Impaired brain development in the rat following prenatal exposure to methylazoxymethanol acetate at gestational day 17 and neurotrophin distribution

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Several neuropsychiatric disorders, including schizophrenia, are the consequence of a disrupted development of the CNS. Accordingly, intrauterine exposure to toxins may increase the risk for psychopathology. We investigated whether prenatal exposure of rats to the neurotoxin methylaxoxymethanol acetate led to long-term changes in cerebral neurotrophin levels. We measured the brain levels of nerve growth factor and brain derived neurotrophic factor in young adult and adult rats. Decreased nerve growth factor or brain derived neurotrophic factor were found in the parietal cortex accompanied by altered neurotrophin content in the hippocampus and entorhinal cortex. The present study is the first to show long-lasting effects of a single prenatal exposure to a neurotoxin on adult levels of neurotrophins in brain regions implicated in neuropsychiatric disorders. *NeuroReport* 15:1791–1795 © 2004 Lippincott Williams & Wilkins.

Key words: BDNF; MAM; NGF; Schizophrenia

INTRODUCTION

Methylazoxymethanol acetate (MAM) exposure during development affects brain cytoarchitecture, behavior and neurotrophins in the rat. MAM alkylates the DNA of proliferating neurons, killing the mitotic cells [1]. If administered at gestational day (GD) 11 or 12, MAM induces disrupted development of the entorhinal-hippocampal axis [2–4], whereas at GD15 and 16 the hypothalamus and selective striatal, cortical, hippocampal and thalamic areas are particularly affected [1] (see Table 1 for further information). Disrupted development in these brain areas is associated with specific behavioral impairments. One potential mechanism through which this neurotoxin may disrupt development may be via changes in nerve growth factor (NGF) or brain derived neurotrophic factor (BDNF) levels. NGF and BDNF are neurotrophins that play key roles in the development, maintenance and function of the peripheral and central nervous system [5], regulating neural processes including synaptic function and plasticity, as well as impacting neuronal survival. Rats exposed to MAM at GD11 and GD12 when tested as adults exhibited high levels of NGF and BDNF in the entorhinal cortex but reduced levels in the hippocampus and parietal cortex (GD15) [3,4,6]; this supports a potential role for NGF and BDNF in the cellular atrophy observed following prenatal exposure to MAM. One time point that is of particular interest is the effect of MAM on GD17 because MAM administration at this time point may lead to disruption of brain circuits of known relevance to schizophrenia [7,8]. Adult rats exposed at GD17 to MAM show small-to-moderate reductions in the thickness of limbic and paralimbic cortices. Furthermore, these rats have significant deficits in cognitive tasks that depend on prefrontal-and hippocampal-striatal circuits and behave as rats with frontal lesions. Thus the aim of the present study was to investigate the changes in NGF and BDNF following exposure *in utero* to MAM at GD17 in those brain areas sensitive to MAM-induced cellular ablation such as the hippocampus and cortex. We predicted that prenatal MAM administration would induce selective disruption of both NGF or BDNF levels of the rat brain.

MATERIALS AND METHODS

Subjects and treatments: Timed pregnant Fisher 344 rats (*Rattus norvegicus*) were obtained from Harlan, USA; animals were mated over a period of 4 h, which was defined as day 0 of gestation (GD0). Females with a vaginal plug

Table I. Brain cellular ablation or atrophy induced by a single administration of MAM at different time points of rat gestation (see [1,2] for further details).

