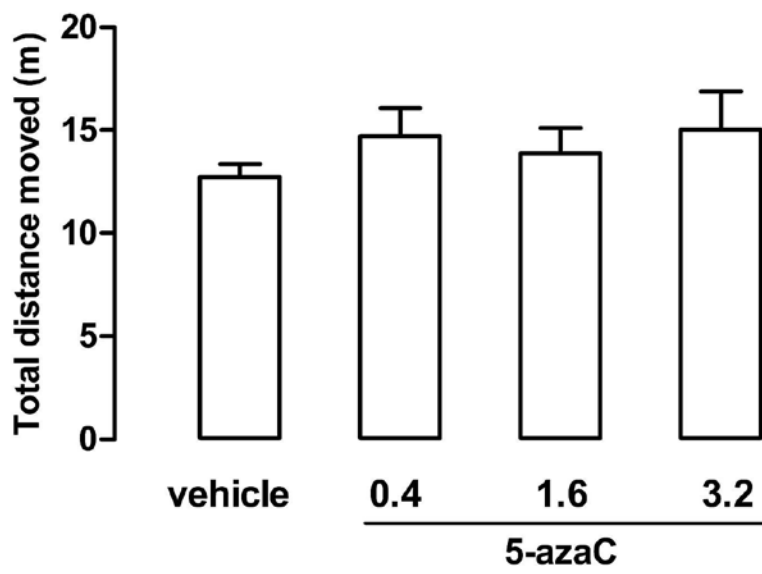
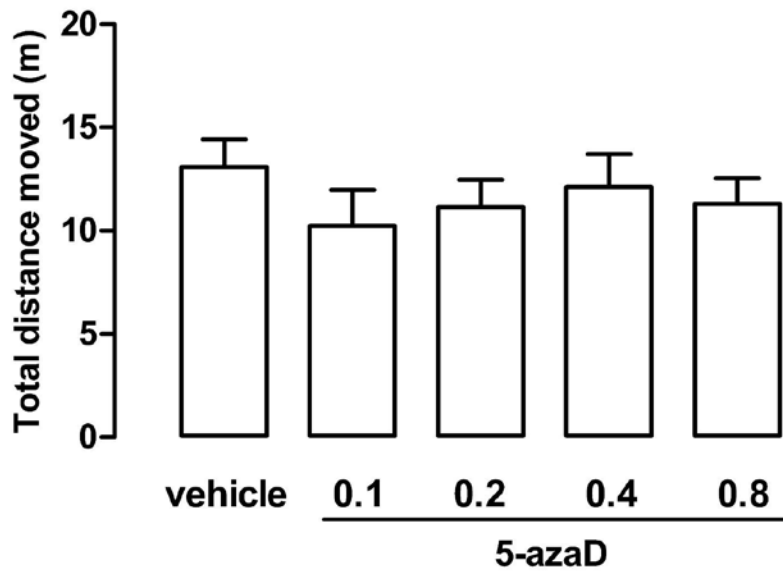


