

Disruption of Neurogenesis on Gestational Day 17 in the Rat Causes Behavioral Changes Relevant to Positive and Negative Schizophrenia Symptoms and Alters Amphetamine-Induced Dopamine Release in Nucleus Accumbens

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Gestational disruption of neurodevelopment has been proposed to lead to pathophysiological changes similar to those underlying schizophrenia. We induced such disruption by treating pregnant rat dams with methylazoxymethanol acetate (MAM) on gestational day 17 (GD17). Total brain size and that of the prefrontal cortex and hippocampus were reduced in adult rats exposed prenatally to MAM. When locomotor activity was assessed in an open field, MAM-exposed rats were hyper-responsive to a mild stress and to amphetamine (2 mg/kg, s.c.). They also engaged in less social interaction than controls. We studied, by microdialysis, the effect of amphetamine on extracellular dopamine in the nucleus accumbens and the medial prefrontal cortex of freely moving control and MAM-exposed rats. Amphetamine (2 mg/kg, s.c.) induced an increase in dopamine release that was larger in the nucleus accumbens of MAM-exposed rats than in controls, whereas no difference was seen in the medial prefrontal cortex. In controls, amphetamine infused into the medial prefrontal cortex (50 μ M) led to a slight decrease in extracellular dopamine in the nucleus accumbens. This effect was absent in MAM-exposed rats, where a transient increase in nucleus accumbens dopamine levels was seen after amphetamine infusion. These results show that the late gestational disruption of neurogenesis in the rat leads to behavioral changes that mimic positive and negative schizophrenia symptoms, and also to a dysregulation of subcortical dopamine neurotransmission. This study contributes to the evaluation of the validity of the prenatal MAM GD17 treatment in rats as an animal model for schizophrenia.

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INTRODUCTION

Epidemiological studies show that gestational or perinatal disturbances increase the risk of developing schizophrenia. This is true for a wide range of perturbations including maternal starvation (Susser *et al*, 1996), maternal infections (Buka *et al*, 2001a; Buka *et al*, 2001b; Brown *et al*, 2000;

Mednick *et al*, 1988), Rhesus blood-type incompatibility (Hollister *et al*, 1996), and perinatal anoxic birth injuries (Zornberg *et al*, 2000; Rosso *et al*, 2000). That such diverse perturbations are associated with psychosis supports the neurodevelopmental theory of schizophrenia. Schizophrenia could be caused by brain abnormalities that occur during development and are a result of an interaction between pre- or perinatal environmental factors and a genetic predisposition. The mechanisms triggering the developmental perturbation may be less important than the timing of it and the subsequent specific disruption of brain areas, such as the prefrontal and temporal cortices (Akbarian *et al*, 1993a; Akbarian *et al*, 1993b).

Disruption of neurogenesis in rats during gestation has been proposed as a developmental model of schizophrenia (Moore and Grace 1997; Ghajarnia *et al*, 1998; Grace and Moore, 1998; Talamini *et al*, 1999; Talamini *et al*, 1998). The disruption can be induced by administering the antimetabolic

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compound methylazoxymethanol acetate (MAM). MAM treatment on gestational day 17 (GD17) is likely to have a major impact on the brain structures that are actively developing at this time, in particular the development of the hippocampus. GD17 is the time of peak proliferation of cells in this region (Bayer and Altman, 1995). In agreement with this, MAM administration on GD17 has been shown to reduce the thickness of the hippocampus, thalamus, and several cortical regions (Moore and Grace, 1997). These structures are also morphologically altered in schizophrenia (Pakkenberg, 1992; Bogerts *et al*, 1993; Vogeley *et al*, 2000; Thompson *et al*, 2001).

Previous studies have found that the adult offspring of MAM GD17-treated rats show enhanced sensitivity to phencyclidine (PCP) in a locomotor activity test, as well as deficits in prepulse inhibition (Ghajarnia *et al*, 1998). Our own preliminary behavioral studies comparing MAM treatment on GD9 to GD17 of gestation also suggested that GD17 MAM treatment (and to some extent GD15 MAM treatment) induced cognitive changes in rats (Didriksen *et al*, 1999). These results suggest that animals exposed to MAM at a specific time during limbic and cortical development show behavioral abnormalities that may reflect the cognitive deficits and positive symptoms seen in schizophrenia. In order to further validate this model as relevant to positive schizophrenia symptoms, in the present study we examined the sensitivity of MAM-treated rats to amphetamine. Furthermore, we explored the relevance of this model in terms of the negative symptoms of the disease. The negative symptom cluster is difficult to examine in preclinical models, although it has been suggested that certain negative symptoms, such as anhedonia and social withdrawal, can be modelled in rats (Ellenbroek and Cools, 2000). In this study, we examined whether the MAM-treated animals show social withdrawal. For this, we used the social interaction test that has been extensively validated in our laboratory (Sams-Dodd, 1999, 1995). Deficits in this social interaction test have previously been described in preclinical models of schizophrenia such as subchronic PCP treatment in rats (Sams-Dodd, 1999, 1995, 1996) and in rats with a neonatal lesion of the ventral hippocampus (Sams-Dodd *et al*, 1997).

We also examined the neurochemical changes associated with the response to amphetamine in MAM-treated rats. An increased response to amphetamine in animals is an interesting parallel to clinical observations. Thus, high doses of amphetamine are needed to induce psychotic episodes in volunteers, whereas schizophrenic patients are sensitive to lower doses (Lieberman *et al*, 1987). The reaction to amphetamine is comparable to the spontaneous psychosis that occurs during the active period of the disease (Laruelle, 2000), suggesting that dopaminergic hyperactivity is part of the pathophysiology of the episodes. In line with the dopamine hypothesis of schizophrenia, new imaging techniques have confirmed that the hypersensitivity to amphetamine (emergence or worsening of psychotic symptoms) is correlated with increased dopamine release in the striatum (Laruelle *et al*, 1999; Breier *et al*, 1997). Cortical malfunction is present in schizophrenia, and it has been argued that this could be the primary cause of altered subcortical dopamine transmission (Weinberger and Lipska, 1995; Carlsson and Carlsson, 1990; Grace, 1991). That a

deficit in prefrontal cortex can lead to a hyperactive subcortical dopamine system is supported by preclinical studies showing that lesions of the prefrontal cortex augment the subcortical dopamine response to stressors (Jaskiw *et al*, 1990; Deutch *et al*, 1990). *N*-acetyl-aspartate (NAA), a marker of neuronal integrity, is present in high concentrations in pyramidal glutamatergic neurons (Moffett *et al*, 1993), and a reduction in NAA may reflect compromised neuronal integrity. Reductions in NAA have been reported in schizophrenia in the hippocampus and the dorsolateral prefrontal cortex (Callicott *et al*, 2000; Bertolino *et al*, 2000b; Bertolino *et al*, 2003). Furthermore, NAA measures in the dorsolateral prefrontal cortex of schizophrenia patients are correlated negatively with dopamine release in the striatum after systemic amphetamine infusion (Bertolino *et al*, 2000a). In order to explore these interactions, we studied the effects of amphetamine on extracellular dopamine in the nucleus accumbens and the prefrontal cortex by microdialysis, after either systemic administration or local administration of the drug in the prefrontal cortex.

