Clozapine or Haloperidol in rats prenatally exposed to methylazoxymethanol, a compound inducing entorhinal-hippocampal deficits, alter brain and blood neurotrophins' concentrations

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Summary. Rats exposed during prenatal life to methylazoxymethanol (MAM) display in postnatal age structural and behavioral deficits resembling those observed in schizophrenic patients. These deficits are associated with significant changes in brain nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), particularly in the hippocampus and entorhinal cortex. In the present study, we used the MAM model to investigate in young rats the effect of antipsychotics, Clozapine and Haloperidol, on brain and blood NGF and BDNF presence. Young animals were used because administration of antipsychotics during adolescence is a common feature of intervention. The results showed that administration of Clozapine and Haloperidol causes significant changes in the concentration of NGF and BDNF in the brain and bloodstream of MAM-treated rats. These findings indicate that these drugs may affect the synthesis and release of neurotrophins in the central nervous system and in the blood circulation. In addition, the MAM model can be a useful tool to investigate the biochemical and molecular mechanisms regarding the effects of antipsychotics.

Key words: NGF, BDNF, MAM, haloperidol, clozapine, schizophrenia.

Riassunto *(Clozapina o Aloperidolo in ratti esposti in fase prenatale a Metiazossimetanolo, un composto che induce danni nella corteccia entorinale e nell'ippocampo, altera le concentrazioni nel sangue e nel cervello delle neurotrofine)*. Il trattamento prenatale con metilazossimetanolo (MAM) determina nel ratto alterazioni morfologiche e comportamentali comparabili a ciò che si osserva nel paziente schizofrenico. Questo complesso di eventi è associato con cambiamenti dei livelli basali di *nerve growth factor* (NGF) e di *brain derived neurotrophic factor* (BDNF) nel cervello del ratto, in particolare nell'ippocampo e nella corteccia entorinale. In questo lavoro abbiamo studiato nel modello animale MAM di ratto giovane gli effetti di antipsicotici come la clozapina e l'aloperidolo sulla presenza nel cervello di NGF e BDNF dal momento che questi farmaci sono comunemente utilizzati sugli adolescenti. I risultati mostrano che sia la clozapina e l'aloperidolo modificano i livelli di NGF e BDNF nel sangue e nel cervello e indicano che questi farmaci possono far cambiare la sintesi e il rilascio delle neurotrofine nel sistema nervoso centrale e nel sistema circolatorio. I dati evidenziano anche che il modello MAM può essere un approccio utile allo studio degli antipsicotici.

Parole chiave: NGF, BDNF, metilazossimetanolo, aloperidolo, clozapina, schizofrenia.

INTRODUCTION

Epidemological studies indicate that gestational and perinatal alterations in brain neurogenesis may increase the risk of developing behavioral and/or neuropathological deficits leading to schizophrenia during early and late post-natal life [1]. This neurodevelopmental disorder is characterized by impaired cognitive and social performances, disruption of brain cytoarchitectural and neural plasticity in the limbic system, particularly in the entorhinal cortex and hippocampus [2-4]. There is also evidence that maternal starvation, infection, and anoxic birth injury can lead during postnatal life to behavioral and brain structural deficits resembling those observed in schizophrenia [5-7].

It has been shown [8, 9] that interference with neurogenesis in the mediotemporal allocortex of the rat embryos, during the earliest stages of cortical proliferation, results not only in a thickness reduction of

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the adult entorhinal cortex and hippocampus [9, 10] but also in other morphological characteristics that may be compared with the changes observed in the hippocampal-entorhinal axis of patients with schizophrenia disease [11]. An animal model displaying these structural brain deficits was obtained administering a single injection of methylazoxymethanol acetate (MAM) in pregnant rat on gestational day 12 (GD12) or 11 when the entorhinal-hippocampal axis was supposed to be in major cell proliferation [12]. Indeed in the rat it has been reported that prenatal MAM administration of [13, 14], at a specific time point of prenatal limbic and cortical development, causes cell loss and reduces the thickness of the hippocampus, the entorhinal cortex and other brain structures [8, 15-17]. MAM (reviewed in [13]) is a short-acting alkylating agent that methylates nucleic acids leading to the death of cells actively replicating DNA. In the rat the reaction of MAM with nucleic acids lasts 2-24 h after injection and is maximal at 12 h. As the different regions of the brain proliferate at overlapping but different times, it is possible, in principle, to produce relatively specific damage, depending on the exact time of drug administration. Prenatal MAM administration induces also loss of neurons, impaired neuroplasticity, cognitive deficits, and alterations in the basal levels of neurotrophins [18, 19] and these changes are long lasting since they were found at different ages *(*see *Table 1)*.

