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SHORT COMMUNICATION
Decrease in parvalbumin-expressing neurons in the hippocampus and increased phencyclidine-induced locomotor activity in the rat methylazoxymethanol (MAM) model of schizophrenia

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Abstract

Treatment of rats with methylazoxymethanol (MAM) on gestational day (GD)17 disrupts corticolimbic development in the offspring (MAM-GD17 rats) and leads to abnormalities in adult MAM-GD17 rats resembling those described in schizophrenic patients. The underlying changes in specific cortical and limbic cell populations remain to be characterised. In schizophrenia, decreases in inhibitory γ-aminobutyric acid (GABA)-containing interneurons that express the calcium-binding protein parvalbumin have been reported in the prefrontal cortex and hippocampus. In this study we analysed the expression of parvalbumin (PV), calretinin (CR) and calbindin (CB) in the prefrontal cortex and hippocampus of MAM-GD17 rats. Exposure *in utero* to MAM led to a significant decrease in the number of neurons expressing PV in the hippocampus, but not the prefrontal cortex. Neurons expressing CR or CB were not affected in either structure. The neurochemical changes in MAM-GD17 rats were accompagnied by increased hyperlocomotion after administration of phencyclidine (PCP), analogous to the hypersensitivity of schizophrenic patients to PCP. Therefore, the developmental MAM-GD17 model reproduces key neurochemical and behavioural features that reflect cortical and subcortical dysfunction in schizophrenia, and could be a useful tool in the development of new antipsychotic drugs.

Introduction

Disruption of neurogenesis in rats on gestational day (GD)17 by the antimitotic compound methylazoxymethanol (MAM) has been proposed as a developmental animal model of schizophrenia (Moore *et al*., 2001; Grace, 2003; Fiore *et al*., 2004; Flagstad *et al*., 2004; Gourevitch *et al*., 2004; Flagstad *et al*., 2005; Lavin *et al*., 2005; Moore *et al*., 2005). Unlike treatment with MAM on other GDs (Jongen-Rêlo *et al*., 2004), treatment on GD17 affects mainly the development of the hippocampus in the offspring (MAM-GD17 rats), which results in a compensatory rewiring of corticolimbic circuits (Grace *et al*., 1998; Lavin *et al*., 2005). In MAM-GD17 rats there is a decreased size of cortical and limbic areas (Moore *et al*., 2001; Gourevitch *et al*., 2004; Moore *et al*., 2005), and animals present with behavioural abnormalities in early adulthood that are reminiscent of schizophrenia (Flagstad *et al*., 2004; Gourevitch *et al*., 2004; Flagstad *et al*., 2005).

Schizophrenia is associated with significant changes in γ-aminobutyric acid (GABA)-containing interneurons in cortical and limbic areas, and such changes may constitute a key pathogenic mechanism underlying the altered neuronal excitability in schizophrenia (for a recent review, see Lewis *et al*., 2005). GABAergic interneuron subpopulations can be distinguished by the nonoverlapping expression of the calcium-binding proteins parvalbumin (PV), calretinin (CR) and calbindin (CB) (Andressen *et al*., 1993). Some post-mortem studies have shown that the number of GABAergic interneurons expressing PV is decreased in both the prefrontal cortex and the hippocampus of schizophrenic subjects, whereas other studies have reported lower levels of PV mRNA, but not neuron numbers, in the prefrontal cortex (for review, see Reynolds *et al*., 2001). No changes have been reported in the density of CR-expressing neurons, whereas conflicting results have been obtained for CB-expressing neurons (Daviss & Lewis, 1995; Beasley *et al*., 2002). A decrease in PV expression has also been reported in animal models of schizophrenia, such as those based on the administration of phencyclidine (PCP; Cochran *et al*., 2002; Cochran *et al*., 2003; Reynolds *et al*., 2004) or ketamine (Keilhoff *et al*., 2004). To investigate whether changes in PV expression can be found in the MAM-GD17 model of schizophrenia, we analysed in the current study the expression of PV, CR and CB in the prefrontal cortex and hippocampus of MAM-GD17 rats. A decrease in PV expression would consolidate the value of this animal model, and would also confirm a link between abnormal neurodevelopment and the significant changes in interneurons reported in schizophrenia.