(Prenatal) Day of the injection	Brain area disrupted
11	Entorhinal cortex
12	Hippocampus entorhinal cortex
13	Hypothalamus, Striatum thalamus, Cerebellum cortex
14	Hypothalamus, Cortex hippocampus
15	Striatum, Thalamus, Hypothalamus cortex, Hippocampus
16–19	Striatum, Cortex hippocampus, Olfactory bulbs

were separated from the males and transported to the laboratories. Upon arrival at the laboratory, animals were housed in a temperature and humidity controlled environment (temperature $21 \pm 1^{\circ}C$, relative humidity $60 \pm 10\%$), with white lights on 07.00–19.00 h in Plexiglas boxes with a metal top and sawdust as bedding. Regular rat pellet food and water were available *ad lib*. Either MAM (22 mg/kg, i.p.) or saline was administered to the pregnant rats at GD17 [2,3]. At birth all litters were culled to four males and four females and fostered to the biological dams following minor modifications of previously described behavioral procedures [2,3]. Post-weaning treated male adult rats (age 3 or 9 months) were used for the neurotrophin studies (n=3 for each group, one animal per litter) and all efforts were taken to reduce the number of the experimental subjects. Experiments were made following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals (USPHS).

Animals were euthanasized at 3 or 9 months of age with an overdose of pentobarbital; the brain was quickly removed, the tissues were dissected out, weighed to analyze differences between groups and stored at $-70^{\circ}C$ until assayed. Tissues were then homogenized and centrifuged at 8500 r.p.m. and the supernatant was used for the assays of NGF and BDNF.

NGF and BDNF determination: NGF and BDNF evaluation was carried out in the hippocampus, entorhinal cortex, striatum, hypothalamus, frontal cortex and parietal cortex of the rat brain with ELISA kits NGF Emaxtm ImmunoAssay System number G7631 and BDNF Emaxtm ImmunoAssay System number G6891 (Promega, Madison, WI, USA) following the instructions provided by the manufacturer. Tissues were homogenized in the kit calibration buffer and centrifuged. The brain tissues were homogenized with ultrasonication in extraction buffer 0.2% Triton. Briefly, 96-well immunoplates were coated with 100 µl/well monoclonal anti-mouse-NGF/BDNF antibody. After overnight incubation at $4^{\circ}C$, the plates were washed three times with buffer and the samples were incubated in the coated wells (100 µl each) for 2 h at room temperature with shaking. After an additional five washes the immobilized antigen was incubated with an anti-human NGF/BDNF antibody for 2 h at room temperature with shaking. The plates were washed again with wash buffer, and then incubated with an anti-IgY HRP for 1 h at room temperature. After another wash the plates were incubated with a TMB/Peroxidase substrate solution for 15 min and then phosphoric acid 1 M (100 µl/well) was added to the wells. 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 standards (ranging from 7.8 to 500 pg/ml purified mouse NGF/BDNF) incubated under similar conditions in each assay. Under these conditions, the recovery of NGF in our assay ranged from 80 to 90%. The sensitivity of the assay was about 3 pg/g wet tissue and cross-reactivity with other related neurotrophic factors (neurotrophin-3 and neurotrophin-4) was $<3\%$. Data are represented as pg/g wet tissue and all assays were performed in triplicate for NGF and in duplicate for BDNF.

Data analysis: Statistical analyses were performed using 2-way ANOVA with prenatal treatment (saline *vs* MAM) and age (3 months *vs* 9 months) as factors by using StatView for Macintosh. Post-hoc analysis was were performed using Tukey's test.