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are neurotrophins promoting the differentiation and survival of a number of brain developing neurons. NGF and BDNF contribute to the survival and plasticity of adult neurons in

Table 1 | *Some biochemical and behavioral parameters analyzed following prenatal MAM given in rats at gestational day 11 (GD11), 12 (GD12); ↑↑ means increasing, ↓↓ means decreasing, ↔ means no changes, NE means not examined*

Age	Young [51] 24 days	Young adult [15] 60 days	Fully adult [10, 64] 6 months	Aged [18] > 1 year
NGF Entorhinal cortex				
GD12	↑↑	↑↑	↑↑	↑↑
GD11	NE	↔	↔	↔
BDNF Entorhinal cortex				
GD12	↑ (p=0.08)	NE	↑↑	↔
GD11	NE	NE	↔	↔
NGF Hippocampus				
GD12	↑↑	↑ (p=0.07)	↑↑	↓↓
GD11	NE	↔	↔	↔
BDNF Hippocampus				
GD12	↑↑	NE	↑↑	↓↓
GD11	NE	NE	↔	↓↓
ChAT Meynert's nuclei				
GD12	NE	NE	↔	↓↓
GD11	NE	NE	↔	↓↓
ChAT Septum				
GD12	NE	NE	↔	↓↓
GD11	NE	NE	↔	↔
Learning performance in the water maze				
GD11	NE	NE	↓↓	↓↓
GD12	NE	NE	NE	↓↓
Passive avoidance response				
GD11	NE	↓↓	NE	NE
GD12	NE	↓↓	NE	NE
TrkA Entorhinal cortex				
GD12	↑↑	NE	NE	NE
GD11	NE	NE	NE	NE
TrkA Hippocampus				
GD12	↑↑	NE	NE	NE
GD11	NE	NE	NE	NE
TrkB Entorhinal cortex				
GD12	↑↑	NE	NE	NE
GD11	NE	NE	NE	NE
TrkB Hippocampus				
GD12	↑↑	NE	NE	NE
GD11	NE	NE	NE	NE

both normal conditions and following injuries [20-22]. Neurotrophins are known to play also a crucial role on survival and plasticity of forebrain cholinergic neurons involved in cognitive processes undergoing degeneration in memory-loss-associated disorders, including Alzheimer and schizophrenia [23-27].

It has been reported that the basal level of NGF in the bloodstream undergoes significant changes in patients affected by schizophrenia [28-30], after Haloperidol administration [31, 32] or electro-convulsive therapy [33] and that schizophrenic patients have low NGF levels [32, 34] or presence of NGF autoantibodies [35]. Moreover, it has been postulated that modulation of the synthesis and secretion of neurotrophins, such as NGF and BDNF by antidepressants might be a novel approach to treat depression and other brain diseases including Alzheimer and Parkinson [36, 37]. These observations raised the question as whether a short or acute administration of antipsychotics as Clozapine or Haloperidol, drugs that are widely used in psychiatric disorders [38-40], might affect the synthesis and release of NGF and BDNF in the blood and brain regions in young rats since antipsychotics are widely used during childhood for treating psychiatric disorders. To further explore the functional role of these neurotrophins, we also investigated the expression of their main receptors, TrkA and TrkB respectively, and the presence of granulocytes in the blood since these cells are known to be affected by antipsychotics administration [41].

METHODS

Subject housing and MAM treatment

Gestating Sprague Dawley rats (*Rattus norvegicus*) were obtained from an animal farm (Charles River, Italy), which mated animals over a period of 4 h, on the day considered as day 0 of gestation (GD0). Dams with a vaginal plug were separated from the males and transported to the laboratories. Upon arrival at the laboratory, animals were housed in a room with air conditioned (temperature 21 ± 1 °C, relative humidity 60 ± 10%), with white lights on from 7:00 a.m. up to 7:00 p.m. in Plexiglas boxes with a metal top and sawdust as bedding. Regular rat pellet food and water were available *ad libitum*. According to procedures previously described [15, 42], pregnant rats were randomly divided in the MAM and Saline groups. Ten pregnant rats for the MAM group underwent a single intraperitoneal injection of MAM (22 mg/kg) on gestational day 12 (GD12). This period of gestation is crucial for the neurogenesis of the hippocampal-entorhinal axis and alteration during this time may selectively impair cell migration and also may lead to cell death. MAM was purchased from NCI Chemical Carcinogen Reference Standard Repository Midwest Research Institute, Kansas City, MO, USA. As controls we used dams (10 rats) which received saline solution (22 mg/kg i.p.) on GD12. At birth animals were fostered to the biological dams following behavioral procedures previously described [42] and used also for other MAM studies [8, 18]. Litters were also reduced to 3 males and 3 females.

Male animals prenatally administered with MAM or saline were tested at a month of age. Six groups were considered: MAM-Cloza, MAM-Halo, MAM-Controls, Sal-Cloza, Sal-Halo and Sal-Controls. The first 3 groups received MAM *in utero*, the second 3 groups received saline *in utero*. At a month of age MAM and SAL rats were administered daily for 8 consecutive days with vehicle, Clozapine (Cloza) or Haloperidol (Halo). MAM-Controls and Sal-Controls (n = 10 for both) were injected i.p. with vehicle (0.8% acetic acid in glucose). MAM-Halo and Sal-Halo (n = 10 for both) received i.p. 2 mg/kg of haloperidol (Janssen-Cilag, Cologno Monzese, Italy). MAM-Cloza and Sal-Cloza (n = 10 for both) received i.p. 20 mg/kg of clozapine (Novartis Pharma, Origgio, Italy). All procedures involving chemicals' preparation, dosages and administration were performed following methods previously described [43, 44] involving the effects of antipsychotics in the hippocampal region. We used young animals because the awareness of the onset during childhood of psychiatric disorders is rapidly increasing. This issues includes administration of antipsychotics during adolescence that is at the present time a common method of intervention with times and dosages of administration extremely variables [45]. Female rats were used for other experiments.