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The presence of behavioural abnormalities was also investigated in MAM-GD17 rats from the same batch, by assessing the effect of an acute administration of PCP on locomotor activity. PCP is an antagonist of the *N*-methyl-D-aspartate (NMDA) receptors, and can induce schizophrenia-like symptoms in both animals and humans (for review, see Halberstadt, 1995). GABAergic and glutamatergic deficits may be linked in schizophrenia (for review, see Coyle, 2004) and we hypothesised that the effects of PCP could be exacerbated in the MAM-GD17 rats which have a deficit in certain GABAergic neurons.

Materials and methods

Animals

Pregnant Wistar rats were obtained from Harlan (Netherlands) on GD10 and were housed individually. On GD17, the dams were treated with MAM acetate (National Cancer Institute Chemical Carcinogen Reference Standard Repository) at a dose of 22 mg/kg, or saline injected intraperitoneally (i.p.). Male pups were weaned 30 days after birth and were housed in pairs under a 12-h light-dark cycle (lights on 06.00 h), at 21 ± 2 °C, with food and water available *ad libitum*. All experiments were carried out under a license from the Danish Ministry of Justice, and in accordance with the Danish law regulating experiments on animals. Experiments were carried out when the animals were 3–6 months old. Animals that were the offspring of the same batch of treated dams were used for the behavioural and immunohistochemical analysis.

PCP hyperactivity

Because PCP can induce changes in PV expression (see Introduction), the behavioural analysis was carried out in a set of rats different from that used for immunohistochemistry. Animals were moved to the room where they were tested 24 h before the test. The hyperactivity experiment was run in normal light conditions, during the light phase, with the observer blind to the experimental conditions. After subcutaneous (s.c.) injection of saline or PCP (0, 1.25, 2.5 or 5 mg/kg), the rats were placed individually in test cages (macrolon type III, 42.5 × 26.5 × 18.5 cm, equipped with four infrared light sources and photocells located 4 cm above the bottom of the cages, which was covered with a thin layer of sawdust). Locomotor activity was measured for 2 h. Eight rats were tested per group.

Immunohistochemistry

Ten rats each of the MAM-GD17 and vehicle groups were anaesthesised with Avertin (tribromethanol, 680 mg/kg i.p.), and perfused transcardially with ice-cold phosphate-buffered saline followed by 4% paraformaldehyde containing 15% of a saturated picric acid solution. Brains were postfixed for 4–6 h at 4 °C, then cryoprotected in 30% sucrose at 4 °C. Sections 40 μm thick were cut sequentially in sets of four sections. The tissue was stained using the diaminobenzidine immunoperoxidase method, and primary antibodies for either PV (1 : 5000), CR (1 : 2000) or CB (1 : 10000), according to the manufacturer's instructions (Swant, Bellinzona, Switzerland). A fourth section in each set was stained with Cresyl Violet.

Image analysis

The semiquantitative analysis was performed blind using the C.A.S.T. stereology software (Olympus, Denmark). The planes of the areas chosen for analysis correspond to the following coordinates in the brain atlas of Paxinos & Watson (1998): AP from bregma, 3.7–2.7 mm for the prefrontal cortex (as depicted in Fig. 1G and H) and −2.8 to −3.3 mm for the hippocampus (see also Fig. 1A and B). The areas of the prefrontal cortex and the hippocampus were measured bilaterally in four Cresyl Violet-stained sections per area. To quantify the neuronal disarray in the hippocampus after MAM exposure, the stratum oriens was delineated at low (0.5×) magnification, and the dispersed cells detected in the field were counted at high magnification (20×). The number of sites where the pyramidal cell layer was totally disrupted was also noted in each section. In the prefrontal cortex the total cell number was determined using the 40× objective, a counting frame with an area of 4113 μm^2 and a counting interval of 413 μm in the *X* and *Y* planes. At least 150 cells were sampled per prefrontal cortical area. Due to the difference in cell populations and the cell disarray, the total cell number could not be reliably assessed in the hippocampus.