MATERIALS AND METHODS

Animals

Pregnant dams (Wistar, Brl) were obtained from the animal provider (M&B, Denmark) on gestational day (GD) 10 or GD14 and were housed individually in polycarbonate cages (42.5 × 26.6 cm). On GD17, the dams were treated with 22 mg/kg MAM or saline intraperitoneally (i.p.). Within 4 days after birth, the litters were culled to 10, by removal of females only. Pups were weaned 30 days after birth, and housed in pairs. Only the male pups were included in the studies, and each animal was tested only once.

The experiments were performed on several batches of control or MAM-treated animals. They consisted of: (1) assessment of body and brain weight; (2) assessment of the weight of the prefrontal cortex, hippocampus, nucleus accumbens, and striatum, and histological analysis; (3) open field test; (4) social interaction test; (5) microdialysis studies. In all the experiments, animals were randomly assigned from a large number of litters. All experiments were carried out in animals between postnatal days 58 and 90.

Animals were housed in the animal room in macrolon cages (42.5 × 26.6 cm). The animal room was kept at 21 ± 2°C with a relative humidity of 55 ± 5% and animals had free access to water and commercial food pellets. The rats used for behavioral experiments were tested on postnatal day 58–60 and were kept on a reversed light/dark cycle (light on 1800–0600 hours). They were moved to the experimental room 1 day before testing.

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Drugs and Administration

MAM (obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository)

and D-amphetamine hemisulfate (Sigma, Denmark) were dissolved in saline. Compounds were given in a volume of 5 ml/kg. MAM or its vehicle were given i.p., and D-amphetamine or saline were given subcutaneously (s.c.). D-amphetamine hemisulfate (Sigma, Denmark) for systemic injections was dissolved in isotonic saline. D-amphetamine used for local infusion through the microdialysis probe was dissolved in Ringer solution.

Measurement of Body and Brain Weight

On postnatal day 90, rats were weighed and then killed by decapitation, and the brain (including the cerebellum) was removed and weighed. On a separate batch of control and MAM-exposed animals, the medial prefrontal cortex, hippocampus, nucleus accumbens, and striatum were dissected out and weighed. The prefrontal cortex corresponded to an area that included the rostral pole of the brain, and was delimited medially by the interhemispheric fissure, laterally by the corpus callosum and caudally extended to AP +2.7 according to Paxinos and Watson (1998). The nucleus accumbens (core and shell), striatum, and hippocampus were dissected from the remaining bloc of tissue. After weighing, these structures were used in separate studies not reported here.

Immunohistochemistry

Brains from three control and three MAM-exposed animals were frozen on dry ice and 12 μ m serial sections were cut. Some sections were stained with cresyl violet and others were processed for immunostaining for the neuronal nuclei antigen (NeuN). Immunolabelling was performed using a protocol based on the avidin-biotin-peroxidase technique. Briefly, sections were rehydrated prior to fixation in 4% paraformaldehyde. Endogenous peroxidase activity was quenched by incubation of the sections in 0.3% hydrogen peroxide for 15 min. Sections were incubated with mouse anti-NeuN antibody (1:50; Chemicon, Temecula, CA) overnight at +4°C and then incubated with biotinylated anti-mouse IgG antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Then, sections were incubated in avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) for 30 min and 3',3'-diaminobenzidine was used as a chromogen. Sections were counterstained with hematoxylin before mounting.

General Experimental Procedure-Behavioral Studies

The tests were performed in an open field arena (150 \times 100 \times 40 cm) made of black Plexiglas. The bedding was gray gravel (Dust-Free Cat-Litter) to provide a contrasting background to the rats and reduce reflections. Other rats had been exposed to the same gravel before testing to provide a constant odor level in the arena. Behavior was recorded by a video camera that was placed above the arena and was connected to a S-VHS videocassette recorder. The videotapes were analyzed using the EthoVision[®] program (Noldus Information Technologies).

Open Field Test

The room was illuminated with bright light (during the dark period of the light/dark cycle). At the beginning of a trial, rats were placed in the center of the arena and the behavior exhibited during a 30 min habituation period was recorded. The animals were then removed, given an injection of vehicle or D-amphetamine (2 mg/kg s.c. measured as salt, corresponding to approximately 1.5 mg/kg base) and put back in the arena, and behavior was recorded for another 90 min. The habituation period was analyzed in 5-min intervals, whereas the 90 min period was analyzed in 15-min intervals. The distance traveled in the arena by the animals was used as a global measure of motor activity (pixel movements), but to ensure that the motor activity recorded was related to real locomotor movements in space and not due to repeated pixel changes within a limited space, the data was filtered, removing all distances below 10 cm. The EthoVision[®] program was set to record the activity with a temporal resolution of five samples/s.

Social Interaction Test

The detailed description of this model has been published previously (Sams-Dodd, 1995). The test was carried out during the dark period of the light/dark cycle. A week prior to testing, half of the group of MAM rats and half of the control group were dyed with black hair color (Polycolor 890) to allow computer-imaging recognition from the videotapes. Color was applied to the rear part of the body and covered approximately 50% of the rat. The room where the test was carried out was dimly illuminated with red light. At the initiation of a trial, two unfamiliar rats (which had been given the same treatment, that is, MAM or vehicle, but one dyed black) were placed in adjacent corners, and the behavior exhibited during a 10-min-trial was recorded (temporal resolution of 3.125 samples/s).

The following parameters were analyzed: (a) distance traveled (based on pixel changes and data not filtered for small repetitive movements), (b) percent time spent in the center of the arena (center defined as a central square covering 33% of the arena), and (c) time spent in active and passive social interaction. Social interaction was defined as the time spent by the two rats in proximity of each other (20 cm). This measure was then subdivided into passive and active social interaction for each rat, based upon whether the animal actively moved or not (see for details (Sams-Dodd, 1995, 1996). In addition, the mean duration of proximity and frequency of proximity were examined. The criterion for beginning an episode of proximity was that animals were within 20 cm of each other. A period of proximity ended when the rats moved more than 25 cm apart.

Microdialysis

At PD 60-90, intracerebral guide cannulae (CMA/12) were stereotaxically implanted in rats placed in a stereotaxic frame with the incisor bar set at -2.0 mm. Anaesthesia was induced with Hypnorm[®]/Dormicum[®] 2 ml/kg i.p. (fentanyl citrate, 0.079 mg/ml; fluanisone, 2.5 mg/ml; midazolam,

1.25 mg/ml). The local anesthetic xylocain was applied to the periosteum during surgery.