All efforts were made to minimize and reduce animal suffering and for limiting the number of animal used. All animal experiments were carried out following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC). The Haloperidol has been taken into account since it represents a typical antipsychotic acting mainly on D2 receptors. Clozapine has been used since it is the first member of the atypical antipsychotics with a wide mechanism of action binding both dopaminergic and serotoninergic receptors [39]. The distinctive binding abilities of the 2 molecules lead to different clinical applications and to different effects on neurotrophins.

Animal sacrifice, and tissue dissection

Rats were killed with a guillotine, the brain quickly removed and the hippocampus, entorhinal cortex and striatum dissected out using a rat brain matrix (ASI Instruments, Inc. Co. USA) [see also the methodology described by Cuello, 46] and stored at -70 °C until used.

NGF and BDNF determination

NGF/BDNF evaluation (n = 5 for group, one animal per litter) was carried out in the hippocampus, entorhinal cortex and striatum of the rat brain with a ELISA kits "NGF Emaxtm ImmunoAssay System number G7631" and "BDNF Emaxtm ImmunoAssay System number G6891" by Promega, (Madison, WI,

USA) following the instructions provided by the manufacturer. The brain tissues were homogenized with ultrasonication in extraction buffer (Tris-acetate 20 mM, pH 7.5, NaCl 150 mM, EDTA 1mM, EGTA 1 mM, Sodium-Pyrophosphate 2.5 mM, Ortovanadate 1 mM, β-Glycerolphosphate 1 mM, NaF 100mM, PMSF 1 mM, Leupeptin 1 μg/ml) and centrifuged at 4 °C for 10 min, 13 000 rpm and supernatants were recovered (EDTA, Ethylenediamine-Tetraacetic acid; EGTA, Ethyleneglycol-Tetraacetic acid; PMSF, Phenylmethylsulfonyl Fluoride). Briefly, 96-well immunoplates were coated with 100 μl per well of polyclonal anti-NGF antibody. After an overnight incubation at 4 °C, the plates were washed one time with wash buffer (Tris-HCl 20 mM, pH 7.6, NaCl 150 mM, 0.05% Tween® 20) and then blocked for 1 hour with block & sample 1x buffer provided by manufacturer (200 μl for well). After washing the samples were incubated in the coated wells (100 μl each) for 6 h at room temperature with shaking. After an additional five washes the immobilized antigen was incubated with an anti-NGF monoclonal antibody overnight at 4 °C. The plates were washed again with wash buffer, and then incubated with an anti-rat IgG HRP (Horseradish Peroxydase) conjugate (100 μl/well) for 2.5 h at room temperature. After washing the plates were incubated with a TMB/Peroxidase substrate solution for 15 min (100 μl/well) (TMB, Tetramethyl Benzidine) provided by the manufacturer. Reaction was then stopped with 100 μl/well 1N HCl. The colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, PBI International, USA). NGF/BDNF concentrations were determined, from the regression line for the NGF/BDNF standard (ranging from 7.8 to 500 pg/ml purified NGF or BDNF) incubated under similar conditions in each assay. Under these conditions, the recovery of NGF or BDNF in our assay ranged from 80 to 90%. The NGF sensitivity of the assay was about 3 pg/g of wet tissue and cross-reactivity with other related neurotrophic factor (BDNF, Neurotrophin-3 and Neurotrophin-4) was less than 3%. The BDNF sensitivity of the assay was about 15 pg/ml of wet tissue and cross-reactivity with other related neurotrophic factor (NGF, Neurotrophin-3 and Neurotrophin-4) was less than 3%. Data are represented as pg/g wet tissue and all assays were performed in duplicate.

Western blotting analysis for TrkA, TrkB

The hippocampus, entorhinal cortex and striatum (n = 5 for group, one animal per litter) were homogenized in sample buffer (0.01 M TRIS-HCl buffer pH 7.6, containing 0.25 M sucrose, 0.1 M NaCl, 1 mM EDTA, and 1 mM PMSF) and centrifuged at 10 000 g for 10 min at 4 °C. The supernatants were then used for western blotting. Samples (30 μg total protein) were dissolved with loading buffer (0.1 M TRIS-HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% SDS-PAGE, and electrophoretically transferred to polyvinylidene fluoride membrane, (PVDF) for 3 h. The membranes were incubated for 40 min at room temperature with blocking buffer (10% non-fat dry milk, 10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20). Membranes were washed three times for 10 min each at room temperature in Tris-buffered saline and Tween (TTBS) (10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20) followed by an incubation for 2 h at room temperature with anti-TrkA 1:1000 (provided by Santa Cruz, CA, USA, catalog number 763:sc-118), anti-TrkB 1:1000 (provided by BD Biosciences Pharmingen, San Jose CA, USA, catalog number 610101). As shown in the instructions TrkA antibody does not react with TrkB or TrkC. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with horseradish peroxydase-conjugated anti-rabbit IgG for TrkA or anti-mouse IgG for TrkB (Cell Signalling, Beverly, Mass., USA) as secondary antibodies. The blots were developed with enhanced chemiluminescent (ECL) assay (Amersham Bioscience) as chromophore. Similar results were obtained in 5 independent Western blot runs. Band densitometry evaluation, expressed as arbitrary units of gray level, of five different gel runs/blot, was made on a Macintosh computer with the public domain NIH Image program (developed at NIH, Bethesda, MD; available from: http://rsb.info.nih.gov/nih-image/), which determines the OD of the bands with a gray scale thresholding operation. The OD of β-actin bands was used as a normalizing factor. The optical density of β-actin bands was used as an internal control for difference in sample loading. For each blotting, normalized values were expressed as percentage of relative normalized controls and used for the statistical analyses.