figure 2

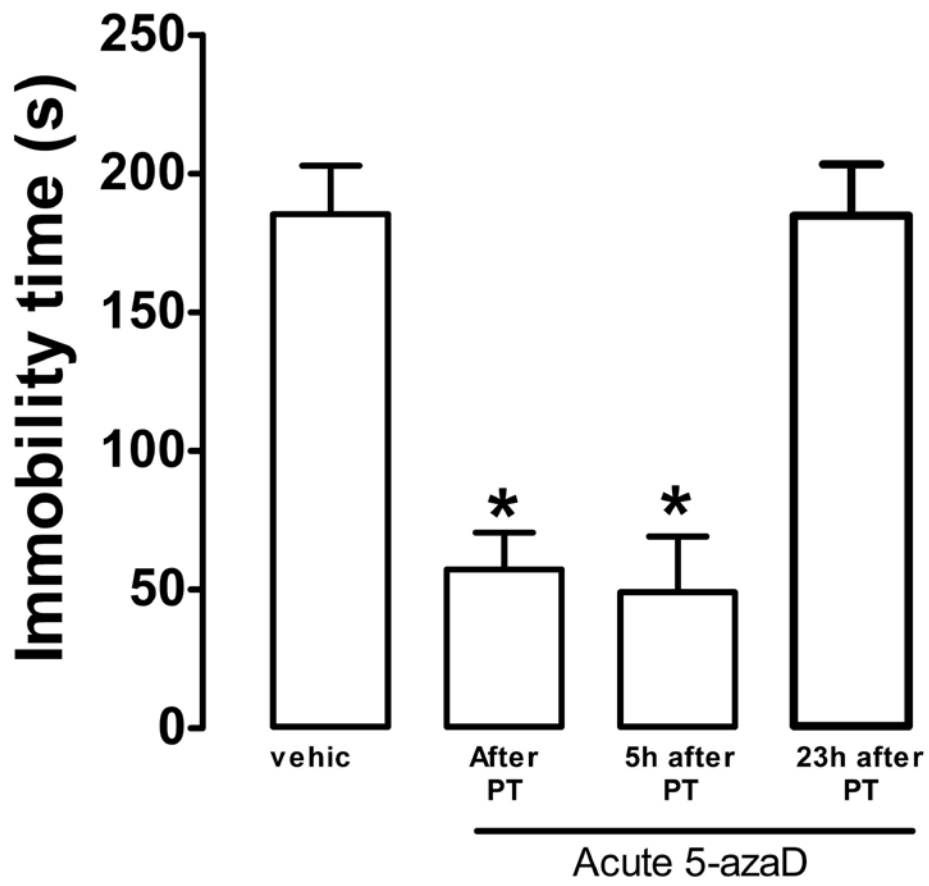


figure 3

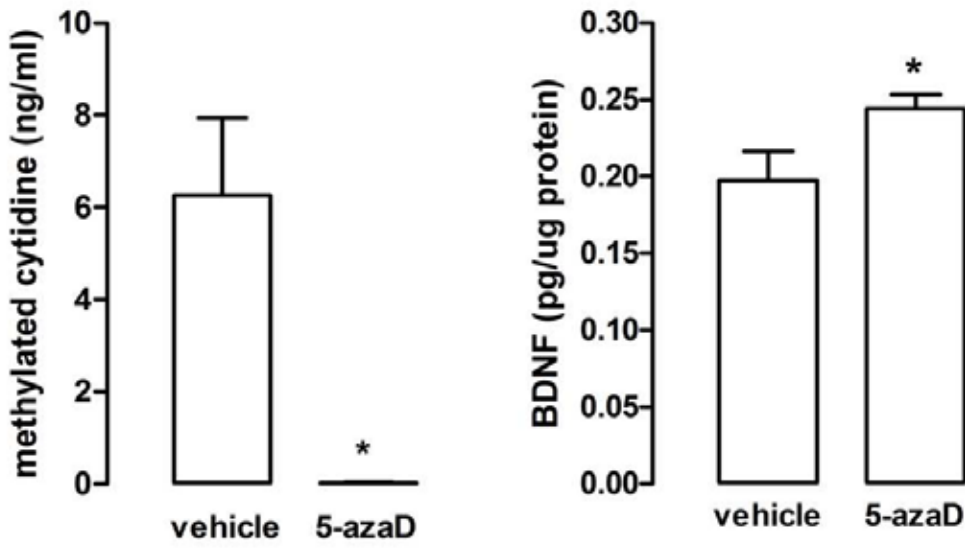


figure 4

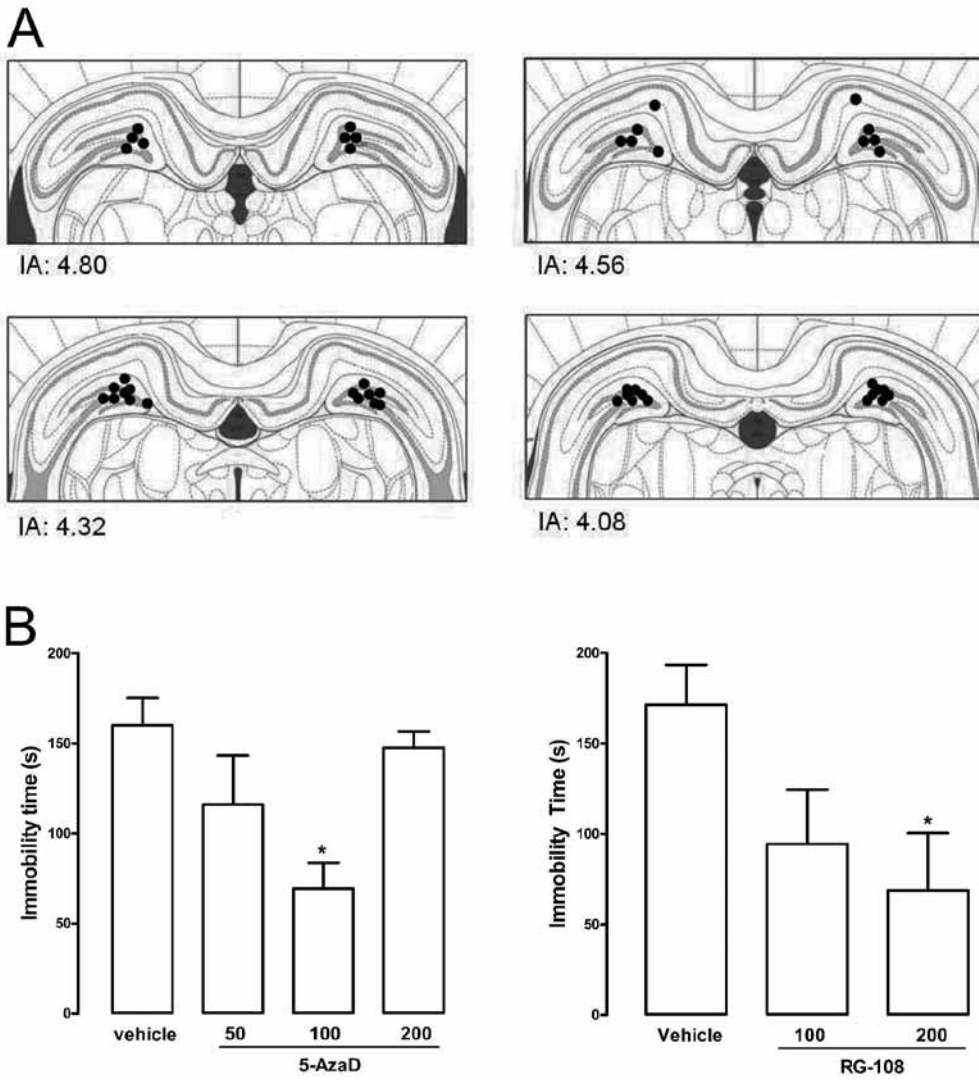