The guide cannula for the nucleus accumbens was implanted in the right hemisphere at AP: +1.7 mm, L: 0.8 mm, V: -6 mm from the brain surface (Paxinos and Watson, 1998). The guide cannula for the medial prefrontal cortex was implanted in the right hemisphere at an angle of 20 degrees, at AP: +3.8, L: 0.7, V: -0.35. In the experiment where guide cannulae were inserted both in the NAc and the mPFC, the two cannulae were placed contralaterally (ie cannulae placed in opposite hemispheres). The probes were attached to the bone with dental cement using stainless steel screws. The animals were treated with Rimadyl® (carprofen 50 mg/ml), 0.01 ml/100 g s.c. for pain management after surgery.

General Dialysis Protocol

The rats were allowed to recover from surgery for 2 days. They were then placed in a hemispheric bowl and the microdialysis probes were inserted in the guide cannulae (CMA/12, 0.5 mm diameter, 2 mm probe length for nucleus accumbens and 4 mm probe length for the medial prefrontal cortex). The probe was connected to a microinfusion pump via a dual-channel liquid swivel, which allows the animals to move freely in the cage during the experiment. The rats had free access to food and water in the test bowls. The dialysis probe was perfused with Ringer solution (145 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂) at a constant flow rate of 1 µl/min. The perfusate was collected in vials, which contained 5 µl 5 mM perchloric acid to prevent dopamine breakdown.

Single-Probe Protocol

The perfusate was discarded during the initial 100 min and then collected in 20-min intervals. The first four fractions represented the baseline level. Rats were then given D-amphetamine (2 mg/kg, s.c.) and fractions were collected for 160 min.

Dual-Probe Protocol

After placement of the probes in the nucleus accumbens and the medial prefrontal cortex, animals were left to habituate in the test bowl with the probes perfused with Ringer solution at a rate of 0.2 µl/min over night. On the next day, the perfusion rate was increased to 1 µl/min. The perfusate from the first 2 h was discarded. Three 30-min fractions were then collected to establish the baseline level. Then, the prefrontal probe was perfused with Ringer solution containing D-amphetamine (50 µM) for 1 h, before continuing with ordinary Ringer solution. Six 30-min fractions were collected during and after the D-amphetamine infusion, both from the nucleus accumbens and the prefrontal cortex. The choice of the concentration of amphetamine was dictated by our intention to use conditions similar to those used by Karreman and Moghaddam (1996).

Sample Analysis

The samples were stored at -80°C until analysis. Dopamine was analyzed by HPLC with electrochemical detection. The

levels of dopamine in the dialysates were not corrected for probe recovery. Dopamine was separated by reverse phase liquid chromatography (ODS 80 × 4.6 mm, 3 µm) using a mobile phase consisting of 75 mM NaH₂PO₄, 1.8 mM sodium dodecyl sulfate, 20 µM EDTA, 15 % acetonitrile, and 10% methanol (pH 5.6) at a flow rate of 0.7 ml/min. Electrochemical detection used a coulometric detector, with the potential set at E1 = -75 mV and E2 = 220 mV; guard cell = 350 mV (Coulchem II, ESA). Data were collected and analyzed using Chromeleon Clinet 6.30 software (Dionex).

Histology

After completion of the microdialysis procedure, the brains were removed under terminal anesthesia and frozen using solid carbon dioxide and stored at -80°C. Sections were cut on a cryostat and stained with cresyl violet. Probe placement was determined using the stereotaxic atlas for guidance (Paxinos and Watson, 1998). Data from inaccurately placed probes was excluded from the study.

Statistics

In the open field test, the difference between groups during habituation was analyzed by two-way repeated measures ANOVA. The distance traveled after saline or D-amphetamine injection was analyzed by three-way repeated measures ANOVA (pretreatment × drug × time) and Student-Newman-Keuls *post hoc* test. Body weights were analyzed by *t*-test and brain weights were analyzed by ANCOVA with body weight as covariate. The data from the social interaction test were analyzed by *t*-test except the social interaction (passive and active), which were analyzed by ANCOVA with traveled distance (sum of the traveled distance of the pair of rats examined) as covariate. In the microdialysis experiments, the level of dopamine was expressed as percentage of baseline values, defined as the average of the three baseline samples before D-amphetamine injection/infusion. The analysis of the data was carried out using two-way repeated measures ANOVA, followed by a *post hoc* Fisher's Protected Least Significant Difference test. Differences were considered significant when *P* was less than 0.05.

RESULTS

Measurement of Body and Brain Weight

The gestation of MAM-treated dams proceeded normally and it terminated on the 21st-22nd day. MAM-exposed dams did not present any abnormal type of behavior and they could not be physically distinguished from control dams, throughout gestation. The overall appearance of the control and MAM-exposed offspring was healthy and no differences were noted in litter size and offspring gender ratio. As shown in Figure 1, MAM-treated rats had 5.6% lower mean body weights than the controls at PD 90. This difference was significant (*P* < 0.05). The brain size was reduced by approximately 11.1% in MAM-exposed rats as compared to the controls. The ANCOVA analysis showed that the difference between MAM and controls was significant (*F*(1,21) = 44.1, *P* < 0.001), and that

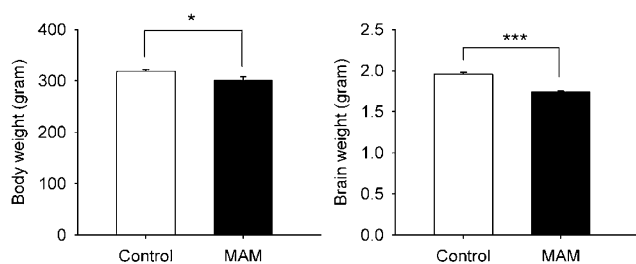


Figure 1 Body weight (left) and brain weight (right) of animals treated with MAM at GD17 vs controls ($n = 12$ per group). Results are shown as mean \pm SEM. * $P < 0.05$ and *** $P < 0.001$ vs controls.

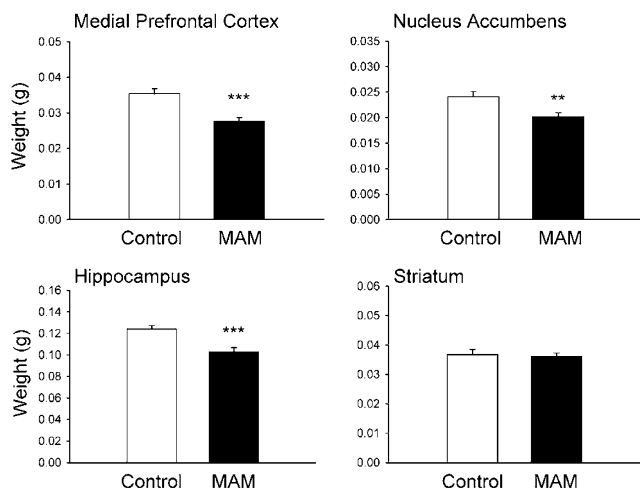


Figure 2 Weight of brain structures in control and MAM-exposed rats. Results are expressed as means \pm SEM of 14 animals per group. ** $P < 0.01$ and *** $P < 0.001$ vs controls.

the contribution from the body weight was not significant ($F(1,21) = 0.07$).