Biological activity and quantitative evaluation of NGF released in the bloodstream

The rat pheochromocytoma PC-12 cell line was used in a functional assay and quantitative evaluation of NGF capable to induce neurite outgrowth as previously described [47, 48]. Briefly the serum of control, Halo, and Cloza MAM-treated rats was diluted between 1:2 and 1:50 and added to PC-12 cell cultured in RPMI with 5% of fetal bovine serum and 10% of horse serum. After 24 and 48 h in culture, cells bearing neurites were scored. The quantitative evaluation was determined according to the standard NGF dose response curve obtained by exposing PC-12 cells to different concentrations of purified NGF [47].

Blood collection, cell staining and granulocyte counting

The blood was collected at the moment of animal sacrifice. One hundred mg of blood taken from each rats was used to make glass smears. Blood smears were stained with May-Grunwald-Gimsa method, dried, covered with mounting medium and the granulocytes present in 10 different fields at magnification 40X were counted and compared.

Statistical analysis

Data were analyzed by two-way ANOVA considering as effects the prenatal MAM and the drugs' exposure. Post-hoc comparisons were performed using the Tukey-Kramer method and a difference of 0.05 or less was considered statistically significant.

RESULTS

Effect of prenatal MAM administration and drug exposure on rat body weight

In *Figure 1* is shown that prenatal MAM affected the body weight of young rats (the lines with the black geometrical drawings). ANOVA revealed that MAM *per se* decreased the rat body weight [$F(1,40) = 15.71$; $F(7,280) = 19.43$, $ps < 0.01$ for the main effect of prenatal MAM administration and for the interaction prenatal MAM x repeated measures, respectively]. ANOVA did not evidence any significant effect of antipsychotics' treatment. However, the body weight of both MAM rats and Saline rats (the lines with the triangles) and administered postnatally with vehicle is highly comparable and no statistical differences were found.

Effect of Haloperidol and Clozapine on NGF levels

As shown in *Figure 2*, Haloperidol and Clozapine administration alters brain NGF concentration. In the hippocampus an increase was evidenced by statistical analysis [$F(1.24) = 39.51$, $p < 0.01$ for the main effect of prenatal MAM] which resulted to be higher mainly following Haloperidol [$F(2.24) = 12.47$, $p = 0.05$ in the interaction prenatal MAM x drug exposure; $p < 0.05$ in post-hocs]. In the entorhinal cortex ANOVA showed a significant increase in NGF in MAM animals [$F(1,24) = 150.48$; $F(2.24) = 5.90$; $ps < 0.05$, main effect of MAM and interaction prenatal MAM x drug exposure, respectively] with a more pronounced effect following Clozapine administration [$F(1.24) = 23.17$; $p < 0.05$ for the effect of drug administration; $p < 0.05$ in post-hocs]. In the striatum again prenatal MAM induced elevation in NGF values [$F(1.24) = 6.58$, $p = 0.02$ in the ANOVA for the

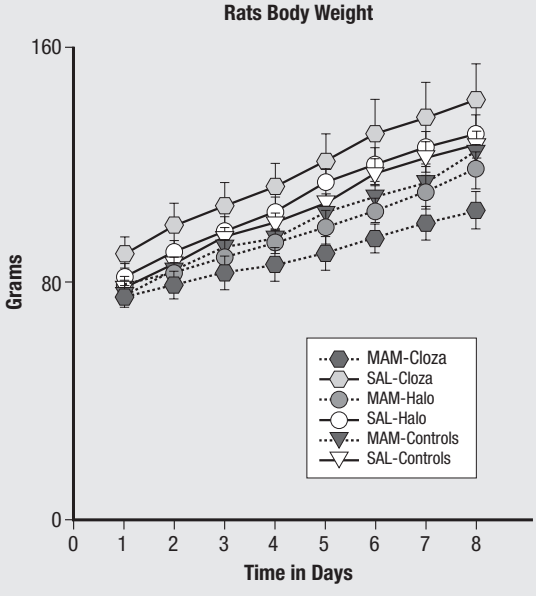


Fig. 1 | *Rat body weight in grams in 30-days old rats prenatally exposed to 20 mg/kg of MAM at gestational day 12 and administered with Clozapine (Cloza) or Haloperidol (Halo) for 8 days.*