To quantify cells expressing the calcium-binding proteins, the areas of the prefrontal cortex and hippocampus were first delineated at low magnification (0.5×) and cells were then counted bilaterally in four sections per structure (adjacent to the Nissl-stained sections) using the 20× objective. In addition to being a marker for interneurons, CB is also expressed by glutamatergic cells, especially granule cells in the hilus; these latter can be easily distinguished from the other CB-positive cell populations. Because we were interested in GABAergic interneurons, these cells in the hilus were excluded for the quantification of CB staining in the hippocampus. With this exception, cells expressing calcium-binding proteins were counted in all hippocampal fields between the stereotaxic boundaries chosen. As we did not see differences between subfields, we chose to cumulate the counts and not present them separately. All immunoreactive cells were counted regardless of staining intensity. In the prefrontal cortex, because the total cell density (cells/mm^2 ± SEM) was the same in both MAM-treated and control rats (see Results), the results were expressed as numbers of immunoreactive cells/mm^2. In the hippocampus, the total cell density was assumed to be the same and the results were also expressed as numbers of immunoreactive cells/mm^2 ± SEM.

Statistical data analysis

The results from the behavioural testing were analysed using three-way ANOVA, with gestational treatment (MAM or saline) and the PCP dose (0, 1.25, 2.5 or 5 mg/kg) as independent factors, and time after drug administration as a repeated measure. *Post hoc* analyses used Fisher's protected least-significant-difference test.

For the cell counts and area measurements, statistical comparisons were carried out using Student's *t*-test or one-way ANOVA, followed by Bonferroni's *post hoc* comparisons.

Results

As it has been reported that MAM-GD17 rats have a reduced brain weight and size and display histological abnormalities (Gourevitch *et al*., 2004; Moore *et al*., 2005), we further confirmed this phenotype by the present analysis. The analysis of the selected areas in the prefrontal cortex and hippocampus showed a significant decrease in MAM-GD17 animals compared to vehicle-controls, from 2.94 ± 0.14 to 2.33 ± 0.11 mm^2 (20%) in the prefrontal cortex, and from 3.49 ± 0.07 to 2.88 ± 0.04 mm^2 (17%) in the hippocampus, respectively (both $P < 0.001$ vs. controls). In the prefrontal cortex, the total cell density was not different between MAM-GD17 and control rats