There were significant differences in the weight of the prefrontal cortex, hippocampus, and nucleus accumbens in MAM-exposed animals vs controls, as shown in Figure 2. No difference was seen in the weight of the striatum between the two groups. The analysis of cresyl violet and NeuN staining confirmed the presence of an incompletely developed hippocampus and that of cell disarrays in MAM-exposed brains (Figure 3a–d). No obvious structural abnormalities were noted in the medial prefrontal cortex (Figure 3e) or in any other prefrontal cortical fields (data not shown).

Open Field Test

The habituation phase of the open field test did not reveal any significant differences between the groups ($F(1,29) = 0.7$, ns), as shown in Figure 4.

The analysis of the behavior after saline or D-amphetamine injection revealed an effect of MAM treatment ($F(1,27) = 5.1$, $P < 0.05$), of D-amphetamine ($F(1,27) = 93.9$, $P < 0.001$), and of time ($F(5,135) = 20.3$, $P < 0.001$). In addition a time \times prenatal treatment \times drug interaction was observed ($F(5(135) = 5.4$, $P < 0.001$). The *post hoc* test showed that MAM-exposed rats were significantly more

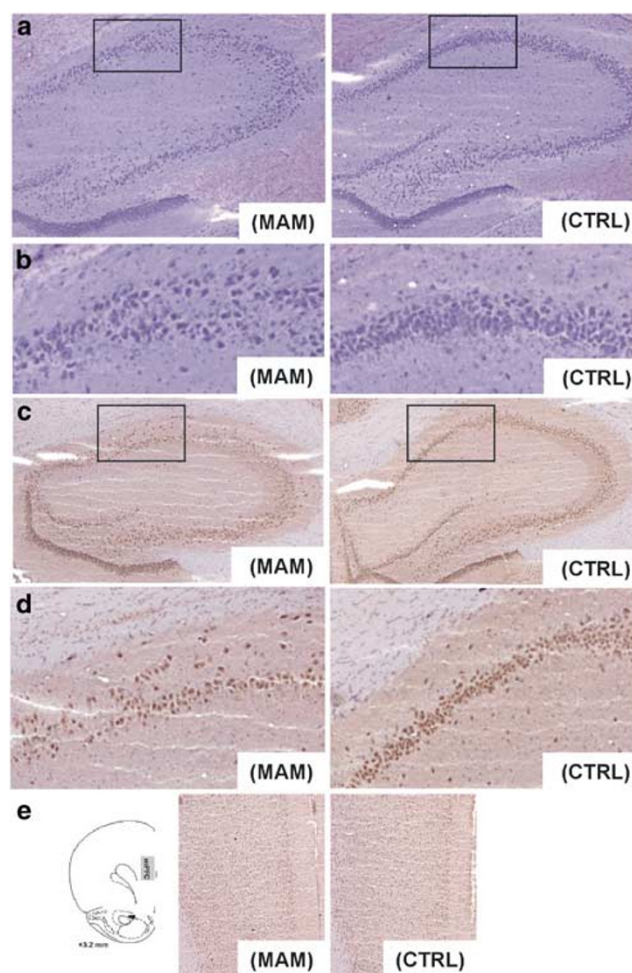


Figure 3 Cresyl violet (a, b) and NeuN (c, d, e) staining of sections from control and MAM-treated rats. The sets of images in (b and d) highlight the cell disorganization in the CA1 region in the treated animals. Section (e) is taken at the level of the mPFC, with the diagram indicating the field shown (coordinates of the section +3.2 mm according to Paxinos and Watson, 1998). Original magnifications: (a, c, e) $\times 40$; (b, d) $\times 200$.

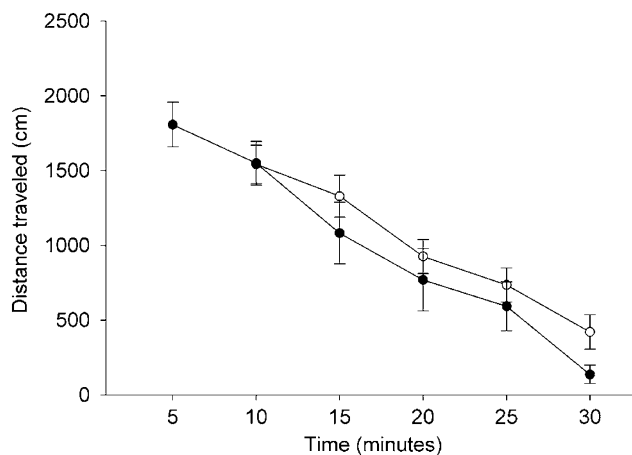


Figure 4 (a) Distance traveled by control and MAM rats during a habituation period of 30 min in the open field. Filled circles represent MAM-treated rats ($n = 15$) and open circles represent control rats ($n = 16$). Results are expressed as means \pm SEM.

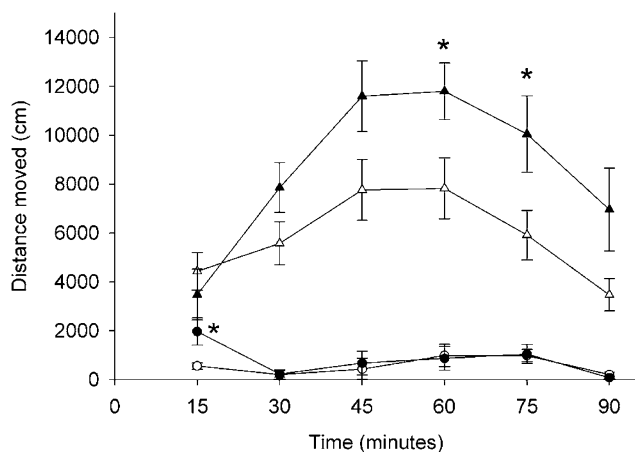


Figure 5 Effect of saline or amphetamine 2 mg/kg s.c. on the distance traveled in the open field. Filled circles represent MAM+saline ($n=8$). Open circles represent control+saline ($n=8$). Filled triangles represent MAM+amphetamine ($n=7$). Open triangles represent control+amphetamine ($n=8$). Results are expressed as means \pm SEM. * $P<0.05$ vs respective controls.

active in the first time interval (15 min) after saline injection ($P<0.05$). There was no difference between the groups in the period 15–90 min after the saline injection (Figure 5). In addition, MAM-treated rats showed behavioral hypersensitivity to the effect of *D*-amphetamine in the fourth and fifth time interval ($P<0.05$), corresponding to the interval from 45 to 75 min after injection (Figure 5).