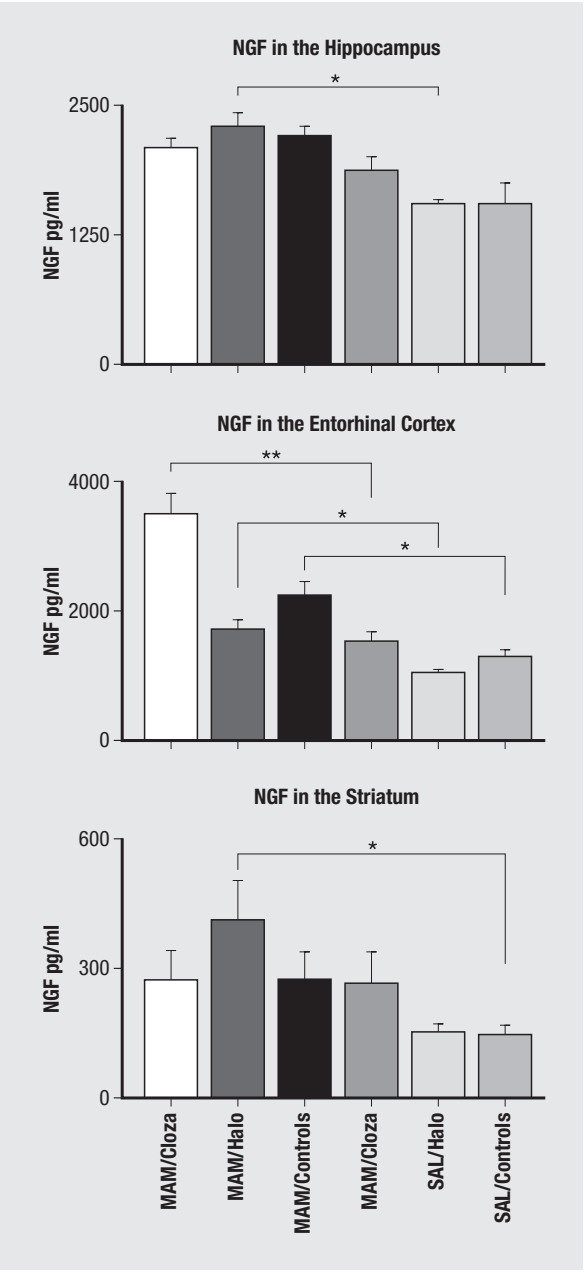
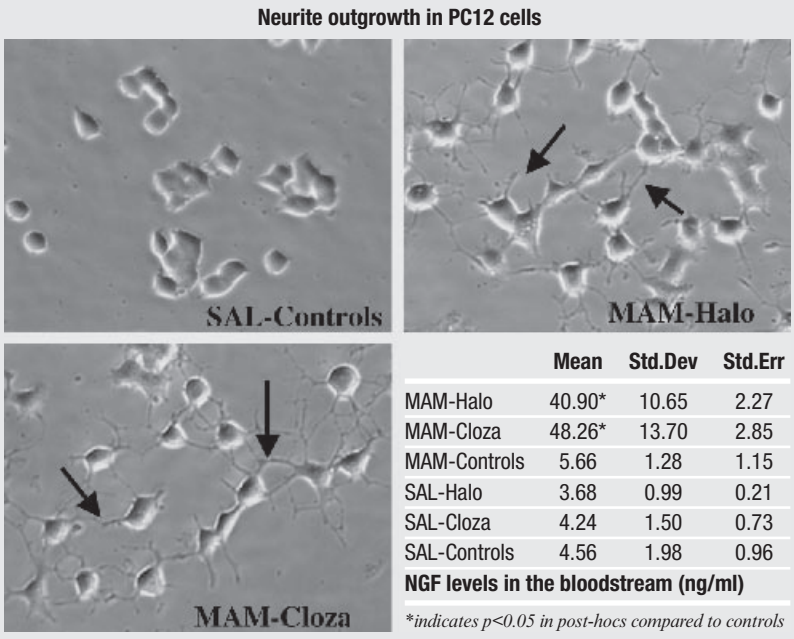


Fig. 2 | *Mean concentration (± SEM) of NGF in the hippocampus, entorhinal cortex and striatum of saline and MAM treated rats receiving Clozapine (Cloza) or Haloperidol (Halo). Asterisks indicate significant differences between groups (* = p < 0.05; ** = p < 0.01).*



	Mean	Std.Dev	Std.Err
MAM-Halo	40.90*	10.65	2.27
MAM-Cloza	48.26*	13.70	2.85
MAM-Controls	5.66	1.28	1.15
SAL-Halo	3.68	0.99	0.21
SAL-Cloza	4.24	1.50	0.73
SAL-Controls	4.56	1.98	0.96

NGF levels in the bloodstream (ng/ml)

**indicates p<0.05 in post-hocs compared to controls*

Fig. 3 | *Neurite outgrowth (arrows) in PC12 cells following exposure to blood serum of a control animal (SAL-Control), a MAM rat receiving Haloperidol (MAM-Halo) and a MAM rat receiving Clozapine (MAM-Cloza). Pictures of the other groups were not included. The figure shows also the NGF levels in the bloodstream. Asterisks indicate significant differences between groups (* = p < 0.05).*

effect of prenatal MAM] with significant post-hocs ($p < 0.05$) in MAM-Halo *vs* SAL-Controls.

Figure 3 reports the effect of serum extract on the expression of neuritis outgrowth from PC-12 cells. The table indicates that prenatal MAM and administration of Clozapine or Haloperidol causes a significant increase in NGF in the serum [$F(1.24) = 398.41$; $F(2.24) = 15.24$, $ps < 0.05$ in the ANOVA for the main effect of both prenatal MAM and drug exposure, respectively]. The NGF present in the bloodstream was biologically active, since it stimulated the growth of neuritis (arrows) in MAM-Halo rats or MAM-Cloza rats compared to SAL-Controls rats in a classic biological PC-12 cell assay within 48 h. In *Figure 3* microphotographs from MAM-Controls, SAL-Halo and SAL-Cloza rats were not included.