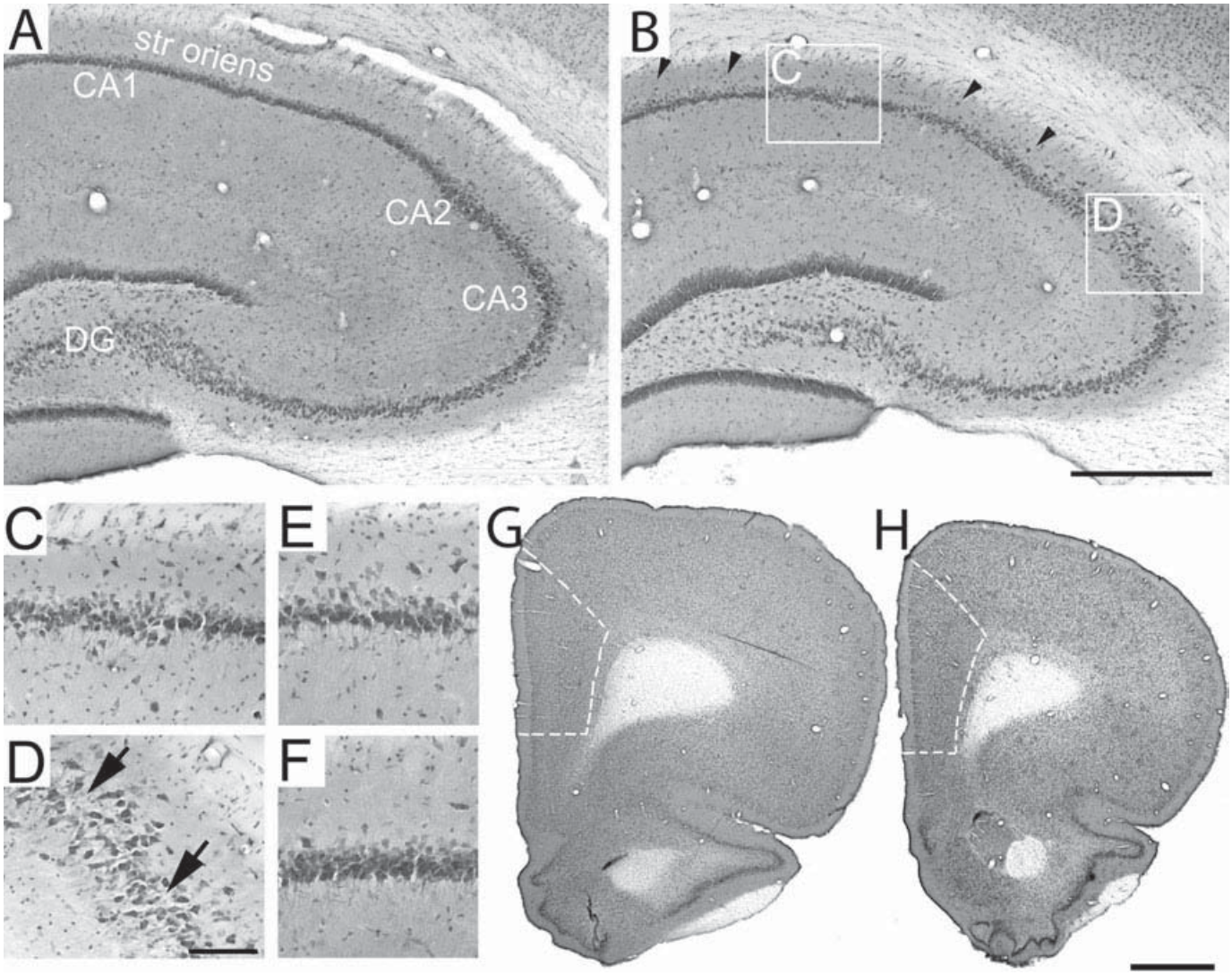

FIG. 1. Structural changes in the hippocampus and prefrontal cortex in Cresyl Violet-stained sections from MAM-GD17 and vehicle rats. (A–F) Hippocampus: low magnifications of the hippocampus of (A) a vehicle-treated rat and (B) a MAM-GD17 rat are shown for comparison. The smaller size and the pyramidal cell dispersion in CA1–3 of the hippocampus of the MAM-GD17 rat (B) as compared to the control (A) is evident. The arrowheads in B mark cells in the stratum oriens. The boxes in B encompass the regions of (C) the CA1 and (D) CA2 magnified in C and D, respectively, showing a close-up view of the pyramidal cell dispersion. The arrowheads in D indicate locations where the integrity of the pyramidal cell layer is disrupted. For comparison, a representative magnification of a section of the pyramidal cell layer in the hippocampus of (E) a MAM-GD17 rat is shown with (F) that of a vehicle rat. (G and H) Prefrontal cortex: low magnification overviews of prefrontal cortical sections of (G) a vehicle rat and (H) a MAM-GD17 are shown. The broken lines encompass the prefrontal cortical area measured for the area quantification. Note the overall smaller size of the section in the MAM-GD17 rat (H) in comparison to the vehicle rat (G). Scale bars, 500 μm (A and B), 100 μm (C–F), 1 mm (G and H).