Social Interaction Test

In the social interaction test (Figure 6), MAM-exposed rats displayed significantly less active social interaction than the controls ($F(1,25)=12.29$, $P<0.01$). The ANCOVA analysis showed that the contribution from the effect of MAM on the distance traveled was not significant ($F(1,25)=3.4$, $P=0.077$). In contrast with the active interaction, no significant difference was seen in the passive interaction between the two groups. However, the time spent in passive interaction by both groups was very low: controls = 3.6 ± 0.90 s and MAM-treated = 1.6 ± 0.47 s, respectively.

The mean duration of proximity was decreased in MAM rats compared to the controls ($P<0.05$). The proximity frequency was also decreased, but this difference did not reach statistical significance. Thus, the decrease in time spent in social interaction reflected both shorter durations of proximity and a tendency to have fewer episodes of interaction. In addition, MAM rats were hyperactive, as they traveled a significantly larger distance in the 10 min test ($P<0.001$). There were no differences in the percent time spent in the central zone.

Microdialysis Baselines

As apparent from Figures 7–10, the baseline was stable when collection of the samples was initiated. Concerning variability, in a few samples in the experiments, we had a

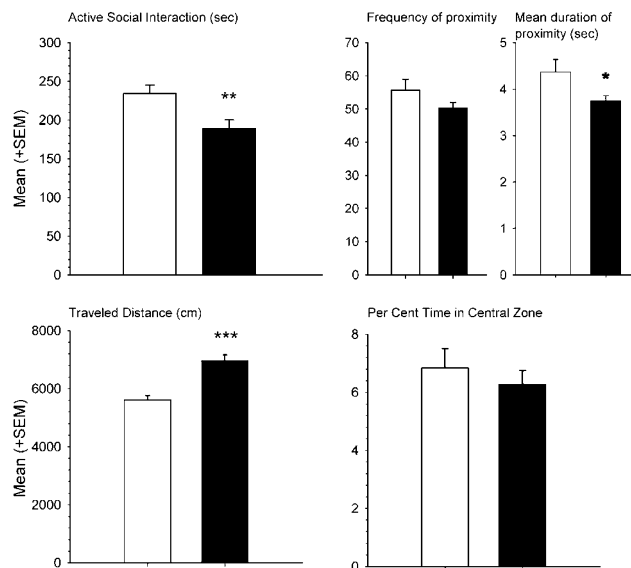


Figure 6 Effect of MAM vs saline treatment on GD17 on the behavior of rats in the social interaction test. Each treatment group included 14 pairs of rats that had been subjected to the same treatment. Filled bars represent MAM-treated rats and open bars represent controls. Results are expressed as means \pm SEM. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs controls.

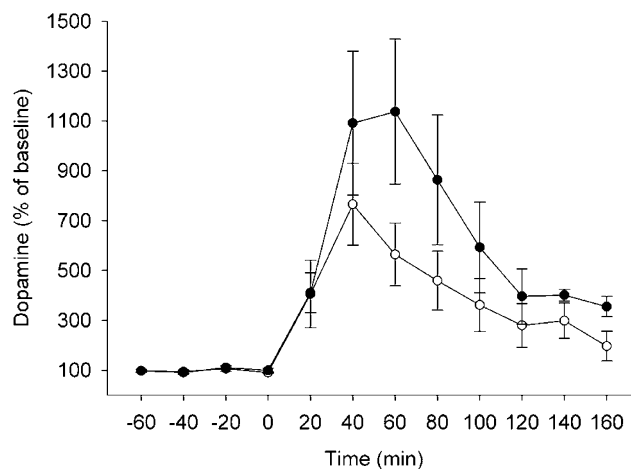


Figure 7 Effect of amphetamine (2 mg/kg, s.c.) on extracellular dopamine in NAc of controls (white circles, $n=6$) and rats exposed to MAM at GD17 (black circles, $n=5$). Amphetamine was injected at time 0. Results are expressed as means \pm SEM. The basal levels (fmol/20 μ l) were 31.8 ± 11.98 in controls and 26.36 ± 6.13 in MAM rats.

deviation from the mean of 25%, but these were very rare, and most data points were within 10% of the mean.

Effect of Systemically Administered *D*-Amphetamine on Dopamine in the Nucleus Accumbens and the Medial Prefrontal Cortex

As shown in Figure 7, the administration of *D*-amphetamine (2 mg/kg s.c.) induced a significant increase in dopamine concentration in the dialysate from the nucleus accumbens ($F(11,99)=18.95$, $P<0.001$). The basal level of extracellular dopamine was slightly decreased (-18%) in MAM rats vs controls, but the decrease was not statistically significant.

Additionally, there was a significant time \times group (controls vs MAM) interaction ($F(11,99) = 1.97$, $P < 0.05$), the MAM treated rats had increased dopamine release in the nucleus accumbens (not significant in *post hoc* analysis). Figure 8 shows that a significant increase in dopamine was also seen in the medial prefrontal cortex ($F(11,121) = 19.4$, $P < 0.001$). No effect of the group and no time \times group interactions were seen in this structure.

Effect of Amphetamine Infusion in Prefrontal Cortex

The infusion of amphetamine (50 μ M) through the prefrontal probe increased the level of dopamine significantly in the prefrontal cortex (Figure 9) ($F(8,64) = 82.8$, $P < 0.001$). Additionally, there was a significant interaction between group (control or MAM) and time ($F(8,64) = 2.4$,

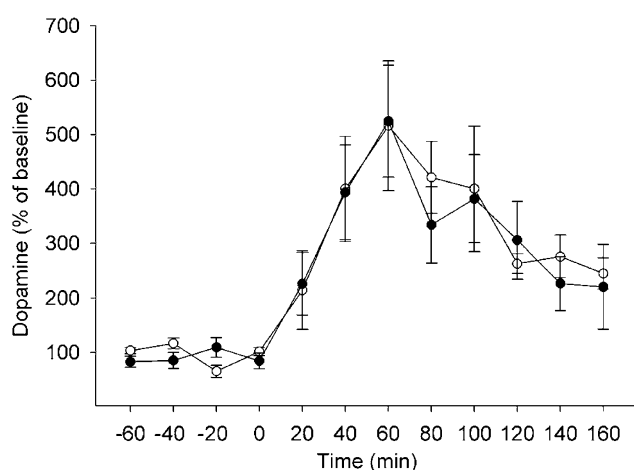


Figure 8 Effect of amphetamine (2 mg/kg, s.c.) on extracellular dopamine in mPFC of controls (white circles, $n = 6$) and rats exposed to MAM at GD17 (black circles, $n = 7$). The amphetamine was injected at time 0. Results are expressed as means \pm SEM. The basal levels (fmol/20 μ l) were 4.72 ± 0.22 in controls and 5.78 ± 0.62 in MAM rats.