Effect of Haloperidol and Clozapine on brain BDNF levels

Figure 4 shows that in the entorhinal cortex statistical analysis showed an interaction MAM exposure x drug exposure [$F(2.24) = 3.85$, $p = 0.01$] caused by the elevated levels of BDNF in animals exposed prenatally to MAM and administered postnatally to Clozapine. In the striatum MAM exposure in fetus increases the levels of BDNF following Haloperidol treatment, while decreasing after Clozapine administration [$F(2.24) = 11.35$, $p < 0.01$ in the ANOVA for the interaction prenatal MAM x drug exposure; $p < 0.05$ in post-hoc comparisons].

Effect of Haloperidol and Clozapine on NGF/BDNF receptors

To assess whether antipsychotic drugs would influence the NGF and BDNF-target cells, brain tissues

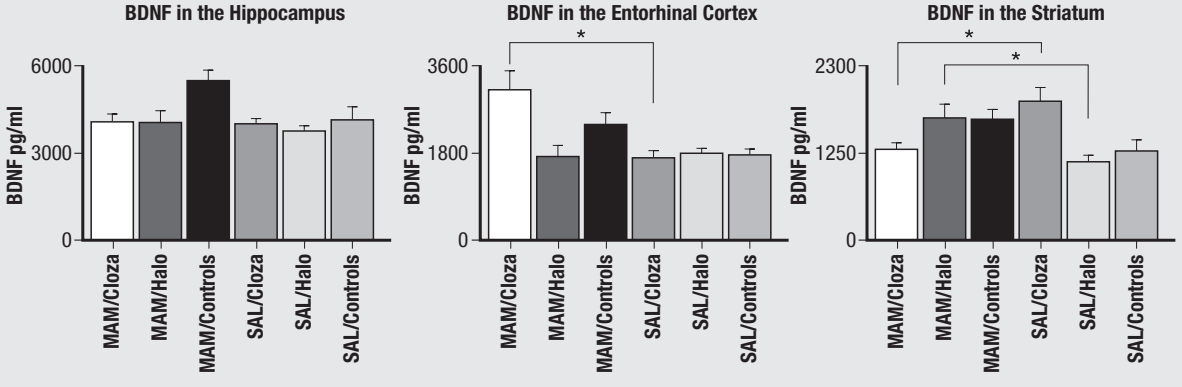


Fig. 4 | *Mean concentration (± SEM) of BDNF in the hippocampus, entorhinal cortex and striatum of saline and MAM treated rats receiving Clozapine (Cloza) or Haloperidol (Halo). Asterisks indicate significant differences between groups (* = p < 0.05).*