(3169 and 3164 cells/mm^2, respectively). In the hippocampus, the total cell density could not be assessed due to structural changes, i.e. a marked dispersion of pyramidal cells that was observed in the CA1-3 region in the hippocampus of MAM-GD17 rats (Fig. 1A and C–E). As a consequence, significantly more cells (94 ± 7.4 as compared to 59.0 ± 7.7 in vehicle controls), were present in the stratum oriens of MAM-GD17 rats ($P < 0.01$, Fig. 1E and F). Complete disruption of the tightly packed pyramidal cell layer was seen at 2.6 ± 0.2 locations per section in MAM-GD17 rats, as compared to 0.1 ± 0.1 in vehicle controls ($P < 0.001$, Fig. 1A and D). These histological changes are a useful internal control for the MAM phenotype of individual rats derived from different litters.

Rats derived from several different litters were examined for the number of PV-immunoreactive cells. This was significantly decreased in MAM-GD17 rats in the hippocampus from 9.5 ± 0.2 to 6.8 ± 0.2 cells/mm^2 (28%, Fig. 2A–D and F). In the prefrontal cortex, the number of PV-immunoreactive cells/mm^2 was not different between MAM-GD17 and control rats (Fig. 2E).

In contrast to the decrease in PV-expressing cells, no changes in the numbers of either CR- or CB-immunoreactive cells were found in either structure (Fig. 2E and F).

Rats from the same litters used for the histological analysis were challenged with PCP and evaluated for locomotor activity. PCP induced an increase in locomotor activity at the highest dose tested (5 mg/kg), but not at 2.5 or 1.25 mg/kg, in both control and MAM-GD17 rats. However, the effect was more marked in the latter group ($P < 0.01$ for the difference between control and MAM; Fig. 3).