RESULTS

There were no significant differences between the weights of brain regions dissected when comparing between treatment groups. Rats exposed prenatally to MAM were found to exhibit significant differences in neurotrophic factor immunoreactivity in the hippocampus, entorhinal, frontal and parietal cortex (Fig. 1). ANOVA revealed a significant interaction between MAM and age in the hippocampus for both NGF and BDNF ($p<0.01$) with higher levels in 9-month-old MAM rats than in their respective age-matched controls; this was at least partially due to the failure to observe the normal decrease in NGF and BDNF content observed at 9 months of age in controls ($p<0.05$, *post-hoc*, Fig. 1). An interaction between MAM and age in the entorhinal cortex for NGF ($p<0.01$) was due to lower values in 3-month-old MAM rats compared to their respective controls ($p<0.01$ in *post-hoc* comparison); however, no differences were found for BDNF. Statistical analyses did not show significant differences between groups for both NGF and BDNF in the frontal cortex. In contrast, in the parietal cortex NGF and BDNF were significantly lower ($p<0.01$; ANOVA, MAM $\times$ age) in 9-month-old MAM rats than in their respective controls ($p<0.05$ in *post*-hoc comparisons). Within the striatum (Fig. 2) the highest levels of NGF and BDNF were found in 9-month-old rats with no significant differences between groups for NGF but with higher levels ($p<0.01$; ANOVA, MAM $\times$ age) of BDNF in 9-month-old MAM rats compared to their age-matched controls ($p<0.05$ in *post-hoc* comparisons). In the hypothalamus (Fig. 2), highly variable values were found for NGF; nonetheless, significantly lower levels of NGF ($p<0.01$; ANOVA, MAM $\times$ age) were observed in 3-month-old MAM animals with respect to their controls ($p<0.05$ in *post-hoc* comparisons) whereas no differences between groups were found for BDNF.

DISCUSSION

The findings of the present study demonstrate that prenatal MAM exposure at GD17 produces long-lasting effects on NGF and BDNF levels in the rat brain. Indeed the major effects were found in 9-month-old rats. In the parietal cortex