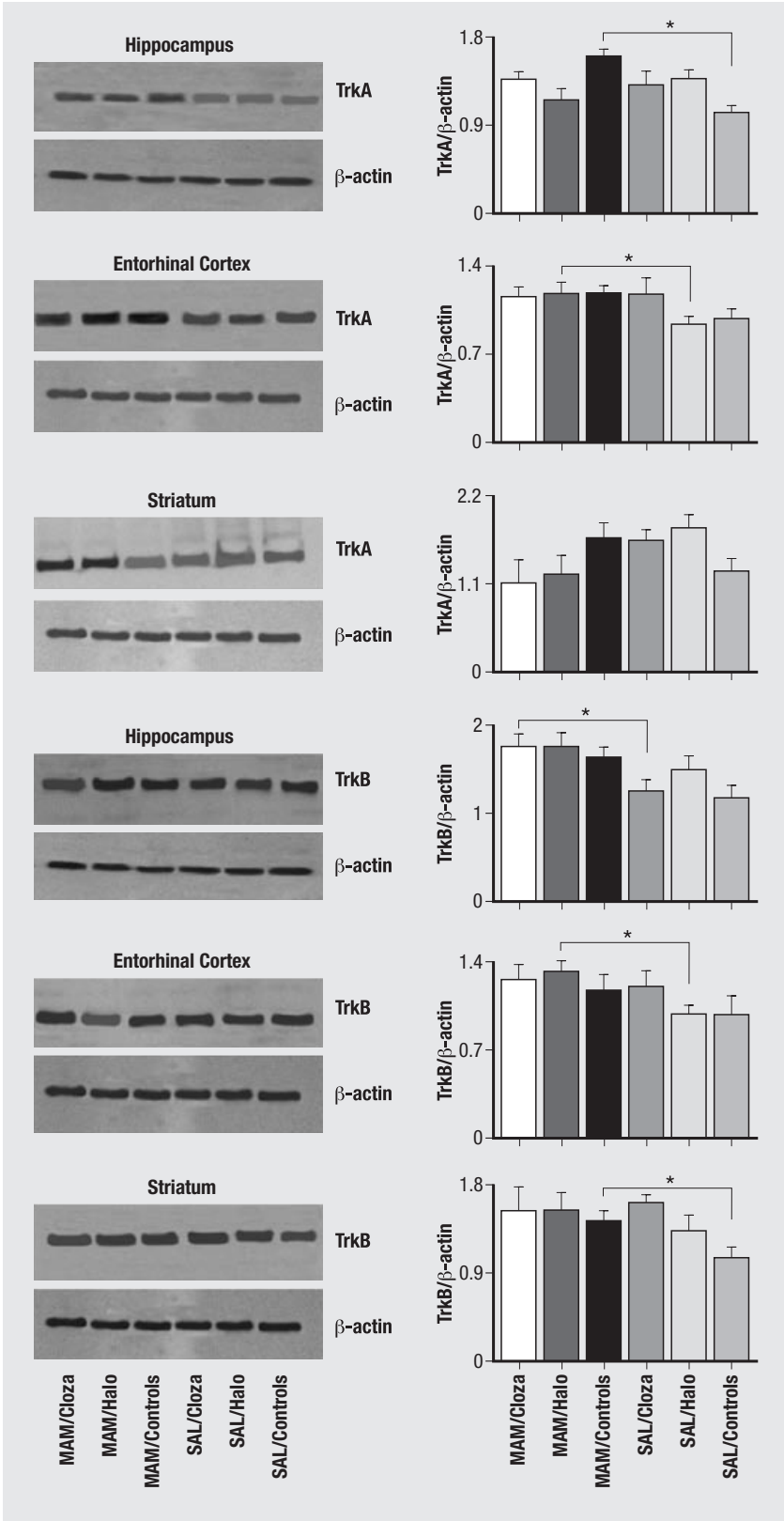


Fig. 5 | *Representative Western Blottings for TrkA and TrkB in the hippocampus, entorhinal cortex and striatum of saline and MAM treated rats receiving Clozapine (Cloza) or Haloperidol (Halo). Asterisks indicate significant differences between groups (* = $p < 0.05$). Values are ± SEM and are normalized for β-actin.*

of Haloperidol and Clozapine treated MAM rats and their respective controls were used for testing TrkA and TrkB protein presence. Western Blotting analyses reported in *Figure 5* showed that in the hippocampus TrkA expression was higher following prenatal MAM exposure [$F(1.24) = 23.86$, $p < 0.05$ for the main effect of prenatal MAM administration] due to high values in MAM-Controls ($p < 0.05$ in post-hocs). Similar

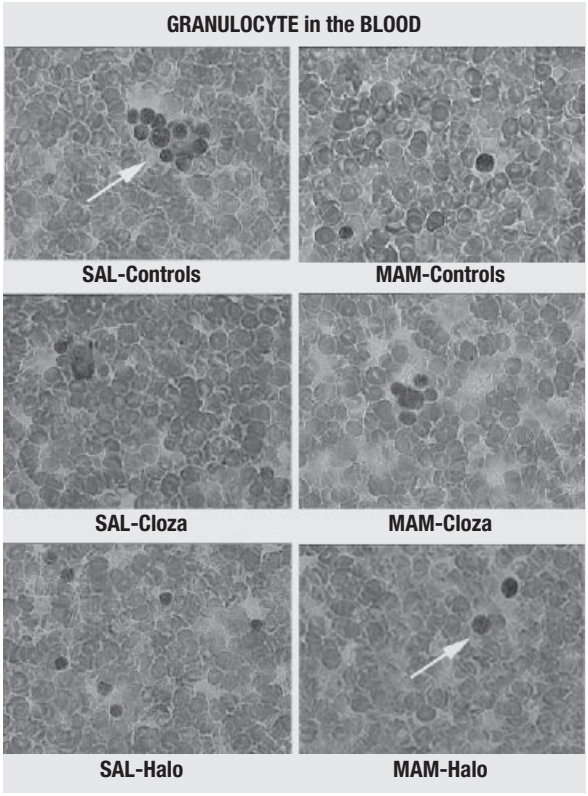


Fig. 6 | *Blood May-Grunwald-Gimsa granulocyte (arrows) staining of rats prenatally exposed to 20 mg/kg of MAM at gestational day 12 and administered with Clozapine (Cloza) or Haloperidol (Halo) for 8 days.*

changes were revealed for TrkB [$F(1.24) = 40.30$, $p < 0.05$ for the main effect of prenatal MAM administration] with high values following Clozapine exposure ($p < 0.05$ in post-hocs). In the entorhinal cortex both TrkA and TrkB increased in MAM rats administered with Haloperidol compared to normal rats administered with Haloperidol [$F(1.24) = 9.68$; $F(1.24) = 13.90$, $ps < 0.05$ for the main effect of MAM administration and in post-hocs]. In the striatum no changes were found for TrkA whereas for TrkB a significant difference was observed between MAM rats and controls [$F(1.24) = 19.33$; $ps < 0.05$ for the main effect of MAM exposure and in post-hocs].

Haloperidol and Clozapine effects on circulating granulocytes

Recent findings reported that antipsychotic drugs administration in human causes changes in granulocytes [41]. To further explore the validity of rat MAM model, we investigated the effect of Haloperidol and Clozapine on the distribution of circulating granulocytes. As shown in *Figures 6* and *7*, the number of granulocytes (arrows in *Figure 6*) in the blood of young rats was affected by both prenatal MAM exposure and administration of Clozapine or Haloperidol. Indeed, granulocyte number resulted *(Figure 7)* to be lower in MAM rats [$F(1.24) = 22.47$; $p < 0.01$ for the main effect of MAM in the ANOVA], with significant differences between groups in post-hocs in rats administered with Clozapine or Haloperidol [$F(2.24) = 7.57$; $p < 0.01$ for the main effect of drug exposure in the ANOVA].