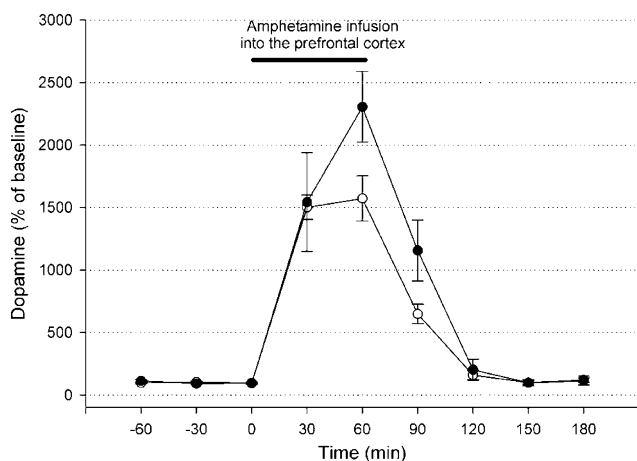


Figure 9 Effect of the local infusion of amphetamine (50 μ M) through the mPFC dialysis probe on extracellular dopamine in mPFC of controls (white circles, $n = 6$) and MAM-exposed rats (black circles, $n = 4$). The drug was infused for 60 min starting at time 0. Results are expressed as means \pm SEM. The basal levels (fmol/20 μ l) were 7.5 ± 1.52 in controls, and 5.09 ± 1.3 in MAM rats.

$P < 0.05$), which reflected the larger dopamine response in MAM-treated rats. The *post hoc* test revealed trends towards increased dopamine response in the periods 30–60 min ($P = 0.09$) and 60–90 min ($P = 0.06$) after initiation of infusion. The dopamine level returned to baseline within an hour after termination of the D-amphetamine infusion.

The effect of the infusion of D-amphetamine in the prefrontal cortex on extracellular dopamine in the nucleus accumbens is depicted in Figure 10. A time \times group (control or MAM) interaction ($F(8,72) = 2.1$, $P < 0.05$) was found. The effect of the D-amphetamine infusion in control rats was a decrease in extracellular dopamine in the nucleus accumbens to approximately 80% of the baseline level. In MAM rats, the effect was an increase in dopamine in the fraction collected in the period immediately after termination of D-amphetamine infusion. These differences resulted in a highly significant difference between MAM and controls in the dialysates collected from 60 to 90 min ($P < 0.001$) after initiation of the infusion of D-amphetamine into the prefrontal cortex. Additionally, a small difference was seen in the first baseline fraction ($P < 0.05$).

In this dual-probe experiment, the basal level of extracellular dopamine in the nucleus accumbens was decreased (–50%) in MAM rats vs controls and this difference in baseline was statistically significant ($P < 0.05$).

DISCUSSION

We investigated the effect of the prenatal treatment with the toxin MAM at GD17 on the brain weight and behavior of adult rats in tests relevant for the positive and negative symptoms of schizophrenia. In parallel, we analyzed changes in dopamine release in the nucleus accumbens and the medial prefrontal cortex after stimulation with amphetamine, in order to further assess possible cortico-limbic dysfunction.

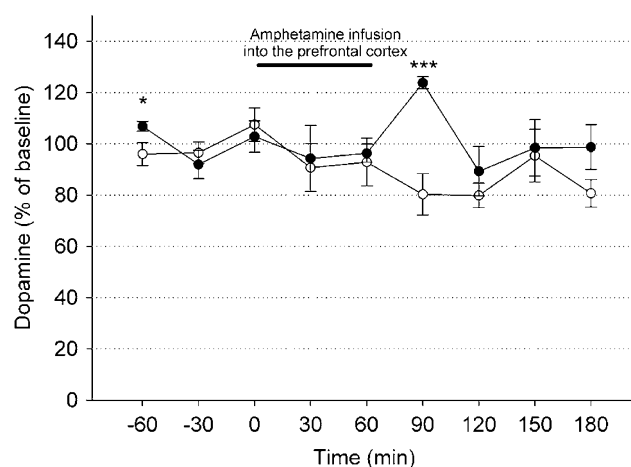


Figure 10 Effect of the local infusion of amphetamine (50 μ M) through the mPFC dialysis probe on extracellular dopamine in the NAc of controls (white circles, $n = 5$) and MAM-exposed rats (black circles, $n = 6$). The drug was infused for 60 min starting at time 0. * $P < 0.05$ and *** $P < 0.001$ vs controls. The basal levels (fmol/20 μ l) were 17.1 ± 3.6 in controls and 8.5 ± 0.97 in MAM rats. The difference in basal levels was significant ($P < 0.05$).

Brain and Body Weight

In agreement with an earlier report (Balduini *et al*, 1991) there was no effect of MAM treatment on litter size and composition, suggesting no adverse effects of the toxin on gestation. Furthermore, MAM treatment only caused a small decrease in body weight at PD 90. The MAM treatment on GD17 reduced the weight of the brain by approximately 11%, an effect that remained significant after controlling for the change in body weight. This reduction is markedly less than the 40% reduction reported after treatment at GD15 (Balduini *et al*, 1991). More recently, a reduction of 21% in brain weight at postnatal day 85 was reported after GD 15 MAM treatment (Jongen-Rêlo *et al*, 2004). Interestingly, the latter authors reported variability in data obtained in their own laboratory when MAM treatment was repeated in two different batches of animals at a given gestational date. As suggested by the authors, this variability may be due to the difficulty of timing accurately and precisely the pregnancy onset in rats, which may lead to slight shifts in the timing of exposure to the toxin.

The weight analysis of several brain structures showed significant decreases in MAM-exposed animals compared to controls, ranging from -22% (prefrontal cortex) to -17% (hippocampus) and -16% (nucleus accumbens). This is in accord with the anatomical changes quantified by stereology, reported after treatment with this compound at GD17 by Moore and Grace (1997). Furthermore, our qualitative examination of selected stained sections showed hippocampal abnormalities in MAM animals due to dysgenesis of this structure. The abnormalities induced by treatment at GD17 appeared to be different from the white matter and CA1 heterotopias described after treatment with MAM at GD14 (Chevassus-Au-Louis *et al*, 1998). The striatum was unaffected in terms of weight, although we cannot rule out more subtle alterations induced by the toxin in this structure. In MAM animals, cell disarray was evident in the CA1 hippocampal region, a region where structural abnormalities have been reported in schizophrenic patients (Jonsson *et al*, 1999). However, the type of structural abnormalities seen in the brain of schizophrenic patients are complex and possibly widespread, affecting neuronal size, neuronal arrangement as well as the characteristics of the neuropil (Harrison, 1999; McCarley *et al*, 1999).

Considering the normal neurodevelopment of the rat, it is also very likely that the administration of MAM at GD17 disrupts not only the development of structures of immediate relevance for corticolimbic pathways (eg entorhinal cortex, hippocampus, subiculum) but also other structures whose development includes GD17 as a critical period, such as the pontine precerebellar nuclei, parts of the inferior colliculus, or of the hypothalamus (Bayer and Altman, 1995). The structural changes induced by MAM at GD17 remain to be fully characterized histologically, adding information beyond the crude decreases in weight observed in this study. The induction of changes that are widespread may be an insurmountable limitation of not only animal models that use toxins such as MAM, but also of lesion models. Thus, the ventral hippocampus excitotoxic lesion, one of the most well-studied animal models of schizophrenia, has been shown to lead to widespread structural changes in rat brain (Halim and Swerdlow, 2000).