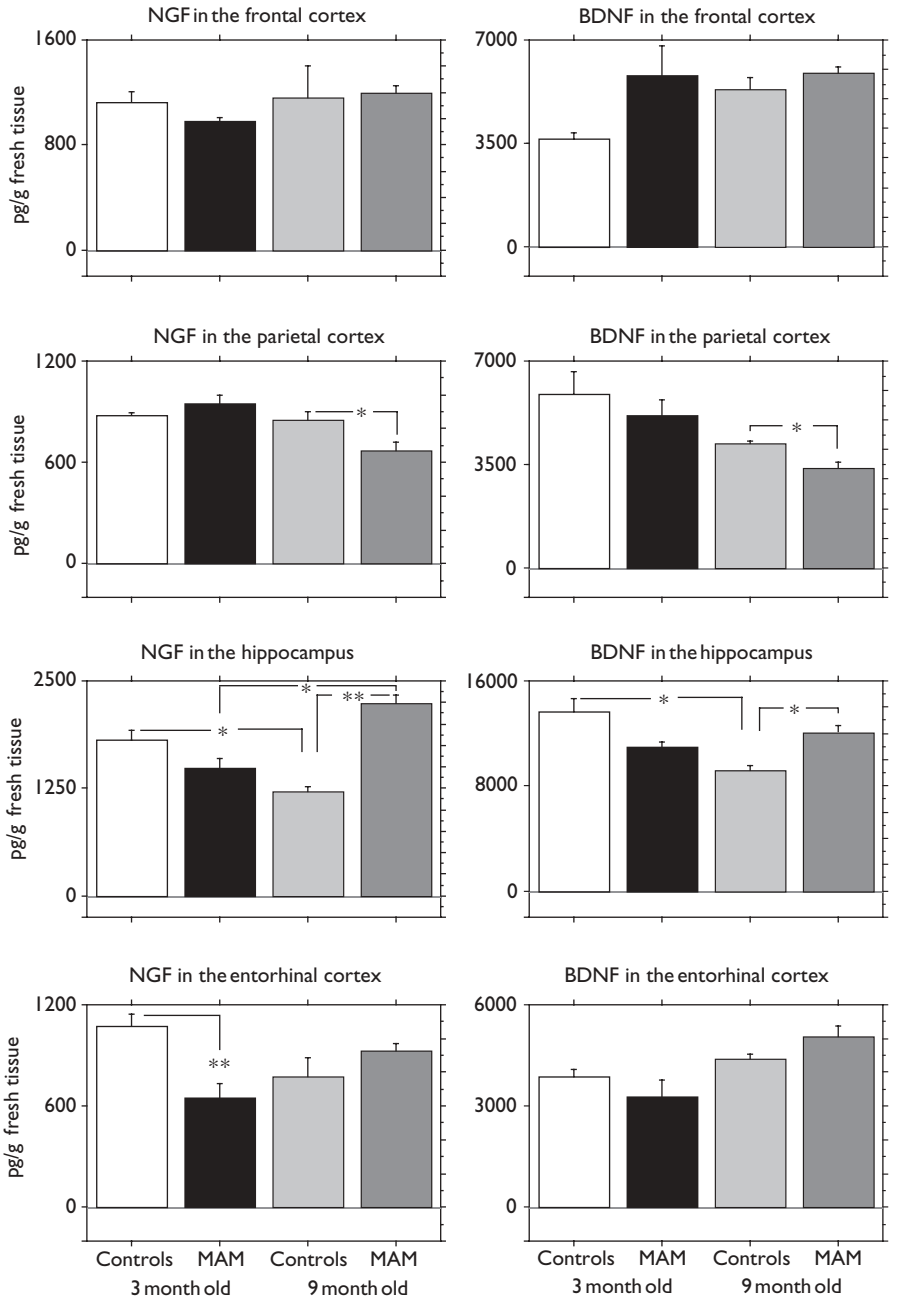

Fig. 1. NGF and BDNF in the hippocampus and in the frontal, parietal and entorhinal cortex of rats exposed *in utero* to MAM (GD17). Data represent mean levels (±s.e.m.). Significant differences between groups: $*p < 0.05$; $**p < 0.01$.

NGF and BDNF were found to be significantly lower in MAM-treated rats than in controls. This is consistent with previous studies of MAM administered at GD15 which revealed a decrease in cortical neurotrophin that was associated with atrophy of some cholinergic nuclei of the basal forebrain [6]. In contrast, in the hippocampus both NGF and BDNF were significantly higher in 9-month-old MAM-treated rats whereas there was a decrease in NGF in the entorhinal cortex of young MAM-treated animals. Similar results were found in the hippocampus when MAM was administered at GD11 and GD12 [4]. Since neurotrophins are produced in neurons and glial cells [9], one interpretation of these findings is that the observed elevation in neurotrophin levels in selected structures of the limbic system may be due to a compensatory sustained overproduction if these factors to counteract the mitotoxin-induced impaired development in these regions [5]. The data on NGF and BDNF concentrations in the striatum and hypothalamus are novel and seem to represent a specific response to the MAM treatment. The decrease in hypothalamic NGF may reflect an impaired coping reaction to stressful situation; a condition known to be present in these rats and which may contribute to the observed deficits in cognition and social interactions [7,8].