DISCUSSION

Animal models are considered useful tools for studying and characterizing pathophysiological events and for identifying pharmacological therapies of human diseases. Since Weinberger's reports described that schizophrenia is largely regarded as a neurodevelopmental disorder with specific alterations of the neuronal architecture in the hippocampal formation and cortex [49, 50], several laboratories tried to develop animal models with these characteristics. Indeed, Talamini and other researchers showed that MAM administration in rats during gestation [8, 16-18] induces abnormalities in the limbic system resembling some morphological and behavioral findings observed in schizophrenia [2]. In subsequent years, we evidenced changes at behavioral, cellular, biochemical and molecular levels in the limbic system of adult rats prenatally treated with MAM suggesting that one mechanism through which the brain is affected involves the dysregulation of the synthesis and secretion of neurotrophic factors such as NGF and BDNF [15, 18, 51].

In the present study we demonstrate that a short and acute administration of Clozapine or Haloperidol did not induce either mortality in MAM rat nor differences in body weight due to both drugs findings associated with brain changes in NGF/BDNF levels and in the expression of their main receptors, TrkA and TrkB. We also found decreased presence of blood granulocytes. The administration of Clozapine elevates NGF levels in the blood stream and entorhinal cortex, and the concentration of BDNF in the en-

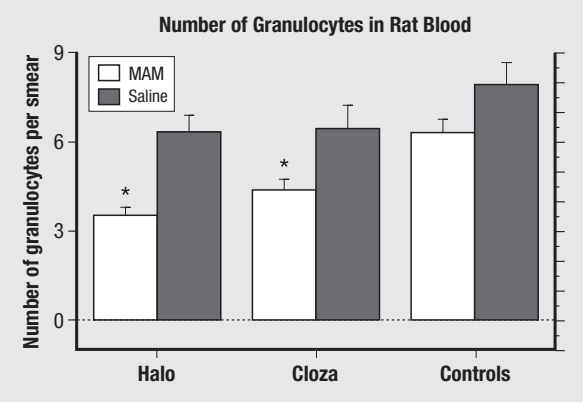


Fig. 7 | *Mean granulocyte number per smear (± SEM) of rats prenatally exposed to 20 mg/kg of MAM at gestational day 12 and administered with Clozapine (Cloza) or Haloperidol (Halo) for 8 days. Asterisks indicate significant differences between groups (* = $p < 0.05$).*

torhinal cortex increases after Clozapine administration. NGF increased in the hippocampus following Haloperidol while Haloperidol administration per se enhanced the presence of both NGF and BDNF in the striatum. Given that these neurotrophins are markedly involved in promoting and maintaining brain neuron plasticity Clozapine or Haloperidol may produce their effects modulating the synthesis and release of NGF/BDNF. It was also found that Clozapine or Haloperidol administration may influence the expression of TrkA and TrkB in MAM rat. TrkB in MAM rats increased in the hippocampus following Clozapine. Haloperidol elevated TrkA/TrkB expression in the entorhinal cortex which is a highly vulnerable structure in the schizophrenic brain. Findings previously released in the rat found hippocampal changes in TrkB expression [52] following Clozapine or Haloperidol [34, 53, 54]. In humans the expression of TrkB appears to be reduced with or without neuroleptic treatment [55] in post-mortem schizophrenic brains while in schizophrenic patients it has been shown that the basal levels of NGF and BDNF undergo through significant alterations [29, 32, 55, 56]. It has also been reported that the second-generation of antipsychotics, Olanzapine, Quetiapine, and Clozapine enhances neurite outgrowth induced by NGF in PC12 cells [57]. Chronic exposure to Haloperidol, but not the atypical antipsychotics Risperidone or Clozapine, alters the presence of brain choline acetyltransferase [34, 58] that is regulated by NGF [59]. Reduction in NGF or BDNF following Haloperidol was found in the rat hippocampus and striatum [60]. Moreover, a time-course study on the effect of Haloperidol demonstrated elevated NGF values in the hippocampus after 1 or 2 weeks of treatment [61]. As for the data with PC12, our novel data showing that antipsychotic administration stimulates the synthesis of biological active NGF may represent an innovative approach to investigate the mechanism of action of antipsychotic drugs and their implication in brain neurodegenerative/neuroprotective events. Administration of antipsychotic drugs has been shown to influence the presence of cells of the immune system, including granulocytes [41]. In the present study we found that the administration of Haloperidol and Clozapine in rats prenatally exposed to MAM decreased the number of circulating granulocytes indicating that the MAM rat model may represent a tool for investigating undesired side-effects of antipsychotics on immune cells.

Antipsychotic drugs are normally used to treat schizophrenia, however, their usefulness in treating other psychiatric disorders and neurodegenerative diseases, such as Alzheimer and Parkinson has been thoroughly explored [36, 37, 62]. Therefore, the modulation of brain neurotrophins' synthesis by antidepressants might be a novel approach to identify biochemical and molecular mechanisms and the possible therapy for brain cell deficits induced by neuropsychiatric disorders [63].

Based on these findings, our working hypothesis is that the MAM rat model may represent a tool for investigating undesired side effects of antipsychotics and these results may represent a further step in the effort to unravel the molecular events involved in "schizophrenia-like" diseases.

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