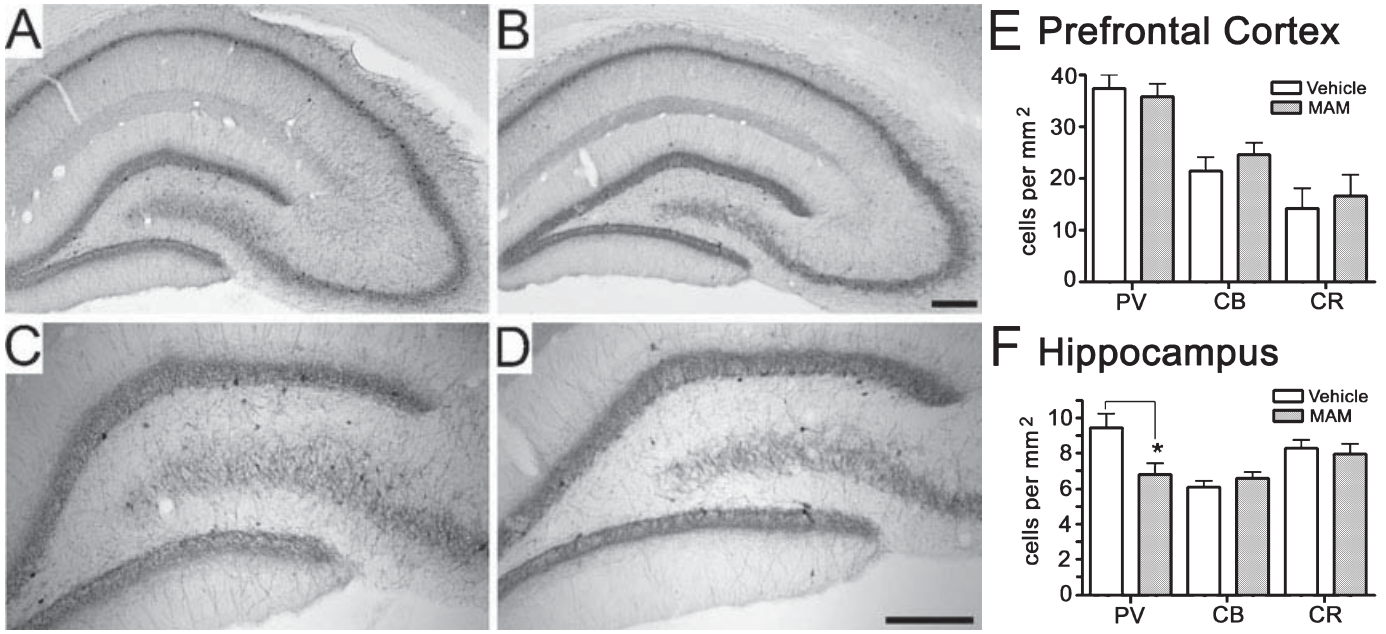

FIG. 2. PV immunoreactivity in the prefrontal cortex and hippocampus. (A–D) PV staining in the hippocampus: representative hippocampal sections of (A) a vehicle-treated and (B) a MAM-GD17-treated rat show a decrease in the numbers of PV-immunoreactive neurons across the hippocampal subfields. A magnified view of the dentate gyrus of these sections is shown in C (vehicle) and D (MAM-GD17). (E and F) Quantification of PV, CR and CB neuron numbers. The number of PV-immunoreactive cells was not significantly decreased in the MAM-GD17 rats in (E) the prefrontal cortex, but was in (F) the hippocampus ($P < 0.05$), as compared to vehicle rats, whereas the numbers of CR- and CB-immunoreactive cells were not changed in either structure. Scale bars, 100 μm (in B for A and B, and in D for D and E).

Discussion

In this study we show that gestational exposure to MAM on GD17 leads to a decrease in the number of PV-immunoreactive cells in the hippocampus. This change occurs in parallel with a behavioural hypersensitivity to PCP. Both results are similar to observations made in schizophrenic patients, and contribute to the characterization and validation of the MAM-GD17 animal model of schizophrenia.

The area of the prefrontal cortex was decreased in MAM-GD17 rats, but the total cell density was not different between MAM-GD17-treated rats and control rats. Similarly, the density of PV-immunoreactive cells was not significantly different in the prefrontal cortex of MAM-GD17-treated rats. In contrast, in the hippocampus the magnitude of the decrease in the number of PV-positive neurons was higher than the decrease in area; therefore the density of PV-positive neurons was significantly lower, suggesting that the loss of PV-positive neurons and decrease in area are not entirely interdependent phenomena. The total cell number in the hippocampus could not be assessed in the current study. Therefore, it can not be entirely ruled out that, unlike in the prefrontal cortex, the total cell density in the hippocampus of the MAM-GD17-treated rats is also lower and the decrease in PV cell number proportional