Open Field Experiment

Our results suggest that the disturbance of neurodevelopment during late gestation in rats causes behavioral hypersensitivity to a mild stress (ie handling and saline injection) as the MAM animals were hyperactive in the first 15 min of exploration of the open field following injection. Animals were also more responsive to a D-amphetamine challenge. This result is reminiscent of the findings in schizophrenic patients, who are hypersensitive to D-amphetamine, both on a behavioral level, and in terms of subcortical dopamine release (Laruelle *et al*, 1999; Lieberman *et al*, 1990; Breier *et al*, 1997). The hypersensitivity of MAM GD17 animals to a mild stress is also in accordance with stress susceptibility in schizophrenia, where psychological stress may precipitate or exacerbate psychotic symptoms (Day *et al*, 1987; Norman and Malla, 1993).

The mechanisms by which the subcortical dopamine output is increased in schizophrenia are unknown. It has been hypothesized that prefrontal cortical dysfunction could lead to a lack of regulation of subcortical dopamine activity (Weinberger, 1987). Electrophysiological data also show that the response to dopamine is altered in cortical neurons in MAM-treated rats. Thus, the cortical pyramidal neurons of control rats respond to a local application of dopamine by lowering the firing frequency, whereas no such effect is observed in MAM-treated rats (Lavin and Grace, 1997). This may lead to an altered regulation of subcortical dopamine activity, through a dysfunctional corticoaccumbens glutamatergic projection (Grace, 2000). The critical role of dopamine in the prefrontal cortex is also supported by recent observations that show that D_{1/5} agonists reverse amphetamine-induced hyperactivity, probably through a medial prefrontal cortex-mediated mechanism (Isacson *et al*, 2004).

Monoamines within the prefrontal cortex have a regulatory action on dopamine transmission in the nucleus accumbens and its behavioral outcome. Dopamine inhibits whereas noradrenaline has a putative disinhibiting effect on the behavioral activation resulting from increased dopamine transmission in the nucleus accumbens, as shown by the blockade of behavioral responses induced by amphetamine, with the α -1 adrenoceptor antagonist prazosin (Blanc *et al*, 1994; Darracq *et al*, 1998). Therefore, an imbalance between dopamine and noradrenaline in the prefrontal cortex could contribute to the observed abnormal limbic-mediated behaviors. Interestingly, MAM treatment has been shown to lead to an increased level of noradrenaline in the cortex (Johnston *et al*, 1988).

Social Interaction

Our experiments showed that MAM treatment at GD17 caused disruption of social interaction. This was mainly a consequence of a lower duration of social interaction episodes. In addition, the study showed that MAM-treated rats were hyperactive during the test.

The analysis of the data showed that the deficit in social interaction was not a consequence of the increased distance traveled by MAM-exposed animals, as the difference between the groups was maintained even when controlling for the increased distance traveled. This is in line with the

evidence from previous studies in our laboratory (using different treatments) which have shown that a dissociation between distance traveled and social interaction does exist, for example, subchronic haloperidol inhibits the hyperactivity caused by subchronic PCP without ameliorating the deficit in social interaction (Sams-Dodd, 1999). Thus, the decrease in social interaction and the increase in locomotor activity are independent phenomena. We found no evidence of an anxiogenic effect of the MAM treatment, since the percent time spent in the central zone of the open field was not changed significantly. Thus, the decreased time spent in social interaction does not appear to be an artefact due to the rats displaying a higher level of anxiety.

The negative symptoms of schizophrenia have in some studies been correlated with prefrontal dysfunction, and with extensive gray and white matter abnormalities in cortico-thalamo-cortical circuits (Gur *et al*, 2000; Lieberman *et al*, 2001; Mathalon *et al*, 2001; Nopoulos *et al*, 2001; Paillere-Martinot *et al*, 2001; Sigmundsson *et al*, 2001; Wible *et al*, 2001). Few clinical studies have specifically explored the biological basis of social dysfunction in schizophrenia, but one study suggests that morphological abnormalities of the ventral frontal cortex could be connected to social dysfunction (Chemerinski *et al*, 2002). In the absence of an extensive histopathological examination of the brain of the MAM-treated rats, it is not possible to hypothesize which structural deficits underlie the deficit in social interaction in these animals.

The effects of other neurodevelopmental perturbations in rats have also been examined in the social interaction test. Neonatal lesion of the ventral hippocampus led to deficits in this test (Sams-Dodd *et al*, 1997). The interaction deficit was apparent both before (postnatal day 35) and after (postnatal day 56) puberty, whereas hyperactivity only emerged after puberty. This shows great resemblance to the emergence of symptoms of schizophrenia, as negative symptoms may become apparent already during childhood, prior to positive symptoms (Done *et al*, 1994; Auerbach *et al*, 1993). The deficit described after ventral hippocampal lesion in active social interaction was comparable to the observations in the present study. A deficit was reported in the same model by other authors (Becker and Grecksch, 2003). Interestingly, this group also showed that this deficit is sensitive to the semichronic treatment with haloperidol and clozapine. However, when examining the effect of neonatal ventral hippocampal damage on play and other types of social behavior, Wolterink *et al* (2001) were unable to confirm social dysfunction. On the other hand, in another proposed preclinical model of schizophrenia, that is, lesions to the amygdala at postnatal day 7 or postnatal day 21 (Hanlon and Sutherland, 2000), the same group found significant disturbances in social behavior (Wolterink *et al*, 2001). The rats in the present study not only showed disruption of social interaction but also increased motor activity during the test, probably reflecting increased subcortical dopamine activity. This was in accord with our observations in the open field and after the amphetamine challenge. In yet another preclinical model of schizophrenia, that is, electrical sensitization of the mesolimbic dopaminergic cells in the ventral tegmental area, electrical stimulations once daily for 70 days resulted in a hypersensitive response to dopaminergic stimulation as well as

disturbed social interaction between stimulations (Glenthøj *et al*, 1993). This suggests that sensitization to dopaminergic stimulation as shown in the amphetamine challenge could be directly linked to the deficits of MAM-treated rats in the social interaction test. A balanced subcortical and cortical dopamine activity seems to be important for normal social interactions, but the exact impact of the behavioral (subcortical) hyperactivity on the observed abnormal social interactions is not known. The clinical findings and possibly also the observations in a model such as the MAM GD17 rats rather point to schizophrenia and schizophrenia-like disturbances as supraregional disorders of multiple, distributed circuits, than a disorder in which each symptom is caused by disruption of function in specific brain regions.