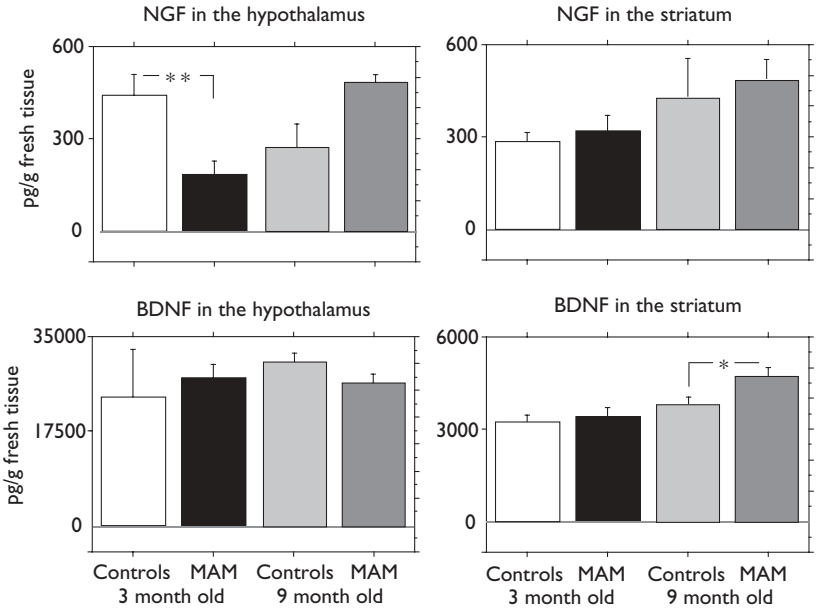

Fig. 2. NGF and BDNF in the striatum and hypothalamus of rats exposed *in utero* to MAM (GDI7). Data represent mean levels (±s.e.m.). Significant differences between groups: $*p < 0.05$; $**p < 0.01$.

The experimental disruption of the limbic cortex during different stages of development could be used as a tool to analyze the interactions between abnormal ontogenesis, neurotrophins and neuropsychiatric disorders. There is accumulating evidence that several neuropsychiatric disorders, including schizophrenia, may be related to a neurodevelopmental pathogenesis of the limbic cortex [10,11], with key roles for neurotrophins in this disruption [12,13]. Our initial studies demonstrated that rats in which MAM was administered at GD11 or 12 exhibited several characteristics consistent with that observed in schizophrenia, and which selectively affected neurotrophin levels within the entorhinal-hippocampal axis [14]. The present MAM model exhibits both anatomical and behavioral characteristics that appear to yield a more representative model of this disorder [8], and which also exhibits some alterations in neurotrophins that are consistent with those observed in schizophrenia [15,16]. Other studies have also provided evidence for a temporal limbic cortical involvement in the pathophysiology of schizophrenia [17]. Thus, rats with postnatal lesions of the entorhinal cortex exhibit increased basal concentrations of dopamine as well as of methamphetamine-induced release of dopamine in the amygdala [18]. It is also of note that the changes in BDNF and NGF observed after MAM exposure at GD17 show changes in the adult rat with differences at 3 *vs* 9 months. Considering that schizophrenia often begins at early adulthood (equivalent to ~3 months of age in the rat) these data also appear to be consistent with the delayed onset of this disorder. Such a delayed onset of alterations has also been reported in a rat model of schizophrenia based on neonatal ventral hippocampus lesions [19]. Therefore, the current results combined with previous observations using MAM models [3,4] show that prenatal toxin exposure leads to changes in adulthood. Our working hypothesis is that damage during specific time points of prenatal development could affect brain morphology and neurotrophin levels as proposed to occur in schizophrenia in humans [13,17,19]. It may also be predicted that NGF and BDNF may show parallel alterations in different brain regions known to be associated with other types of neurological or psychiatric disorders [5,12].

It is interesting to note the data showing an opposite trend for NGF concentrations in the hippocampus when comparing young adult rats (3 months) with adult pre-senescent (9 months) rats. Thus, MAM rats at 9 months of age exhibit significantly higher levels of NGF primarily due to a failure to show the normal NGF reduction observed in controls at that age. One possibility is that this elevation could contribute to the increased presence of progenitor brain cells that are normally present in the aged animal and are known to be regulated by neurotrophins [20–22]. Indeed recent data on neurogenesis [23,24] show that treatment with MAM reduced neural precursor cells in adult rat hippocampus and enhanced the survival of new granule cells in the dentate gyrus. This induced the recovery of proper cell layer structures and decreased dentate granule cell death. Thus, since neurotrophins play a crucial role in neuronal survival and differentiation, it is possible that the reported hippocampal recovery following prenatal MAM exposure [23,24] might be due to the augmented levels of hippocampal neurotrophins observed here.

CONCLUSION

Our study suggests that MAM models of abnormal brain development may provide a powerful model for the study of the etiopathogenesis of neuropsychiatric disorders that are believed to have an origin in disrupted development such as mental retardation, epilepsy and schizophrenia.

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