figure 5

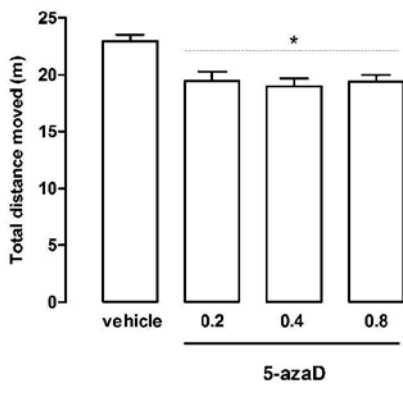
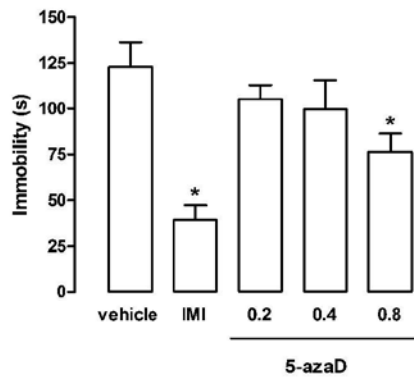
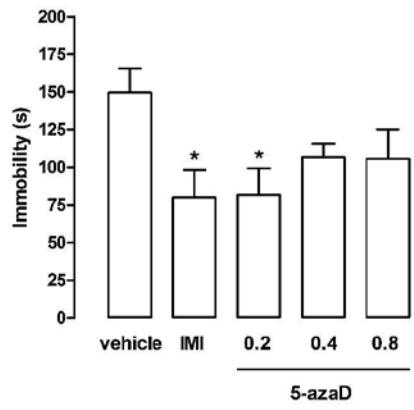


figure 6