Effect of D-Amphetamine on Extracellular Dopamine in Nucleus Accumbens and Prefrontal Cortex

Increased dopamine activity in the nucleus accumbens is associated with behavioral hyperactivity (Kelly *et al*, 1975); therefore, the increased response to the mild stress of handling and to D-amphetamine suggested a hyperactive subcortical dopamine system. We showed by microdialysis that indeed, the dopamine response to systemic amphetamine was elevated in the nucleus accumbens of MAM-exposed rats compared to controls. No such differences were found in the medial prefrontal cortex.

The functional pathology in the subcortical dopamine system in schizophrenia is believed to be the consequence of impaired cortical input (Carlsson, 1988; Weinberger and Lipska, 1995). This is supported by evidence that prefrontal cortical neuronal pathology (reflected in decreased NAA levels) predicts both baseline and evoked striatal dopamine activity (Bertolino *et al*, 1999, 2000a); the greater the prefrontal pathology, the lower the baseline striatal dopamine and the higher the D-amphetamine-induced dopamine response. This is in agreement with the idea of a decreased tonic and increased phasic striatal dopamine transmission, possibly due to a reduction in the activity of the glutamatergic corticostriatal pathway (Grace, 1991; Moore *et al*, 1999). Interestingly, in both the single-probe and dual-probe microdialysis experiments, the basal level of extracellular dopamine in the nucleus accumbens was lower in MAM-exposed rats, the difference being statistically significant in the dual-probe experiments. We also noted that in the dual-probe experiment, the basal dopamine dialysates level was lower than in the single-probe experiment. It is likely that this was due to the longer perfusion time and equilibration (15–18 h) before collection of the samples.

The involvement of the prefrontal cortical dopamine in the regulation of subcortical dopamine release is supported by the observation that lesions of the prefrontal cortical DA system increase the sensitivity of the subcortical dopamine system to stress (King *et al*, 1997; Deutch *et al*, 1990). In contrast, augmentation of dopaminergic transmission in the frontal cortex reduces subcortical dopaminergic activity (Jaskiw *et al*, 1991; Louilot *et al*, 1989; Karreman and Moghaddam, 1996; Kolachana *et al*, 1995). To further clarify the nature of the hypersensitive subcortical dopamine response in the MAM exposed rats, we investigated the subcortical dopamine response after prefrontocortical

D-amphetamine infusion. In agreement with the literature (Karreman and Moghaddam, 1996), we found that this manipulation resulted in a slight decrease in extracellular dopamine in the nucleus accumbens of control rats. The decrease in subcortical dopamine was approximately 20%, which corresponds to previous reports (Karreman and Moghaddam, 1996). The dialysis probes were positioned contralaterally to ensure that the results would not be confounded by diffusion of D-amphetamine from the prefrontal probe to the probe in the nucleus accumbens. Contralateral projections exist between the prefrontal cortex and the striatum (Berendse *et al*, 1992), although these are less dense than the ipsilateral ones. This might be even more pronounced for the nucleus accumbens (Berendse *et al*, 1992). Thus, it is likely that the prefrontocortical regulation of ipsilateral subcortical dopamine is stronger than what was measured contralaterally in our study.

In the MAM-exposed rats, no decrease was observed in extracellular dopamine in the nucleus accumbens after D-amphetamine application in prefrontal cortex. Instead, an increase in subcortical dopamine was observed in the fraction collected in the interval immediately after termination of the D-amphetamine infusion. The mechanism underlying this paradoxical response is unclear, but this supports the existence of a dysfunctional cortical control over subcortical systems. A similar disruption has been described in monkeys with neonatal medial-temporal lobe lesions (Saunders *et al*, 1998). It is also interesting to note that MAM-exposed rats had an increased cortical dopamine response after local D-amphetamine infusion, compared to controls. In contrast, no differences in prefrontal cortex extracellular dopamine were seen between control and MAM rats after systemic D-amphetamine. This discrepancy could be due to the fact that the relatively high concentration of D-amphetamine infused locally mobilized a much larger fraction of cortical dopamine stores (eg D-amphetamine induced an increase in baseline dopamine of approximately 500% after systemic administration, and of 1500% after local infusion). The amplitude of the dopamine response in this situation could be determined at least partly by the size of the tissue stores. According to preliminary observations, dopamine tissue levels appear to be increased in the frontal cortex of MAM-exposed rats (unpublished data).

The prefrontal dopamine response to local amphetamine infusion had a fast onset, whereas the changes seen in extracellular dopamine in the nucleus accumbens were delayed. Therefore, the changes in dopamine in the prefrontal cortex alone may not explain entirely the effects in the nucleus accumbens. The lack of cortical inhibition over the subcortical system may be due to a lack of postsynaptic effect of the released dopamine in the cortex. As mentioned previously, in MAM-exposed rats (GD15–17), the dopamine-mediated inhibition of pyramidal cells in the prefrontal cortex is attenuated (Lavin and Grace, 1997; Grace *et al*, 1998).

In conclusion, our observations indicate that a disturbance in neurodevelopment induced by the antimetabolite compound MAM administered at GD17 leads to several behavioral and neurochemical changes similar to the pathophysiology of schizophrenia. The changes appear to reflect an imbalance between cortical and subcortical

systems, and in particular impairment in the gating processes controlled by cortical and hippocampal afferents to the nucleus accumbens. MAM-treated rats are hyper-reactive to stress or to an amphetamine challenge. This is reminiscent of the reactions of subjects with disturbed cortical development, who show an exaggerated dopamine response and as a consequence an aberrant assignment of salience (Kapur, 2003) leading to the positive symptoms of schizophrenia. Interestingly, this increased reactivity of dopaminergic systems to stress is not seen in one of the other well-studied models of schizophrenia, the ventral hippocampus lesion model (Lillrank *et al*, 1999). In addition, the MAM GD17 rats show decreased social interaction, a finding that in the context of schizophrenia is believed to reflect at least partly the negative symptoms. Therefore, a developmental disturbance that affects the hippocampus, the thalamus, and other cortical regions, in a manner that resembles abnormalities reported in schizophrenia, can bring about behavioral changes in animals reflecting positive as well as negative symptoms of schizophrenia. Our observations after treatment at GD17 also highlight the importance of the choice of the day of exposure to MAM. The use of the administration of MAM to induce neurodevelopmental abnormalities and provide an animal model of schizophrenia has been recently questioned (Jongen-Rêlo *et al*, 2004). However, the negative results reported by these authors after MAM treatment was confined to the GD9-GD15 period. Each gestational period has its own critical importance for selected neural circuits; therefore, the negative results seen at earlier times than GD17 cannot be used to dismiss in general the relevance of this type of manipulation and its potential. A continuation of the neurochemical, behavioral, and pharmacological validation of the MAM GD17 model is required in order to establish its value as an animal model of schizophrenia. Furthermore, as schizophrenia is a clinical syndrome and not a uniform clinical entity (Carpenter *et al*, 1999), it remains to be determined if the MAM model has relevance for a particular schizophrenia subtype.

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