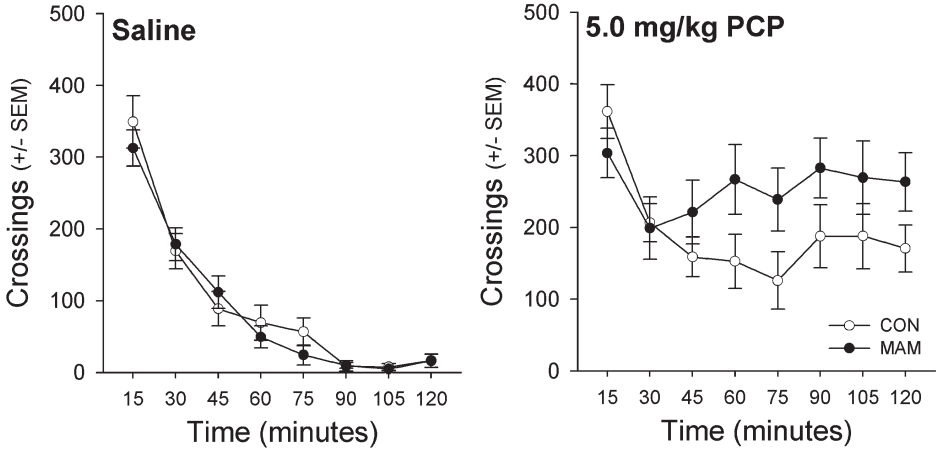

FIG. 3. Effect of PCP on the locomotor activity of vehicle- or MAM-exposed rats. Saline or PCP at increasing doses were injected s.c. and the locomotor activity of the animals was monitored for 2 h. Results are expressed as means ± SEM. The ANOVA revealed a significant effect of time ($F_{7,378} = 109.4$, $P < 0.001$) and drug dose ($F_{3,54} = 26.1$, $P < 0.001$), as well as a time × drug interaction ($F_{21,378} = 6.4$, $P < 0.001$) and a time × drug × gestational treatment interaction ($F_{21,378} = 1.9$, $P = 0.01$). The *post hoc* analysis showed that the effect of 5 mg/kg PCP was significantly different from any of the other doses including the saline group ($P < 0.001$ for all comparisons). In addition, the *post hoc* analysis of the time × drug × gestational interaction showed that only the effect of 5 mg/kg PCP was different between vehicle and MAM-GD17 rats ($P < 0.01$).

to a decrease in total cell number. However, we consider this possibility unlikely because the numbers of CR- and CB-positive neurons were not significantly affected in either structure, despite the decrease in size of the prefrontal cortex and hippocampus areas measured. The area decreases and the hippocampal pyramidal cell dispersion and disarray are in accord with previous findings (Flagstad *et al*., 2004; Gourevitch *et al*., 2004; Flagstad *et al*., 2005; Moore *et al*., 2005). Whether the decrease in size of these regions is similar to the overall decrease in brain volume in MAM-GD17 rats (Balduini *et al*., 1991), or is relatively larger, could not be assessed in the current study. Studies on schizophrenic patients have reported conflicting results, showing either a selective decrease in hippocampal and prefrontal cortical size (Weinberger *et al*., 1983; Cannon & Marco, 1994), or no relative decrease, when the sizes of the areas were normalized to the total brain volume (Tanskanen *et al*., 2005).

The maturation of GABAergic interneurons in the hippocampus has been shown to be orchestrated by brain-derived neurotrophic factor (BDNF) signalling through its high-affinity receptor TrkB (Marty *et al*., 1996). Changes in BDNF levels have been reported in MAM-GD17-treated rats (Fiore *et al*., 2004); therefore it can be speculated that altered BDNF levels underly the hippocampal deficit in the number of PV-positive cells observed in the present study. The PV-positive GABAergic interneurons play a crucial role in hippocampal circuitry by controlling pyramidal cell output (Freund & Buzsaki, 1996). A decrease in the number of these neurons in the hippocampus may contribute to the behavioural abnormalities previously reported in MAM-GD17 rats (Gourevitch *et al*., 2003; Flagstad *et al*., 2004; Flagstad *et al*., 2005). Thus, they may play a critical role in the cognitive dysfunction which may be a core feature of schizophrenia (Lewis *et al*., 2005).

The acute administration of PCP exacerbates symptoms in stabilised schizophrenia patients (Luby *et al*., 1959; Allen & Young, 1978). GABAergic interneurons are suspected of being particularly sensitive to PCP, and in a broader sense to NMDA hypofunction, and this would provide a connection between the GABAergic and glutamatergic deficits reported in schizophrenia (Coyle, 2004). After PCP, MAM-GD17 animals show increased locomotor hyperactivity, a behaviour generally associated with midbrain structures. Interestingly, developmental neuropathological changes in the hippocampus including changes in GABAergic transmission have been shown to lead to decreases in dopamine transporter mRNA expression in the midbrain in adult rats, which could contribute to the locomotor hyperactivity (Lipska *et al*., 2003). Whether such changes also occur in the MAM-GD17 model should be addressed in future experiments.

In conclusion, our results show that the MAM-GD17 model closely mimics abnormalities observed in schizophrenia patients and, in particular, the selective changes in a subpopulation of limbic GABA interneurons that may be at the core of the cognitive impairment that compromises the life of schizophrenic patients.

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Abbreviations

CB, calbindin; CR, calretinin; GABA, γ-aminobutyric acid; GD, gestational day; MAM, methylazoxymethanol; MAM-GD17 rats, rats treated on GD17 with MAM; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; PV, parvalbumin; s.c., subcutaneous.

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